THE CHARACTERISTICS AND REGULATORY NATURE OF 3-O-METHYL-D-GLUCOSE (3OMG) UPTAKE IN BOVINE ADRENOMEDULLARY CHROMAFFIN CELLS

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by

Luisa Bigornia

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Department of Pharmacology and Therapeutics

Faculty of Medicine

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ABSTRACT

The characteristics and regulatory nature of sugar transport were investigated in bovine adrenal chromaffin cells, serving as a model of a homogeneous neuronal cell population. Transport was measured by following the cell/medium distribution of the nonmetabolizable glucose analogue, 3-O-methyl-D-glucose (3OMG). In isolated chromaffin cells, 3OMG uptake had a Km = 8.2 mM and Vmax = 0.69 nmol/mg protein/min, and exhibited saturability, competitive inhibition, countertransport and inhibition by cytochalasin B and phloretin. Thus, sugar transport in adrenal chromaffin cells is mediated by facilitated diffusion, as in other neural tissue preparations and most other animal cell types.

Insulin, hyperosmolarity and secretagogues stimulated 3OMG transport and ^{45}Ca uptake in isolated chromaffin cells, and stimulatory effects on sugar transport were abolished in nominally Ca^{2+}-free medium. In addition, transport activation by insulin and hyperosmolarity was also depressed in the presence of the Ca^{2+}-antagonists, La^{3+} and methoxyverapamil. Thus, sugar transport in isolated chromaffin cells is subject to regulation by several factors, including insulin and secretory stimuli, and in a Ca^{2+}-dependent manner, as in muscle. In contrast to muscle, sugar transport was not affected by factors which enhance Ca^{2+} influx via Na^{+}/Ca^{2+} exchange.

The effects of culturing on characteristics of 3OMG uptake in adrenal chromaffin cells were examined. 3OMG uptake in rapidly growing day 1 cultures had a Vmax = 139 nmol/mg protein/min and Km = 15 mM, and was not altered by insulin. In stationary day 5 cultures, Vmax and Km

of 3OMG uptake decreased to 50 nmol/mg protein/min and 9 mM, respectively. Insulin stimulated sugar transport in day 5 cultures, and this effect was abolished in nominally Ca^{2+}-free medium. Treatment of chromaffin cell cultures with dexamethasone did not inhibit the formation of processes, but 3OMG uptake was reduced in dexamethasone-treated day 5 chromaffin cell cultures in comparison to untreated controls. Thus, saturation kinetics, insulin and Ca^{2+} sensitivity of 3OMG uptake were maintained in culture. Quantitative differences in transport activity between freshly isolated and cultured chromaffin cells may be related to differences in energy requirements at various stages of cell growth and morphologic change.

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AMP, ADP, ATP - adenosine 5'-mono, -di and triphosphate

cAMP - cyclic adenosine 3',5'-monophosphate

ara-C - arabinofuranosylcytosine

ATPase - adenosine triphosphatase

A-V - arteriovenous

BBB - blood brain barrier

$^{45}CaCl_2$ - calcium chloride radioisotope

^{14}C - carbon radioisotope

^{14}C-3OMG - ^{14} carbon labelled-3-0-methyl-D-glucose

CB - cytochalasin B

CBF - cerebral blood flow

CHAPS - 3[(3-cholamidopropyl)-dimethylammonia]1-propane-sulfonate

Ci - curie

CMR_x - cerebral metabolic rate of x

cpm - counts per min

cytosar - cytosine beta-D-arabinofuranoside

CW - cell water

^{o}C - degree centigrade

D600 - methoxyverapamil

dbcAMP - dibutyryl cyclic AMP

dex - dexamethasone

2DG - 2-deoxyglucose

2DG-6-P - 2-deoxyglucose-6-phosphate

DMEM - Dulbecco's Modified Eagle's Medium

DNase - deoxyribonuclease

2,4-DNP - 2,4-dinitrophenol

dpm - disintegrations per min

E - Luciferase

ECS - extracellular space

EDTA - ethylene diamine tetraacetic acid

e.g. - example

EGTA - ethyleneglycol-bis-(beta-aminoethylether)-N'N'-tetraacetic acid

F.C. - final concentration

FDU or FUdR - fluorodeoxyuridine

G-^{3}H - generally labelled with tritium radioisotope

G-6-P - Glucose-6-phosphate

g - gram

g. - gravity

HEPES - N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid

hr - hour

ICS - intracellular space

ID - inhibitory dose

i.e. - that is

IGF - insulin-like growth factors

ins - insulin

I.V. - intravenous

Km - Michaelis constant

l - liter

LH_2 - luciferin

LH_2-AMP - luciferin adenylate

LS - liquid scintillation

M - molar, moles per liter

m - milli or 10^{-3}

mol - mole

mm - millimeter

Mann - mannitol

min - minute

MSA - multiplication-stimulating activity

n - nano or 10^{-9}

NGF - nerve growth factor

3OMG - 3-O-methyl-D-glucose

P - probability

PC - pheochromocytoma

PdBu - 4-beta-phorbol-12,13, dibutyrate

PI - phosphatidyl-inositol

pmol - picomol or 10^{-12}

PS - permeability surface area product

RQ - respiratory quotient

S - substrate concentration

S.D. - standard deviation of the mean

S.E. - standard error of the mean

SV40 - Simian virus 40

TCA - trichloroacetic acid

TPA - 12-O-tetradecanoyl-beta-phorbol-13-acetate

TTX - tetrodotoxin

TWV - total water volume

U - unit

u - micro or 10^{-6}

V - initial rate

Vmax - maximal velocity

VS - virtual distribution space

SECTION I

INTRODUCTION

In most animal tissues, glucose transport into the cell takes place by energy-independent facilitated diffusion (Widdas, 1952; Wilbrandt and Rosenberg, 1961). Exceptions are the absorptive epithelium of the small intestine (Crane, 1965) and of the renal proximal tubule (Kleinzeller and Kotyk, 1961), where glucose uptake occurs by an active Na^+-coupled process. In other cells, the net flux of glucose depends on the concentration gradient across the cell membrane and the kinetic parameters (capacity and affinity) of the carrier. In addition, transport in some cell types can be modulated by metabolic or hormonal factors. Whether this latter regulatory control exists depends on the nature and metabolic requirements of the cell (Elbrink and Bihler, 1975).

In tissues where glucose utilization is variable, such as muscle and adipose tissue, glucose transport is regulated in accordance with the changing energy requirements of the cell. These cells normally have negligible intracellular levels of free glucose, and the rate of glucose metabolism depends on the amount of glucose which gains access to intracellular enzymes. Thus in muscle and adipose tissue, glucose transport is said to be rate-limiting for its utilization, and serves as another site for the regulation of glucose metabolism by hormonal and metabolic factors. These tissues respond to insulin and contain energy reserves in the form of glycogen and triglycerides. They have the capacity for both aerobic and anaerobic metabolism and exhibit the Pasteur effect. Although many different factors may regulate glucose transport, they may activate the transport system by a common mechanism or mediator. A mediatory role for Ca^{2+} in the activation of glucose transport has been proposed (Elbrink and Bihler, 1975; Clausen, 1980).

Although the metabolic rate of the liver is variable, glucose transport is neither rate-limiting nor subject to modulation. The liver's physiological role requires a large capacity of the glucose transport system, which is capable of rapid release as well as uptake of glucose. In tissues where glucose utilization is stable such as the lens of the eye and mature mammalian erythrocyte, free intracellular glucose is present. The transport system has more than sufficient capacity to supply the required substrate and is not subject to regulation. In the brain the rate of glucose metabolism appears to be variable under certain conditions, but whether or not the transport step is also regulated has not yet been definitely established (Lund-Andersen, 1979).

A. GLUCOSE UTILIZATION IN BRAIN AND NERVE

Several studies have shown that glucose is the major metabolic substrate of the brain. Analyses of arterial and cerebral venous blood from experimental animals and man have provided important information on substrate utilization in the brain. In 1929, Himwich and Nahum sampled blood from the superior sagittal sinus in cats and found that the respiratory quotient (RQ) i.e. the ratio between the A-V differences for carbon dioxide and oxygen was close to one. By using the technique of percutaneous puncture of the bulb of the internal jugular, Lennox (1931) could measure the cerebral A-V differences in man, and confirmed that the RQ was close to one. In 1942, Gibbs et al. measured the A-V differences of oxygen, carbon dioxide, glucose and lactate in 50 healthy men, and found that the respiratory quotient of the brain was 0.99. Since only carbohydrates give an RQ of unity, this finding indicated that glucose was the main substrate for the brain. The development of the inert gas technique, which allowed rates of metabolism to be measured later led to a great number of studies of oxygen and glucose consumption in the brain. These studies confirmed that glucose is the main or sole substrate of the brain in vivo. In man, 90-95% of glucose extracted is oxidized whereas 5-20% may be converted to lactic and amino acids (Siesjo, 1978).

Utilization of non-carbohydrate compounds is increased in the immature brain or in starvation as a result of changes in the circulating levels of these substrates and the capacities of their transport processes. The in vivo rates of influx of 3-hydroxybutyrate to brains of adult rats fasted for 4 days were about 7 times the rate observed in normally fed rats (1.13 compared to 0.16 umol/min/g). The

values for acetoacetate were 1.81 and 0.15, a 13-fold increase (Daniel et al., 1971). Transport of 3-hydroxybutyrate and acetoacetate was also low in the newborn rat brain and increased steadily until 3 weeks of age when the animals were weaned and the dietary source of ketone bodies decreased. The adaptation of ketone body transport during development in the rat may be similar in the human. The ketone body concentration in human blood increases from around 90 uM at birth to about 700 uM in the first week of life. It falls steadily thereafter over the first year to 250-300 uM and reaches adult values of around 100-150 uM after 6-7 years (Bachelard, 1983).

Glucose also appears to be the major metabolic substrate in peripheral nerve at rest. The rates of glucose and oxygen uptake and of lactate production were measured at rest and during electrical stimulation in superior cervical ganglia excised from rats (Larrabee and Horowicz, 1955; Horowicz and Larrabee, 1958). At rest, the oxygen equivalent of the difference between glucose and lactate exchanges was equivalent to the observed oxygen uptake. This suggested that the principal fuel oxidized at rest was glucose. However this may not apply to the stimulated nerve. Increasing frequency of neuronal activity was associated with parallel and equivalent increases in the rates of glucose uptake and lactate production but oxygen uptake was also disproportionately elevated during activity. It was suggested that the extra glucose taken up during activity is completely converted to lactate and that some endogenous substrate (perhaps amino acids) may be oxidized and account for the increase in oxygen uptake during activity.

Peripheral nerve may differ from brain in some other aspects of glucose utilization. Hothersall et al. (1982) investigated the activities of enzymes of the glycolytic route, the pentose phosphate pathway, the tricarboxylic acid cycle and lipogenesis in rat sciatic nerve and brain. They found that the contributions of the pentose phosphate pathway and lipogenesis to glucose utilization were substantially higher in sciatic nerve than brain. The relatively high activities of transketolase and transaldolase suggested a special role for these enzymes in sciatic nerve.

In diabetes, hyperglycemia leads to several biochemical and physiological abnormalities in peripheral nerve, capillary pericytes and the lens of the eye, which may contribute to the pathogenesis of diabetic neuropathy, angiopathy and cataract formation. High circulating glucose levels enhance polyol pathway activity, leading to accumulation of sugar alcohols such as sorbitol and to inhibition of active myo-inositol uptake. This results in reduced levels of myo-inositol, a substrate in phosphoinositide metabolism which has been implicated in maintenance of nerve Na^+/K^+-ATPase activity. Subsequent reduction in Na^+ pump activity may be reflected in decreased energy utilization (Brown and Greene, 1984). In tibial nerve fascicle and endoneurial preparations of alloxan-induced diabetic rabbits, energy utilization was reduced to 25-30% of control (Greene and Winegrad, 1981). Reduction in Na^+/K^+-ATPase activity may result in decreased nerve conduction velocity and altered Na^+-dependent cellular processes including decreased Na^+-coupled myo-inositol uptake. Hyperglycemia also increases non-enzymatic glycosylation potentially impairing protein function. While all the details are not yet fully understood, it is

clear that these physiological and biochemical alterations lead to chronically slowed nerve conduction, impaired axonal transport, altered intermediary metabolism, and ultimately structural damage to peripheral nerves (for review, see Brown and Greene, 1984).

B. GLUCOSE TRANSPORT IN NERVOUS TISSUE

Since the brain has limited carbohydrate stores, it is dependent on the minute to minute supply of glucose. Consequently, the metabolism of the brain is critically dependent on the concentration of glucose in the blood. The supply of glucose from the blood circulation to the metabolic enzymes in nerve and glial cells involves transport through the blood brain barrier, diffusion through the brain's extracellular space, transport across the nerve and glial cell membranes and, to a lesser extent, active uptake by the choroid plexus (Lund-Andersen, 1979).

1. Glucose Transport From Blood to Brain

In 1964, Fishman showed that the transport of glucose, 2-deoxyglucose (2DG), and fructose from the blood to cerebrospinal fluid (CSF) in dogs was saturable, and showed stereospecificity, competitive inhibition, and a countertransport effect.

In 1965, Crone investigated the mechanism of transfer of glucose from blood to brain tissue in anesthetized dogs using the indicator dilution technique. The initial unidirectional transfer of glucose was measured after intracarotid injection of glucose + [^{14}C]glucose with subsequent sampling from the superior sagittal sinus. The fraction of glucose which passes into the cerebral tissue fell with increasing concentration of glucose in the concentration range 1 mM to 13 mM. The extraction at low concentrations was almost 50% and that at high concentrations about 10%. The drop in extraction at high concentrations is taken as evidence of a carrier-mediated mechanism, which becomes saturated at concentrations of about 3.9 mM. The transport mechanism appeared to be specific for glucose.

Several studies have since demonstrated that glucose transport from the blood to brain is mediated by a facilitated diffusion mechanism. Insulin had no demonstrable stimulatory effect on the transport mechanism. The net transport proceeds in the direction of the concentration gradient, which normally is in the direction from blood to brain. Thus the characteristics of glucose transfer between the blood and CSF resembled glucose transport in erythrocytes.

Blood Brain Barrier (BBB)

The brain is separated from the blood circulation by a continuous layer of tight-junctioned endothelial cells. In contrast to other capillaries, brain capillaries lack the so-called fenestrations. These tight endothelial cell junctions and the absence of pinocytosis form the basis of the blood brain barrier (BBB), which is almost completely impermeable to macromolecules. Neither the brain capillary basement membrane nor the surrounding astrocytic membrane contribute to the BBB. The permeability of the BBB is determined by the luminal endothelial cell membrane and outer cell membrane. In other words, in its transit from the blood circulation to the brain's extracellular space, glucose crosses two membranes in series i.e. the luminal and contraluminal membranes of the endothelial cells (Oldendorf, 1974, Lund-Andersen, 1979).

The uptake of 2DG was examined in isolated brain capillaries (Goldstein et al., 1977; Mrsulja et al., 1976), and was shown to be saturable with an affinity constant of Km 0.1 mM. These studies support the assumption that the saturation property of glucose transport in vivo refers to the endothelium of the brain capillaries (Lund-Andersen,

1979). The presence of this barrier between the blood and the glial and neuronal cell membranes prevents the direct investigation in vivo or in the intact brain of glucose transport across glial and neuronal cell membranes.

The BBB can be circumvented by perfusion of cerebral ventricles. This method has the drawbacks of: (a) large diffusion distances and (b) possible transit between cerebrospinal fluid and blood across the choroid plexus and across the blood brain barrier in the direction of brain to blood. Ventriculocisternal perfusion studies do not provide quantitative data on glucose transport through glial and neuronal cell membranes. These technical problems have led to the use of in vitro preparations, including tissue slices, isolated synaptosomes, and secondary neural cell cultures (Lund-Andersen, 1979).

2. Glucose Transport in In Vitro Preparations of Nervous Tissue

Brain Slice Preparations

In cerebral cortex slices, glucose was shown to be transported by a carrier-mediated process (Joanny et al., 1969; Fishman et al., 1971; Bachelard, 1971a). However in tissue slice preparations, the cell membranes are not in direct contact with the incubation medium. Between them is interposed the extracellular space through which the substrates must diffuse to reach the cell membranes. Unless the rate of diffusion is high compared to the rate of membrane transport, access to the cell membrane may represent a delaying and possibly rate-limiting factor and measurement of uptake will not reflect unidirectional flux across the cell membranes.

Tissue slices and other neural preparations described above also have the drawback of cell heterogeneity. In addition to neuronal cells, the central and peripheral nervous systems have supporting or satellite cells, which consist of the neuroglial cells in the brain and Schwann cells in the periphery. Neuroglial cells make up half the volume of the brain and outnumber the neurons by ten to one. In the central nervous system, there are four major classes of neuroglia including astrocytes, oligodendrocytes, ependymal cells, microglial cells, which may function in structural support, isolation and electrical insulation of neurons, storage of neurotransmitters, secretory function, repair and regeneration, provision of framework around which subsequent neuronal organization takes place, and a nutritive role (Kuffler and Nicholls, 1976; Alberts et al., 1972).

The role(s) of neuroglial cells have not been clearly defined due to the lack of methods for separating neurons from glial cells. The tissues are so interwoven that it is at present not feasible to separate pure glial and neuronal cell fractions. Several bulk separation procedures such as that of Wilkin et al. (1976) for preparing cells from the immature cerebellum have been described. However, Daniel et al. (1978) showed that sugar transport in the immature rat brain differed in some aspects from that in adult brain. Hence, neural cells isolated from immature brain may not be representative of neural cells in the adult nervous system. Poduslo (1981) developed a method for bulk isolation and maintenance of oligodendroglia as suspension cultures. However, during the tissue dissociation step of this isolation procedure, the processes of the oligodendroglia are shorn off. Although these areas of the cell

membrane presumably reseal with time, it is possible that these freshly isolated oligodendroglia, lacking processes, may differ in various aspects from their in vivo counterparts, which have their processes intact.

Synaptosomes

The lack of a homogeneous neural cell preparation led to the use of synaptosomal preparations which consist of pinched off nerve endings. Synaptosomes represent a population of organelles surrounded by a membrane of neuronal origin. Their surrounding membranes appear to be intact morphologically and biochemically, as they are able to retain K^+ content and lactate dehydrogenase activity. The isolated synaptosomes contain mitochondria and a full complement of glycolytic enzymes so they are capable of glycolysis and respiration. They can thus be regarded as anucleate cells.

Diamond and Fishman (1973) showed that 2DG uptake by rat brain synaptosomes was mediated by a high affinity (0.2-0.3mM), Na^+-independent, saturable transport system which was inhibited by 3-O-methyl-D-glucose (3OMG), D-glucose and phloretin, and was insensitive to insulin. It is important to consider, however, that the membrane surrounding an area as specialized as the synaptic region may not necessarily exhibit properties identical to those in the rest of the neuronal plasma membrane.

Cultured Neuronal and Glial Cells

The shortcomings inherent in the neural preparations described above (BBB, extracellular space, cell heterogeneity) may be

circumvented by the use of cultured neuronal and glial tumour cell lines. These should also permit investigation of possible differences between the transport mechanisms of neuronal and glial cells. Some differences between neurons and glia in glucose uptake properties were first suggested from studies on glucose metabolism in cultured neuroblastoma and glioblastoma cells (Newburgh and Rosenberg, 1972).

The uptake of 3OMG and 2DG by human glioma cells (Edstrom et al., 1975; Walum and Edstrom, 1976a) and mouse neuroblastoma cells (Walum and Edstrom, 1976b) was mediated by a saturable, low affinity (Km ~ 6 mM) transport system, which was competitively inhibited by D-glucose, phloretin and cytochalasin B and insensitive to phlorizin, ouabain, NaCN and iodoacetic acid. Thus similar to other neural tissue preparations, glucose transport in cultured neural cells occurs by a facilitated diffusion mechanism.

The major drawback in using cultured cells is the uncertainty whether a valid comparison can be made with respect to transport and metabolism between isolated cells in culture and cells in intact organized tissue, and between cells of tumor origin and cells of normal origin (Kalckar, 1976). Studies have shown that hexose transport rates are greatly enhanced after transformation of cultures by oncogenic viruses. For both hexose and amino acid analogues, the Vmax of uptake is increased in transformed cultures, the Km being unchanged (Isselbacher, 1972).

Although in the majority of cases, hexose uptake rates seem to be higher in transformed cultures than in their untransformed counterparts, it is not always clear whether uptake rates are regulated upwards by oncogenic transformations, and several exceptions have been

described. African green monkey kidney cells (VERO) and their Simian virus-40 (SV40) infected counterparts did not reveal any difference in uptake rates (Miller et al., 1975) nor did the human cell line W138 (embryonic lung) and its SV40 counterpart (Patterson, et al., 1976) The latter authors point out that the untransformed W138 already had very high uptake rates. Eagle et al. (1961) did not find differences in uptake rates between cells derived from normal tissue and those from malignant tissue. Finally, Anderson and Martin (1976) studying SV40 transformation in cells from mouse brain found no enhancement of uptake rates before or after transformation.

It appears that the glucose concentration in the culture medium affects the transport rate. For example, if the glucose is used up over 24 hours which commonly occurs in transformed cultures, the enhanced glucose uptake may partly be due to glucose starvation. Conversely, very high levels of glucose stimulate hexose uptake in untransformed growing BHK (kidney) cultures and slightly inhibit uptake in their polyoma-transformed counterparts (Patterson et al., 1976).

Other Nervous Tissue Preparations

In comparison with the brain, the superior cervical ganglion may be considered a more simple biological model for nervous tissue. However this model presents several complexities due mainly to the lack of homogeneity in morphology, electrophysiological responses, and metabolism (Dolivo, 1974).

3. Peripheral Nerve

Preparations of whole peripheral nerve suffer from many of the same disadvantages as the brain. Whole nerve preparations are complex in structure and consist of several elements. The epineurial connective tissue constitutes a major fraction of the tissue mass and contains heterogeneous cell types including adipocytes. Similar to the BBB, the perineurial membrane of the nerve fascicle acts as a selectively permeable barrier in vivo (Bradbury and Crowder, 1976) and may provide a diffusion barrier to glucose in the medium in vitro.

Greene et al. (1979) developed and evaluated two preparations of peripheral nerve with the objective of eliminating some of the technical problems associated with whole nerve preparations. These included: (1) an isolated rabbit sciatic nerve fascicle free of epineurium but with an intact perineurial membrane, and (2) a preparation of endoneurial components of the isolated fascicle. Characteristics of glucose transport in axonal segments and in Schwann cells could not be distinguished but those of epineurial components could be excluded. Data obtained using these preparations showed that glucose can provide the total exogenous substrate requirements for the maintenance of steady state energy metabolism in the endoneurial components of resting nerve.

There are few qualitative, much less quantitative studies of glucose transport in peripheral nerve. Hexose transfer in squid giant axon was found to be mediated by an energy-independent, selective, facilitated diffusion mechanism with an apparent Km = 3.6 mM and Vmax = 11.4 pmol/cm^2/sec (Baker and Carruthers, 1984).

In summary, these studies clearly show that as in most animal cells, glucose is transported into neural cells by facilitated diffusion. The next questions to ask concern the regulatory nature of glucose transport into neuronal cells, i.e., is the membrane transport of glucose rate-limiting for cerebral glucose utilization? If so, what are the factors regulating the transport step?

C. REGULATION OF GLUCOSE TRANSPORT IN BRAIN AND NERVE

1. Is Glucose Transport Rate-limiting for its Utilization in Nervous Tissue?

Glucose transport across the cell membrane may determine the rate at which glucose is utilized by the cell. Whether it serves as a rate-limiting step in glucose utilization by neuronal cells depends on the relationship between the rate at which glucose is transported across the cell membrane and the rate at which glucose is phosphorylated within the cell. If the transport rate exceeds the phosphorylation rate, membrane transport cannot play a significant role, and phosphorylation would be rate-limiting. Significant levels of free glucose would be found intracellularly in this situation. The human erythrocyte and liver are examples of this type of relationship between membrane transport and phosphorylation. If on the other hand, the transport rate is less than the phosphorylation rate, membrane transport would be rate-limiting for the cellular utilization of glucose, and intracellular free glucose levels would be very low. Muscle cells normally exhibit glucose transport of this type. If the membrane transport of glucose is rate-limiting, it may then be determined which physiological factors modulate glucose uptake (Elbrink and Bihler, 1975).

It is not clear whether glucose transport is rate-limiting in central nervous tissue (Lund-Andersen, 1979). The question can be approached by comparing the magnitude of transport and phosphorylation rates or by estimating the levels of intracellular free glucose.

The intracellular glucose content was indirectly estimated by assessment of hexokinase activity. When glucose crosses the cell membrane, it is immediately phosphorylated by hexokinase:

Glucose + ATP ⟶ Glucose-6-phosphate (G-6-P) + ADP

Cerebral hexokinase maximally activated in vitro (mouse brain extracts) is capable of phosphorylating glucose at rates of more than 600 umol/g/hr; the overall rate of glucose utilization in the brain is only some 20 umol/g/hr, so it has been argued (Lowry et al., 1964) that over 97% inhibition of hexokinase must occur in vivo. However the products of the hexokinase reaction at their appropriate intracellular concentrations caused only a 65-70% inhibition of enzyme activity. It was, therefore, suggested that the intracellular glucose concentration is so low that it limits the hexokinase reaction (Siesjo, 1978) and hence transport could be rate-limiting. This is also consistent with observations of Gey (1956) who reported brain glucose concentrations of 0.45 umol/g in rats killed by immersion into liquid nitrogen. If the amount of glucose contained in the blood and extracellular fluid is subtracted from this value, the intracellular glucose concentration should be very low.

However, subsequent measurements of glucose concentration in tissue, based on more rapid freezing techniques have given values higher than those reported by Gey (1956). Low glucose values are obtained if the tissue is frozen after decapitation, or if animals larger than mice are frozen by immersion in a coolant. When mice are rapidly frozen in Freon-12 the tissue glucose concentration was about 1.5 umol/g at a plasma glucose concentration of 8 umol/g, corresponding to a blood glucose concentration of 5-6 umol/g (Lowry et al., 1964;

Brunner et al., 1971). At this tissue glucose concentration the intracellular glucose concentration would approach zero only if it is assumed that the CSF glucose concentration is 75% of that in the blood, and that blood and extracellular fluids occupy more than 35% of the brain volume (Siesjo, 1978). As discussed below, these values are too high.

Several facts indicate that the intracellular glucose concentration is considerably higher. First, autolytic changes cannot be entirely avoided even when animals as small as mice are used (Ponten et al., 1973) and the true glucose concentration in the brain tissue of fed mice is probably higher than 2 umol/g. Second, the CSF glucose concentration in rats is 50% of the blood glucose concentration (Lewis et al., 1974). Third, the extracellular fluid volume of brain tissue is probably 15-20% of the tissue volume (Levin et al., 1970). When these facts are taken into account it can be calculated that the intracellular glucose concentration should be about 3 umol/g of intracellular water at a blood glucose concentration of 5-6 umol/g. Direct measurements of microdissected cells demonstrated appreciable amounts of intracellular glucose (Lowry, 1975). Hence these observations are not consistent with transport as the rate-limiting step. It should be noted however that these values are averages in a heterogeneous population and do not exclude the possibility that intracellular glucose levels may be low in some cell types.

Diamond and Fishman (1973) investigated whether glucose transport was rate-limiting for its utilization in synaptosomes. Rat brain synaptosomes were incubated with increasing concentrations of

2DG, and the synaptosomal contents of free and phosphorylated 2DG were determined. Although synaptosomal levels of free 2DG remained constant with increasing concentrations of 2DG, synaptosomal 2DG-6-phosphate content increased linearly with increasing uptake even though total uptake of 2DG was saturable. These observations suggested that 2DG uptake was the rate-limiting step.

In cultures of C-6 astrocytoma (a type of glioma cell) and neuroblastoma cells, Passonneau (1976) examined the effect of the concentration of glucose in the medium on the intracellular concentration of metabolites. When the cells were exposed to medium containing high concentrations of glucose (50 mM), intracellular glucose increased but glucose-6-phosphate, UDP-glucose, and glycogen were not altered compared to corresponding values observed in low (5.5 mM) glucose medium. It was concluded that glucose transport into the cells was occuring faster than its metabolism, and hence was not the rate-limiting step for its utilization. It was generally assumed that in tissues where glucose transport was not rate-limiting for its utilization, insulin will probably have no effect on the membrane transport of glucose (Elbrink and Bihler, 1975). Although Passonneau (1976) found that the membrane transport of glucose was not the rate-limiting step in the utilization of glucose by neuroblastoma and astrocytoma cells, glucose transport in these cells was sensitive to insulin.

Keller et al. (1981) examined glucose transport in confluent monolayers of C1300 neuroblastoma (N2A) and glioma (C6) cells. In neuroblastoma, steady state intracellular glucose concentration reached extracellular levels, while intracellular contents in C6 glioma cells

remained very low. In C6 glioma cells the amount of glycogen measured was much higher than that found in neuroblastoma cells. During the influx period (0 to 40 sec), the transport of glucose did not exceed the phosphorylation rate in C-6 glioma cells, whereas a steady time-dependent increase in glucose content was observed in neuroblastoma cells. While glucose uptake in neuroblastoma cells seems to be regulated at the level of the phosphorylating enzymes, the control point in C6 glioma cells is believed to be membrane transport.

In summary, the bulk of evidence suggests that the transport step appears to be rate-limiting in isolated synaptosomes (Diamond and Fishman, 1973), cultured glioma cells (Edstrom et al., 1975; Walum and Edstrom, 1976a; Walum and Edstrom, 1976b; Cummins et al., 1979; Keller et al., 1981) and in primary astrocyte cultures (Cummins et al., 1979), although not in cultured neuroblastoma cells (Keller et al, 1981). In addition, glial cells, either untransformed astrocytes or transformed glioblastoma cells were shown to have negligible intracellular levels of glucose. Transport appears to be faster than phosphorylation in capillary endothelial cells (Betz et al., 1979) and in neuroblastoma cells, where intracellular glucose can be detected (Keller et al., 1981).

The following considerations suggest that the membrane transport of glucose may be an important regulatory step in cerebral glucose consumption.

If the kinetic properties of transport and phosphorylation are compared, those of transport appear to be more directly compatible with overall rates of glucose consumption. The Km for glucose transport at

the BBB is 6-7 mM, identical to the normal concentration of glucose in the bloodstream. The rate of transport at the BBB at half-maximal capacity is 0.6 to 1.0 umol/min/g and this is close to the overall rate of glucose consumption (0.33 umol/min/g). On the other hand, cerebral hexokinase when maximally activated, is capable of phosphorylating glucose at a rate of more than 10 umol/g/min as described above (Bachelard, 1980).

Hypoglycemia seems to affect transport rather than phosphorylation. At blood glucose concentrations of 5 mM, the theoretical capacity of the BBB uptake process (maximum rate = 0.7 umol/g/min) is in excess of the rate (0.3-0.5 umol/g/min) required to support brain function. However, when the available glucose concentration falls below 2 mM the uptake rate falls below this minimum rate (Bachelard, 1971b). At 2 mM arterial blood glucose, the first symptoms of hypoglycemia begin to appear. In contrast, hexokinase (with a Km 0.04 mM) is still fully saturated at such concentrations of glucose (Bachelard, 1971b, 1980).

Use of the nonmetabolizable glucose analogue, 3OMG eliminates the phosphorylation process from the interpretation of results. As described above, 3OMG, 2DG and D-glucose are all transported by the same carrier. The effects of 3OMG and 2DG after intravenous infusion into monkeys were found to be identical in producing drowsiness and a changed EEG (Meldrum and Horton, 1973). The only difference was that the lowest effective dose of 3OMG (500 mg/kg) was somewhat higher than that of 2DG (300 mg/kg) required to give these first behavioural symptoms of hypoglycemia. It was concluded that symptoms of hypoglycemia resulted from decreased cerebral glucose levels due to

partial inhibition of glucose transport across the BBB by 3OMG and 2DG (Bachelard, 1980).

In contrast, Lund-Andersen (1977) suggested that phosphorylation was the rate-limiting step in cerebral glucose consumption. Their study involved the measurement of 2DG uptake in cortical slices and of the concentrations of 2DG and its phosphorylated form, 2DG-6-phosphate. Since free unphosphorylated 2DG could be detected intracellularly, it was concluded that the transport of 2DG across the cell membrane occured faster than its phosphorylation. However, 2DG has a lower affinity for hexokinase than D-glucose, and hence competes less effectively against D-glucose. Thus if D-glucose is present, free 2DG will accumulate and in the study by Lund-Andersen (1977) both glucose (5-12 mM) and 2DG (0.5-15 mM) were present.

2. Effect of Insulin

Although studies have reported significant amounts of insulin-immunoreactive material in the brain (Gammeltoft et al., 1984), large amounts of "insulin receptors" detected by radiolabelling techniques (Havrankova et al., 1978) and high concentrations of insulin in cerebrospinal fluid detected by radioimmunoassay (Margolis and Altszuler, 1967; Owen et al., 1974), a role of insulin in cerebrospinal function is not known (Bachelard, 1983). Based on the assumption that glucose uptake into brain cells is sensitive to insulin, Butterfield et al. (1966) speculated that insulin sensitivity of the brain may be of significance with respect to the role of the central nervous system in glucose homeostasis. If the brain has a mechanism to regulate glucose released from the liver and thereby regulate the level of blood sugar, the adaptation in diabetics would be to increase glucose output from the liver and raise the blood sugar. Perhaps blood sugar levels would continue to rise until an equilibrium is reached where the blood sugar would be sufficient to return beta cell insulin production and brain glucose uptake to normal.

Data on the influence of insulin on glucose transport into brain cells are controversial. The following studies indicate that insulin has an effect: Gottstein et al. (1965) compared in man hyperglycemia and high insulin level (0.40 U/kg i.v.) to pure hyperglycemia. When glucose was infused together with insulin there was a significant increase in glucose extraction 30 min after infusion. In arteriosclerotic patients, insulin and glucose infusion significantly increased glucose extraction but had no effect on the cerebral metabolic rate and cerebral production of lactic and pyruvic acids. The

authors concluded that the increased glucose uptake was not due to aerobic or anaerobic glucose consumption, but that insulin alleviated a deficient glucose transport in arteriosclerotic patients, and glucose was perhaps consumed for the synthesis of amino acids and proteins (Gottstein et al., 1965). In a later study, the authors infused insulin (0.15 to 0.20 U per kg) into twelve diabetic patients, and despite the fall in blood glucose concentration, there was a highly significant increase in glucose uptake by the brain (Gottstein and Held, 1967).

Butterfield et al. (1966) studied glucose uptake by the brain and peripheral tissues in five healthy men: 1) while fasting, 2) during a glucose infusion, 3) after intravenous insulin, and 4) during a further glucose infusion. The initial glucose infusion increased glucose uptake in both the brain and periphery. Insulin stimulated glucose uptake but the response of the brain to insulin was slower. Under normal physiological conditions, blood sugar levels in man do not fall as rapidly as under these experimental conditions or in the diabetic on daily insulin injections. In addition, the glucose requirement of the brain is relatively constant in comparison with that of muscle, and rapid adaptation is unnecessary. The authors attribute the sluggish response of the brain to the inability of the brain to adapt as rapidly as peripheral tissues to dramatic changes in blood sugar.

Hertz et al. (1982) determined in seven fasting patients the effects of insulin on the BBB transport of glucose using the indicator dilution method. Insulin infused at a dose of 0.40 U/kg body weight increased extraction, unidirectional influx from blood to brain, back flux, and net uptake of glucose (during the initial phase of insulin

infusion); plasma insulin levels of 80uU/ml also caused significant increases in glucose flux of about 20%.

Daniel et al. (1977) showed that in rats, insulin prolonged the transient increase in glucose uptake by the brain, resulting from rapidly induced and sustained hyperglycemia. Rafaelsen (1961a, 1961b) noted that the uptake of glucose by rat spinal cord, isolated cerebellum, and "first cortical slices" (one surface covered by the pia) was increased 10 - 15% in the presence of insulin. Prasannan (1972) found that in rat cerebral cortex slices, insulin enhanced glucose uptake and metabolism in cerebral cortex in vivo, under both aerobic and anaerobic conditions.

Field and Adams (1964) studied the effect of insulin on the uptake of glucose and other sugars by rabbit sciatic nerve in vitro. Insulin stimulated the uptake of glucose by nerves isolated from normal and alloxan diabetic rabbits. Passonneau (1976) found that the addition of insulin to the medium increased intracellular glucose, glucose-6-phosphate and glycogen in cultures of C6-astrocytoma (a type of glial cell).

Several groups have failed to demonstrate an effect of insulin on the transport of hexoses from blood to brain. These studies were done in vivo or in intact brain preparations, and thus results represent transport at the BBB. Crone (1965) found that insulin did not affect glucose transport from blood to brain, as suggested by experiments in which the glucose/fructose ratio in the cerebral blood of pancreatectomized dogs was determined before and after intracarotid injection of insulin. Hertz and Paulson (1983) attribute the lack of insulin effect to insensitivity of the method and possible distortion

of differences in glucose uptake due to the wide scatter of data. Buschiazzo et al. (1970) compared rats with alloxan diabetes (and hence low plasma insulin) with normal rats injected with glucose to produce similar blood sugar levels (but a higher insulin level). They measured the distribution space of L-arabinose (a nonmetabolizable glucose analogue but transported more slowly) in the brain at 30 min and found no effect of insulin. Hertz and Paulson (1983) argued that it was difficult to assess and draw conclusions from the point scatter around the arabinose distribution curve.

Betz et al. (1972) studied the effect of insulin on unidirectional glucose transport across the blood brain barrier in isolated canine brain. In the steady state condition and with the indicator dilution method, which is rapid enough to measure true unidirectional transfer, they were unable to show an effect of insulin (800 uU/ml blood). Hertz and Paulson (1983) argued that the inability to detect an insulin effect may be related to several gross abnormalities of their preparation. These include large unperfused areas and the lack of relation between the cerebral metabolic rate for oxygen (CMR_{O_2}) and glucose (CMR_{glu}), giving a 10-fold range in the oxygen/glucose indices.

Daniel et al. (1975) studied the effect of insulin on: (1) unidirectional glucose influx across the BBB, as measured by following the brain and plasma distribution of [^{14}C]glucose, and (2) cerebral glucose gain, as determined by cerebral A-V differences in glucose levels. They reported that insulin did not affect the unidirectional glucose influx but significantly increased the gain of glucose by the brain. They suggested that the increased gain with no change in influx

may be explained by the ability of insulin to reduce the cellular efflux of glucose. It was possible that insulin increased cellular utilization of glucose and thereby decreased the amount of glucose available for efflux. Hertz and Paulson (1983) claim that the study was invalidated by the non-steady state prevailing at the time of measurement. In their study, Daniel et al. (1975) induced hypoglycemia using insulin for 14 min, and then increased blood glucose to normal levels just before measuring. Hence measurement took place with a normal blood glucose but still subnormal brain glucose, and the increase in glucose gain may represent a rebuilding of brain glucose levels rather than a direct insulin effect. Similarly, the non-steady state situation leads to a lower capillary glucose concentration than in the steady state situation, and thereby to an underestimation of the unidirectional flux. Thus the insulin effect may be disguised (Hertz and Paulson, 1983).

Sloviter and Yamada (1971) examined the effect of insulin on metabolism of an isolated perfused rat brain preparation. Insulin was added to the perfusion fluid or was injected into the rat from which the isolated brain preparation was made. They found that insulin had no effect on the spontaneous electrical activity of the brain, the rate of cerebral glucose consumption and rate of K^+ efflux. Hertz and Paulson (1983) suggested that the perfusion time was too short for insulin to exert any effect in the parenchyma. In addition an increased transport rate is only initially accompanied by a transient increase in net uptake until the brain glucose concentration has been brought up to a new steady state level.

Most of the studies investigating the effect of insulin on sugar transport in nervous tissue were done in vivo or using intact brain preparations. As discussed above, measurement of glucose uptake using these preparations probably reflect glucose transport at the level of brain capillaries, which have been shown to be insensitive to insulin (Goldstein et al., 1977). Hence measurements of sugar transport in vivo or in intact brain preparations may not be truly representative of characteristics of glucose transport into neuronal and glial cells. On the other hand, insulin was shown to stimulate sugar transport in vivo in man (Hertz and Paulson, 1983) and in rat (Daniel et al., 1977), suggesting that insulin may influence glucose transport across the BBB. Thus the question of insulin regulation of glucose uptake into neuronal cells is far from resolved. It is likely that insulin stimulates sugar transport in at least some types of neural cells, possibly glial cells (Passonneau, 1976) and peripheral neurons (Field and Adams, 1964).

3. Other Factors or Conditions Affecting Glucose Transport in Brain and Nerve

Barbiturates

The effects of barbiturates on glucose content of the brain were determined by injecting mice with the drug, rapidly freezing the body or head (after decapitation) of the animal in liquid nitrogen, and preparing brain extracts using methods to minimize changes in the labile components during extraction. Glucose in the brain extract was then determined using enzymic methods (Lowry et al., 1964). Barbiturates increased intracellular glucose but it was not known whether this was a consequence of decreased glucose utilization resulting from inhibition of glycolysis by barbiturates, increased transport due to elevated plasma glucose, or a direct effect of barbiturates on glucose transport (Mayman et al., 1964; Gatfield et al., 1966; Nelson et al., 1968). Strang and Bachelard (1973) investigated the rates of cerebral glucose utilization in rats anesthetized with phenobarbital. They found that phenobarbital stimulated glucose transport but exerted an inhibitory effect on glycolysis, possibly at the hexokinase or phosphofructokinase step. Phillips and Coxan (1976) examined the effects of phenobarbital on 2DG uptake in rat cortical slices. They found that phenobarbital exerts a direct and specific effect on 2DG uptake. The stimulation of glucose transport by barbiturates is associated with increased affinity in vivo (Gjedde and Rasmussen, 1980) and in slices (Phillips and Coxan, 1976) and no consistent change in Vmax (Bachelard, 1983; Phillips and Coxan, 1976).

Anoxia/Ischemia

Betz et al. (1974) investigated the effects of anoxia on net uptake and unidirectional transport of glucose into isolated dog brain. Net uptake was determined from measurement of A-V differences and plasma flow rate per unit weight of brain in the presence and absence of anoxic conditions. Unidirectional transport of glucose was measured using the indicator dilution technique. After the first 2 min of anoxia, net glucose uptake doubled but unidirectional uptake remained unchanged, suggesting a reduction of brain glucose levels. It was concluded that the initial increase was due to a reduction in the rate of unidirectional efflux of glucose from the brain as a result of reduced brain glucose levels. After 10 min of anoxia both unidirectional transport and net uptake were decreased to about 40% of control. This subsequent decrease in net uptake resulted from impaired unidirectional glucose transport. Unidirectional transport was only 59% of the control rate after 1 hr of recovery from 30 min of anoxia. It was suggested that the decrease in glucose transport after anoxic conditions indicates that an energy producing process may in some way regulate the glucose transport system.

Drewes et al. (1973) showed that although brain ATP levels decreased to near zero after 30 min of anoxia, there was always a significant net uptake of glucose. It was suggested that ATP or some form of energy is not absolutely required for transport but rather exerts a modifying effect (Betz et al., 1974).

Electrical Activity

King et al. (1967) investigated the effect of electrically-induced seizures on energy reserves in the cerebral cortex of mice. The animals were rapidly frozen at seven intervals from 3 to 50 sec after the stimulus, and ATP, P-creatine, glucose, glycogen and lactate were measured in the outer (most rapidly frozen) portion of the brain. The electrical stimulus lasting for 1 min resulted in approximately a 3-fold increase in the metabolic rate within the first 3 sec. The observation that lactate increased more than could be accounted for by the reduction of glucose and glycogen content indicated that glucose transport into the brain was increased 8-fold.

Baker and Carruthers (1984) found that electrical activity increased transport of 2DG and D-glucose in squid giant axon, but not of 3OMG suggesting that the effect may be related to hexose metabolism. The ability of electrical activity to stimulate the uptake of 2DG has been applied to the development of a technique using radiolabelled 2DG as an intracellular marker of increased neuronal activity in the brain (Sokoloff et al., 1977). After it is infused into a cranial artery, labelled 2DG is phosphorylated and becomes trapped within the cytosol, and its intracranial position is located by gross or micro-autoradiography. Regions of the brain with increased activity are more heavily labelled than less active regions. It is not known whether the increased activity is due to altered transport and subsequent metabolism or to metabolism alone. Data in the squid giant axon support the latter.

Development

Daniel et al. (1978) showed that the rate of glucose transport across the BBB was low in suckling rats (2 weeks) but increased after weaning to reach its highest level in the young adult rat (7 to 9 weeks), then declined slowly as age increased. There was no change in Km but Vmax was increased from 0.75 to 1.95 umol/min/g. It was suggested that in the young adult rat with a normal level of glucose in the blood, the influx of glucose into the brain exceeds the rate at which it is utilized i.e. there is some efflux of glucose from the brain back into the blood. Thus when the blood sugar is lowered, there is a "margin of safety" before the glucose entering the brain becomes insufficient to meet the needs of the cerebral cells. The lower margin of safety in suckling animals is compensated for by the high influx of ketone bodies which provide an alternative source of energy at this age (Daniel et al., 1978).

The rate of increase of glucose transport across the BBB in the immature brain is further accelerated by thyroid hormone. Moore et al. (1973) examined whether the maturation of the BBB sugar transport system in the infant rat (2 weeks) is influenced by thyroxine. Thyroxine-treated animals showed sugar transport rates about 56% greater than controls and cerebral glucose utilization about 39% greater than controls. Methimazole, an antithyroid drug, delayed the rise in transport activation. Thyroxine stimulation of glucose transport may be due to the increased cerebral blood flow observed in thyroxine-treated animals.

Pathophysiological States

Diabetic patients with increased plasma glucose concentrations may develop cerebral symptoms of hypoglycemia when their plasma glucose is rapidly lowered to normal concentrations, indicative of insufficient transport of glucose from blood to brain. Gjedde and Crone (1981) examined the effect of chronic hyperglycemia on the rate of glucose transport from blood to brain in animals made diabetic with streptozotocin. The animals had been hyperglycemic for 3 weeks and exhibited a 20% reduction of BBB glucose transport in Vmax as compared to normal. It was not known whether this may be due to increased plasma glucose or reduced plasma insulin. In adipose cells isolated from streptozotocin diabetic rats, both basal and maximally insulin-stimulated glucose transport activity were decreased. In addition, the decreased sensitivity to insulin was associated with a reduction in the number of glucose transporters in the intracellular pool (Karnieli et al., 1981).

Christensen et al. (1981) examined the effects of starvation on glucose transport across the BBB in rats. They found that BBB transport was increased 30-80% after 1 to 5 days of starvation. Pardridge and Oldendorf (1975) examined the brain uptake index for glucose and 3-O-methyl-D-[^{14}C]glucose after 2 or 8 days of starvation in rats. The glucose brain uptake index was increased 25%, but the brain uptake for 3-O-methyl-D-[^{14}C]glucose was not changed after 2 or 8 days of fasting. In addition glucose extraction was increased by 0.26-0.31. It was suggested that cerebral glycolysis was accelerated within 2 days of fasting to such an extent that the efflux of glucose was decreased. This indicated that the intracerebral glucose level approaches zero;

this is consistent with observations by Gey (1956) who showed that the intracerebral glucose level dropped to 0.44 umol/g wet weight of brain after only 24 hr of starvation in the rat. Pardridge and Oldendorf (1975) concluded that starvation increased the net uptake or gain of glucose by the brain but had no effect on unidirectional sugar influx.

Blood Flow

Hertz and Paulson (1982) investigated the effects of changes in blood flow on the BBB transport of glucose, phenylalanine, propranolol, and thiourea in awake humans. Two of the substances were studied because of their lipophilic properties, propranolol being highly permeable and thiourea less permeable. The other two substances glucose and phenylalanine were examined because they are transported by carriers. Transport across the BBB was measured in 43 young adults using the indicator dilution method under five different conditions: rest, hyperventilation with low blood flow, hypercapnia due to CO_2 inhalation with high blood flow, and hypo-and hypertension within limits of autoregulation so blood flow was normal. In situations with high cerebral blood flow (CBF), the permeability surface area product (PS) increased for both lipophilic substances and for those transported by a carrier mechanisms. Variations of PS in parallel with CBF suggested that blood flow was increased by capillary recruitment.

pH

In the study described above (Hertz and Paulson, 1982), the permeability surface area product (PS) of all substances tested except glucose decreased in hyperventilation with low blood flow. It was

suggested that the effect of hyperventilation to specifically increase the PS of glucose was related to pH. It has also been shown that glucose transport is decreased in conditions with acidosis (ammonia intoxication) (Laursen et al., 1979; Fishman and Borton, 1969), salicylate poisoning with tissue acidosis (Thurston et al., 1970) and anoxia (Betz et al. (1974). Baker and Carruthers (1984) found that in the squid giant axon, there was no effect on glucose transport when pH is altered externally, but internal acidification reduces sugar efflux.

Temperature

Baker and Carruthers (1984) examined the effects of temperature change on glucose transport in the squid giant axon. Vmax was increased with a temperature change from 0 to 14^{o}C but was decreased beyond 15^{o}C, suggesting denaturation of enzyme systems. The affinity of glucose influx increased 2.4-fold from 0 to 21^{o}C, but that for efflux decreased about 3-fold from 15^{o}C to 21^{o}C. These data suggest that as temperature is increased, the asymmetry of glucose transport is increased. The linearity of the Arrhenius plots for sugar efflux suggested that changes in glucose transport rate did not involve gross bilayer phase transition.

The effects of hypothermia on cerebral glucose levels of rats and mice were examined, and results from several studies were found to be inconsistent. Lowry et al. (1964) reported a decrease in the brain glucose levels of 10-day old hypothermic mice. Mendler (1968) showed that after rats were cooled to 4^{o}C, cerebral glucose was elevated. Similarly, Brunner et al. (1971) found that when the body temperature of mice was reduced from 34^{o}C to 25^{o}C, mouse brain glucose levels

increased by 19%. Sarajas et al. (1968) however reported that the combined effects of hypothermia and phenobarbital or ether had no effect on glucose content of rat brain. It was not determined whether these effects of hypothermia on cerebral glucose levels were related to effects on glucose transport.

Liver Metabolites

Geiger et al. (1954) found that when the isolated brain was perfused with "simplified blood" i.e. with a suspension of washed bovine erythrocytes containing albumin and glucose, the cerebral metabolic rate almost doubled but later declined, tissue glucose decreased, and the electrical activity and functional condition of the preparation deteriorated. The tissue glucose remained low despite the eventual decline in the cerebral metabolic rate or with induced hyperglycemia. Tissue glucose levels were restored with the addition of liver extracts to the perfusion blood or by including the liver in the perfusion circuit. It was concluded that one or several metabolites were necessary to ensure normal glucose transport into the brain. Geiger and Yamasaki (1956) identified these factors as the nucleosides cytidine and uridine, which when added to the perfusion fluid, maintained the perfused brain in good condition with normal carbohydrate metabolism for several hours.

Geiger and co-workers however did not measure glucose transport to the brain directly, but made inferences from tissue glucose concentrations. Gilboe et al. (1970) examined glucose transport to the perfused dog brain and found no difference between perfusion with compatible donor blood or with blood from a live donor. Thus if the

liver metabolites were required for transport, these factors must necessarily be stable in shed blood for several hours. Alternatively, it was suggested that the effects of the liver metabolites on glucose content could be explained by their effects on glucose phosphorylation (Buschiazzo et al., 1970) and may be unrelated to any effect on membrane transport.

Thus, as with insulin, most of the studies described above examined the regulation of glucose transport at the blood brain barrier. These observations have been useful in understanding the effects of various factors on glucose transfer from blood to brain, but have not provided much information on the regulation of glucose transport into neuronal and glial cells.

In summary, although the membrane transport of glucose was found to be rate-limiting in some types of neural cells, currently available in vivo and in vitro neural preparations are encumbered with technical problems that make the qualitative and quantitative analysis of transport regulation exceedingly difficult. Hence, the effects of insulin and other factors on glucose uptake in brain and nerve as yet remain to be clarified. Current evidence suggests that glucose transport serves as a point of regulation by various factors in at least some types of neural cells.

B. ADRENOMEDULLARY CHROMAFFIN CELLS

The adrenal gland is a compound structure consisting of an outer cortex which secretes steroid hormones and an inner medulla which secretes catecholamines. The two components of the organ originate from different embryonic primordia. The medulla differentiates from the neural crest cells along the sympathetic ganglia. In the mouse, the outgrowth of cells from the sympathetic nervous system into the adrenal gland occurs between the 12th and 15th day of development (McPhail and Read, 1942; Fernholm, 1971). Adrenomedullary cells secrete when stimulated by the preganglionic nerve fibers that reach the gland via the splanchnic nerves. Adrenomedullary hormones are not essential for life but they help the organism to respond to emergencies and stressful situations. The parenchyma of the adrenal medulla consists of cells which have the ability to reduce chromate salts and hence are called chromaffin cells. When isolated and grown in culture, adrenal chromaffin cells demonstrate structural and functional similarities to adrenergic neurons.

1. Neurite Outgrowth

Several studies have reported that normal or tumor chromaffin cells undergo changes in their morphology in culture, developing into neuron-like cells. Olson (1970) showed that transplantation of adrenal medullary tissue into the anterior chamber of the eye led to the formation of sympathetic like neurons. Greene and Tischler (1976) examined the effect of nerve growth factor (NGF) on PC_{12}, a clonal cell line established from a transplantable rat adrenal medullary phaeochromocytoma. When the PC_{12} cells in culture were exposed to NGF,

they stopped mitosis and extended long branching neurites. Unsicker and Chamley (1977) found that after 48 hr in culture, adrenal medullary explants prepared from 12 day old rats developed processes with varicosity-like structures. Unsicker et al. (1978) showed that the addition of NGF to cultures of dissociated rat adrenal medullary chromaffin cells caused fiber outgrowth from the chromaffin cells. The number and length of these processes which exhibit varicosities and growth cones similar to those of sympathetic neurons increased steadily from 2 days to 7 days in culture. Many of these made long-lasting contacts with other cells in the culture. Monospecific antibodies to NGF blocked this process of outgrowth from the isolated rat chromaffin cells. Aloe and Levi-Montalcini (1979) reported that pre- and postnatal injections of NGF initiated with one dose on day 17 of pregnancy and continued after birth with daily subcutaneous administration until day 10 of life, induced morphological changes in immature chromaffin cells of rat adrenal medulla.

While rat adrenal chromaffin cells and PC_{12} cell cultures require the presence of NGF for process formation, NGF is not necessary for neurite outgrowth from bovine adrenal chromaffin cell cultures, and antibodies against NGF do not affect process formation in this cell species. It was suggested that the formation of processes in bovine adrenal chromaffin cell cultures is induced by a factor or factors produced by the nonchromaffin adrenal cells present in the culture (Trifaro, 1982).

Trifaro and Lee (1980) studied the morphological changes in the chromaffin cells isolated from bovine adrenal medullae. After 8-16 hr in culture, the chromaffin cells had attached to the dish and had

started to adopt a bipolar shape. By day 1, chromaffin cells had started to develop one or more processes. The length of the processes were linearily related to the age of the culture during the first 9 days in which process length was measured. The processes which display varicosity-like structures contain catecholamines as detected by a fluorescence technique. The other morphological feature of bovine chromaffin cells is the presence of cell contacts. Body-body and process-body contacts were observed. Contacts between chromaffin cells and/or their processes and cortical cells were also observed. It is not known whether these contacts were newly developed functional synapses.

Unsicker et al. (1978) investigated the influence of glucocorticoids on NGF-induced process outgrowth by isolated rat chromaffin cells. They found that the addition of the synthetic glucocorticoid dexamethasone (10^{-5}M) to the cell culture medium abolished the NGF-mediated neurite outgrowth from chromaffin cells and markedly impaired that from cultured dissociated sympathetic ganglia. Since the glucocorticoid concentration achieved in the adrenal medulla is two orders of magnitude greater than in the general circulation (Jones et al., 1977), it has been suggested that this provides the explanation for the lack of fiber outgrowth by medullary chromaffin cells in vivo, in contrast to that seen in adrenomedullary transplants and explants (Unsicker and Chamley, 1977). Recently Soliman et al. (1985) reported that in bovine adrenal chromaffin cell cultures, 10^{-5}M dexamethasone also diminished the development of medullary cells into neuron-like structures. However, observations from several other laboratories have reported no effect of glucocorticoids on the

formation of processes by adult chromaffin cells in culture (Trifaro, personal communication).

2. Neurosecretory Function

Chromaffin cells share in common with endocrine cells the ability to store secretory products in membrane-limited organelles, the secretory granules. Scanning electron microscopy shows spherical structures of approximately 200 nm underneath the plasma membrane. These structures may correspond to the secretory granules within the varicosities and terminal cones, as indicated by catecholamine fluorescence and immunocytochemical studies. The granules store nucleotides and catecholamines at concentrations near 0.15M and 0.7M respectively, along with high concentrations of proteins and important concentrations of lipopolysaccharides, ascorbic acid and other large and small molecules and ions. Labile complexes between the nucleotides and catecholamines appear sufficient to bring the granule contents into iso-osmolarity with the cytoplasm (Trifaro, 1982; Westhead and Livett, 1983).

Isolated chromaffin cell preparations from bovine adrenal medullae contain the catecholamines adrenaline, noradrenaline, and dopamine, and the biosynthetic enzymes tyrosine hydroxylase, dopa decarboxylase, dopamine beta hydroxylase and para N-methyl transferase (Fenwick et al., 1978). Adrenal medullary chromaffin cells are capable of synthesis, storage, and release of catecholamines in a manner analogous to that described for sympathetic neurons.

Amine Uptake

Chromaffin cells in culture show the presence of an uptake mechanism for catecholamines which in many aspects resembles the neuronal uptake transport system in that it is saturable, follows Michaelis-Menten kinetics, and has a high affinity for noradrenaline. The energy source for uptake and sequestration of the catecholamines and nucleotides is a proton-pumping ATPase, much like that of the mitochondrion, providing both a pH gradient and a membrane potential. In comparison with their levels in the cytoplasm, catecholamines are concentrated 2-3 x 10^4-fold in the chromaffin vesicles (Westhead and Livett, 1983).

This uptake process is blocked by low concentrations of desipramine (10^{-8}M to 10^{-7}M) and like neuronal uptake exhibits an absolute Na^+ dependency. The kinetic parameters of this uptake system do not change considerably from day 1 or 2 in culture when neurite outgrowth is insignificant to day 7 in culture when neurites are of considerable length and exhibit varicosities. Thus this amine uptake system does not seem to develop in parallel with the outgrowth of neurite. The physiological significance of this amine uptake system is not known, but it may be a remnant property from chromaffin cell ancestors (Kenigsberg and Trifaro, 1980).

Catecholamine Release

In cultured bovine chromaffin cells, all nicotinic agonists tested (acetylcholine, nicotine, carbamylcholine) caused the release of catecholamines in a dose-dependent manner, whereas pilocarpine, a muscarinic agonist, was ineffective in inducing release.

Acetylcholine-induced catecholamine release was blocked by d-tubocurarine (ID = 5.5 a 10^{-7}M) and by hexamethonium (ID = 8 x 10^{-6}M). This suggests that the cholinergic receptors in bovine adrenal chromaffin cells which mediate the release of catecholamines are of the nicotinic type. Stimulation of chromaffin cells by either a cholinergic agonist or 56 mM KCl produced similar and parallel increases in the output of endogenous catecholamines and of the radioactive noradrenaline from cells previously loaded by incubation with 10^{-7}M [^{3}N]-noradrenaline. The ED_{50} for acetylcholine induced amine release is 2 -3 x 10^{-5}M similar to that obtained in perfused glands (Trifaro, 1982).

As with perfused adrenals, acetylcholine - or depolarization - induced release of catecholamines from cultured bovine chromaffin cells was abolished in Ca^{2+}-free medium or blocked by increasing the extracellular concentration of Mg^{2+}. Substitution of external Na^{+} by choline or sucrose, or substitution of Ca^{2+} by Ba^{2+} produced sharp increases in amine output which were blocked by Mg^{2+}. An increase in intracellular Ca^{2+} appeared to be a necessary requirement for triggering release, as is the case for exocytosis generally (Douglas, 1969; Putney, 1979).

Ca^{2+} may act through a calmodulin-dependent process, since trifluoperazine, a calmodulin inhibitor inhibited catecholamine secretion (Kenigsberg et al., 1982). Calmodulin may mediate the phosphorylation of tyrosine hydroxylase, the rate-limiting step in catecholamine synthesis. The phosphorylation of tyrosine hydroxylase has also been observed in sympathetic ganglia (Westhead and Livett,

1983). Calmodulin may also be involved in phosphorylating the myosin light chain during the activation of myosin by actin. Depolarization-induced stimulation of catecholamine release was associated with increased ^{32}P incorporation into the cell actomyosin. The presence of contractile proteins in chromaffin cells and the association of some of these proteins with the chromaffin granules help to support the hypothesis that a contractile event may be involved in the secretory process in the chromaffin cell (Trifaro, 1982).

The 47,000 dalton protein synexin may regulate the calcium-dependent molecular events underlying membrane fusion and release of hormones and neurotransmitters during exocytosis. Synexin, when activated by low concentrations of calcium, caused isolated chromaffin granules to aggregate forming pentalaminar complexes between adjacent membranes. Upon further addition of low concentrations of arachidonic acid, the vesicles aggregated, then fused into larger (10 um) sacs. Similar large sacs were found in secreting chromaffin cells as evidence of compound piggyback exocytosis. Phenothiazine drugs block both synexin and secretion from chromaffin cells (Creutz, 1981).

Two homeostatic mechanisms appear to regulate catecholamine release: (a) Acetylcholinesterase released from the chromaffin cells may modulate the activation of the cells by acetylcholine. (b) The concentration of acetylcholine may regulate catecholamine release. Low levels of acetylcholine ($<10^{-7}M$) fully activate muscarinic receptors to elevate c-GMP and thereby suppress secretion of the catecholamines (during non-stress conditions). However during rapid splanchnic nerve stimulation (i.e under stress) higher levels of Ach ($> 10^{-5}M$) activate the nicotinic receptors and secretion ensues (Carmichael, 1982).

Substance P and somatostatin inhibited catecholamine release (ID_{50} = 10^{-6}M and 2 x 10^{-5}M, respectively) stimulated by nicotinic agonists but not that in response to 56 mM KCl. This suggested that inhibition was related to an effect on the cholinergic receptor but not on exocytosis. These peptides may have the physiological function of protecting the nicotinic receptor during rapid nerve stimulation, allowing for prolonged secretory response under conditions of stress (Carmichael, 1982).

3. Metabolic Characteristics: Similarities to Brain Metabolism

Millaruelo et al. (1982a) investigated whether adrenal chromaffin cells could be used as a model in the study of energy metabolism of neural tissue. Their objective was to characterize glucose metabolism (isoenzymes and pathways) in adrenal chromaffin cells. The key glycolytic enzyme activities (except for hexokinase), in particular the isoenzyme patterns of the adrenal medulla did not differ much from the brain. However the pattern of glucose utilization was distinct. The brain oxidizes 70-80% of the glucose utilized and only small percentages go through anaerobic glycolysis or the pentose phosphate shunt. In contrast, in adrenal chromaffin cells, 80% of the glucose consumed is converted to lactate and pyruvate and only 3.5% is transformed into CO_2, and as much as 6.7% goes through the pentose phosphate shunt. Following acetylcholine stimulation, glucose utilization and oxidation is increased, probably in response to a higher energy demand in order to replenish chromaffin granules, as the effect is most clearly seen 30-60 min after stimulation. The authors concluded that adrenal chromaffin cells may be a useful model in the study of the effect of stimulation on energy metabolism.

Millaruelo et al. (1982b) also studied glycogen metabolism in bovine adrenal medulla. Total glycogen synthase activities were 452 ± 66 mU/g in whole tissue and 305 ± 108 mU/g in isolated cells. The Km of glycogen synthase for UDP-glucose was 0.67 mM with 13 mM glucose-6-phosphate and 1 mM without this effector. The in vitro inactivation process of glycogen synthase-a was found to be mainly cyclic AMP-dependent, but it also responded to Ca^{2+}. Total glycogen phosphorylase activities were 8.69 ± 1.26 U/g in whole tissue and 2.38 ± 0.30 U/g in isolated cells. During incubation of isolated adrenal chromaffin cells with 5 mM glucose, phosphorylase-a activity was increased; these changes were more marked in fasted cells. [^{14}C]glucose incorporation into glycogen in isolated adrenal chromaffin cells was increased by previous glucose deprivation (fasting). Glycogen content and glycogen synthase and phosphorylase activities were higher in the adrenal medulla than in the brain, suggesting a greater metabolic role of glycogen in the adrenal medulla.

In summary, adrenal chromaffin cells may be regarded as paraneurons or relatives of neurons on the basis of their structure, function, metabolism, and ectodermal origin (Carmichael, 1982; Trifaro, 1982; Westhead and Livett, 1983). These cells, whether freshly isolated or in culture, may provide a useful model for the study of sugar transport and its regulation in nervous tissue.

E. ROLE OF CALCIUM IN THE REGULATION OF SUGAR TRANSPORT

In 1949, Levine et al. first showed that a major action of insulin was to promote glucose uptake in muscle. Subsequent studies have shown that other physiological factors such as muscle contraction and anoxia are also capable of transport activation. Current research is centered on the molecular mechanism by which insulin and other factors activate glucose uptake. To date three major theories have been proposed to explain how sugar transport is regulated.

Based on observations that sugar transport was stimulated by anoxia and uncouplers of oxidative phosphorylation, Randle and Smith (1960) proposed that ATP served as a feedback inhibitor to limit the uptake of glucose by muscle. Although transport activity requires some minimal level of ATP for full expression (Reeves, 1975), ATP does not appear to have a direct role in the regulation of sugar transport. The contraction-induced stimulation of sugar transport was not related to increased workload (i.e. ATP splitting) in skeletal muscle (Holloszy and Narahara, 1965, 1967). The activation of sugar transport in muscle was not always associated with ATP depletion, as shown with ouabain (10^{-5}g/ml) (Bihler, 1968), hyperosmolarity (Clausen et al., 1979; Forsayeth and Gould, 1981), and insulin (Clausen, 1975). It cannot be excluded however that activation of sugar transport may be linked to a redistribution of ATP rather than a decrease in total ATP content (Gould, 1979). A permissive effect of endogenous ATP was demonstrated in the activation of sugar transport by insulin (Haring et al., 1981; Gould, 1979; Suzuki and Kono, 1980) and hyperosmolarity (Forsayeth and Gould, 1980, 1981). ATP may indirectly influence the rate of sugar transport through an effect on energy dependent Ca^{2+} transport systems

or phosphorylation-dephosphorylation reactions which may be required for the activation of sugar transport.

Based on the observation that sulfhydryl blocking reagents inhibited insulin-stimulated sugar transport in the adipocyte, Czech (1976) proposed the "thiol-redox" model for insulin action. According to this model, the action of insulin involves the oxidation of some essential sulfhydryl group(s). Oxidizing agents such as H_2O_2, diamide, and vitamin K5 activate sugar transport in the adipocyte and their action is blocked by sulfhydryl reagents. However, the effects of H_2O_2 on glucose transport may be related to the ability of H_2O_2 to release Ca^{2+} from intracellular storage sites. Sorensen et al. (1980) demonstrated that H_2O_2 produced dose-dependent stimulation of ^{45}Ca and sugar efflux from isolated rat soleus muscles and whole epididymal fat pads. H_2O_2 was also shown to increase the release of Ca^{2+} from isolated mitochondria (Lotscher et al., 1979).

A hypothesis which is currently under intense investigation involves the concept that cellular Ca^{2+} fluxes and distribution may be involved in the action of insulin and other regulators of sugar transport.

Role of Ca^{2+}

The first evidence in support of a role for Ca^{2+} in the regulation of sugar transport was provided by Holloszy and Narahara (1967). In frog sartorius muscle, contractures induced by K^+-depolarization stimulated 3OMG and ^{45}Ca influx, and this effect increased with the concentration of Ca^{2+} in the incubation medium (Holloszy and Narahara, 1967).

In several subsequent studies, the activity of sugar transport in mscle was correlated in terms of time and direction of change with various parameters reflecting a rise in cytoplasmic levels of free Ca^{2+} ions. Such a rise may result from net Ca^{2+} influx and decreased Ca^{2+} efflux across the plasma membrane, and as well from the release of Ca^{2+} from intracellular storage sites such as the sarcoplasmic reticulum, mitochondria, and inner face of the plasma membrane.

Factors which increase transport of glucose and cause corresponding increases in Ca^{2+} uptake include K^+ depolarization induced contractures (Holloszy and Narahara, 1965, 1967), anoxia, metabolic inhibitors (Bihler et al., 1977; Sorensen et al., 1980), hyperosmolarity (Clausen et al., 1970, 1979; Sorensen et al., 1980), the Ca^{2+} ionophore A23187 (Bihler et al., 1980a, 1982), and conditions which inhibit the Na^+ pump: Inhibition of the Na^+ pump by restricting energy supplies through anoxia or uncoupling of oxidative phosphorylation (Bihler, 1972; Bihler et al., 1977) or by high concentrations of ouabain (10^{-5}g/ml) (Bihler, 1968; Bihler and Sawh, 1979) leads to increased levels of internal Na^+ and thus shifts Na^+/Ca^{2+} exchange towards net Ca^{2+} uptake (Baker, 1970; Reuter, 1974; Blaustein, 1974) and the stimulation of sugar transport (Bihler and

Sawh, 1971a, 1971b). Increased Na^+ influx elicited by the ionophore monensin had the same effects (Bihler et al., 1985). Some regulators of sugar transport such as insulin, lose their stimulatory effects if the extracellular medium lacks Ca^{2+} (Bihler and Sawh, 1977, 1980), or contains Ca^{2+} chelators (Bihler and Sawh, 1977, 1980; Haring et al., 1981; Yu et al., 1980), heavy metal antagonists (Bihler and Sawh, 1970; Bihler et al., 1980) or substances like D600 which inhibit Ca^{2+} influx (Bihler and Sawh, 1977, 1980).

The release of Ca^{2+} from intracellular stores may also contribute to an increase in the cytoplasmic level of free Ca^{2+} ion. Measurements of ^{45}Ca efflux indicate the availability of cytosolic Ca^{2+} for efflux (Ashley et al., 1972). If external Ca^{2+} is present and sarcolemmal Ca^{2+} influx is increased, then Ca^{2+} efflux will also be increased. In nominally Ca^{2+}-free medium, there should be minimal sarcolemmal Ca^{2+} influx and any rise in Ca^{2+} efflux reflecting a rise in cytosolic Ca^{2+} should be largely due to release of Ca^{2+} from intracellular stores. ^{45}Ca efflux studies can thus demonstrate the ability of some regulators of sugar transport to release intracellular stores of Ca^{2+}. This would contribute to a rise in cytoplasmic Ca^{2+} levels, and subsequently net efflux of Ca^{2+} across the sarcolemma.

In soleus muscle, Ca^{2+} may be released from the sarcoplasmic reticulum by electrical stimulation, K^+ depolarization, caffeine or veratrine. In addition, 2,4-DNP, H_2O_2, salicylate, and cyanide may induce a rapid loss from the mitochondria. In all instances there is a rapid rise in the efflux of ^{45}Ca preceding or coinciding with the activation of sugar efflux (Sorensen et al., 1980). Vanadate inhibits the Ca^{2+}-activated ATPase of the sarcolemma (Caroni and Carafoli, 1980)

and sarcoplasmic reticulum (Wang et al., 1979), and thus interferes with Ca^{2+} clearance from the cytoplasm. In whole epididymal fat pads, isolated fat cells, extensor digitorum longus and soleus muscles of the rat, vanadate stimulated the efflux of sugar an effect preceded by an increase in ^{45}Ca efflux (Clausen et al., 1981).

Anoxia and metabolic inhibitors cause, through a decrease in energy production, a depression of the active Ca^{2+} uptake system in the mitochondrial membranes (electrophoretic uniporter), and in sarcoplasmic reticulum (Ca^{2+}-ATPase), and consequently a net release of Ca^{2+} from intracellular storage sites. Catecholamine-induced lipolytic activity may result in toxic levels of free fatty acids, which uncouple oxidative phosphorylation, decrease energy reserves (Bihler and Sawh, 1976, 1978) and thereby release intracellular stores of Ca^{2+}.

Conversely, inhibition of sugar transport by free fatty acid oxidation (Randle et al., 1964; Neely et al., 1969; Bihler, 1972) may be related to an increase in cellular energy charge, and subsequently activation of energy dependent Ca^{2+} transport systems in the membranes of the mitochodria, sarcoplasmic reticulum, and plasma membranes, leading to decreased cytoplasmic Ca^{2+} levels (Bihler 1972, 1980).

The ability of insulin to increase Ca^{2+} efflux from muscle preparations (Clausen and Martin, 1977) and further increase in hyperosmolarity-induced tension (Clausen et al., 1974) suggests that insulin may also cause the release of intracellular stores of Ca^{2+}. Studies have shown that insulin can directly alter specific cellular Ca^{2+} pools. Insulin (1.5 mU/ml) diminished the binding of Ca^{2+} to isolated liver membranes (Marinetti et al., 1972) and to artificial

lipid membranes (Kafka and Pak, 1969). Ulrich and Zierler (1985) studied the effect of insulin on Ca^{2+} binding to rat fat cell plasma membrane proteins, solubilized into fractions using 3-[(3-cholamidopropyl)-dimethylammonio] 1-propane-sulfonate (CHAPS), a nondenaturing zwitterionic detergent. They found that insulin-treated fractions consistently bound more Ca^{2+} than control fractions or vice versa. It was suggested that insulin may alter Ca^{2+}-binding properties of plasma membrane proteins and thereby cause relocation of Ca^{2+} which might serve as a transduction signal.

Pershadsingh and McDonald (1979) have shown that insulin inhibits the high affinity Ca^{2+} extrusion pump enzyme, (Ca^{2+} + Mg^{2+})-ATPase in the plasma membrane. They have proposed the following model of insulin action (Pershadsingh and McDonald, 1984): The insulin receptor or one of its subunits is a calmodulin binding protein, and is structurally related to the (Ca^{2+} + Mg^{2+})-ATPase/Ca^{2+} transport complex which in the active form is bound to calmodulin. The binding of insulin to its receptor increases the affinity of the insulin receptor or one of its subunits for calmodulin, causing a competitive dissociation of calmodulin from the (Ca^{2+} + Mg^{2+})-ATPase/Ca^{2+} transport complex. This results in the conversion of the highly active holoenzyme to the less active apoenzyme, leading to a decrease in Ca^{2+} extrusion and an increase in Ca^{2+} concentration in critical microenvironments of the plasma membrane. The increase in cytosolic Ca^{2+} produced by inhibition of the Ca^{2+}ATPase would not only activate Ca^{2+}-dependent target systems but also serve as a negative feedback mechanism. The Ca^{2+}-ATPase would be re-activated by: (1) calmodulin in the presence of high cytosolic Ca^{2+} and (2) the insulin-generated supernatant factor (Jarett et. al., 1980; Kiechle et al., 1981).

In this model, the rise in cytosolic Ca^{2+} levels results in increased binding of Ca^{2+} to the plasma membrane and possibly in Ca^{2+}-dependent gating of K^{+}, explaining the hyperpolarization caused by insulin. Both the increased membrane-bound Ca^{2+} and/or the resultant hyperpolarization may alter membrane viscosity and cause a reorganization of membrane dipoles, resulting in changes in permeability properties and stimulation of organic solute transport systems (Pilch et al., 1980). Zierler and Rogus (1980) have demonstrated that hyperpolarization of only 1.5 mV enhanced glucose transport by 40% in the caudofemoralis muscle of the rat.

Thus there is much evidence, albeit circumstantial, linking changes in Ca^{2+} distribution with changes in the activity of the sugar transport system. This has led to the hypothesis that an increased level of cytosolic Ca^{2+} or increased availability of Ca^{2+} for binding to a specific regulatory site may be instrumental in activating the sugar transport mechanism or process (Elbrink and Bihler, 1975; Clausen, 1980).

However, activation of the sugar transport system may not be directly related to the cytoplasmic concentration of free Ca^{2+}. Glucose transport in frog sartorius muscle remained activated several hours in the cold after contractile activity had ceased and a low cytoplasmic level of free Ca^{2+} had been re-established (Holloszy and Narahara, 1965). In the perifused left atrium of the rat, the contraction-stimulated increase in 3OMG transport persisted for more than 15 min at $37^{o}C$ after contraction had ceased (Bihler and Sawh, 1975). The contraction-stimulated activity of the sugar transport

system also remained enhanced for more than an hour in soleus and extensor digitorum muscles, but not in vas deferens smooth muscle: this memory effect was also demonstrated in skeletal muscle after exposure to hyperosmolar medium (Elbrink and Phipps, 1980). These data suggest that transport regulation may not be directly related to the average free cytosolic Ca^{2+} concentration, but may depend on a membrane-associated regulatory Ca^{2+} binding site in contact with the cytosolic free Ca^{2+} pool.

This hypothetical membrane associated Ca^{2+} binding site may have a general effect on the organization of the plasma membrane, such as the organization of membrane lipids, or it may cause other conformational changes leading to greater mobility or activity of the sugar transport system (Bihler and Sawh, 1975; Elbrink and Phipps, 1980). Alternatively, it may be an allosteric site on the carrier protein (Clausen, 1975; Elbrink and Phipps, 1980). Another perhaps more likely possibility is that Ca^{2+} may be involved in a more indirect manner such as the translocation of new glucose carriers from the intracellular sites to the cell membrane.

The translocation hypothesis, a recently developed, though much favored concept postulates a molecular mechanism whereby insulin increases the maximal capacity of glucose transport. This was based on studies examining the effect of insulin on the cellular distribution of glucose transporters as determined from two experimental approaches. In the study by Suzuki and Kono (1980), subcellular structures of fat cells were fractionated by differential and sucrose gradient centrifugations. The fractionated subcellular structures were solubilized with sodium cholate, and the transport activities

reconstituted into egg lecithin liposomes by sonication, freezing-and-thawing and a second brief sonication. The glucose transport activity in the reconstituted liposomes was estimated by measuring the uptake of D-[^{3}H]glucose in the presence or absence of CB. The difference in glucose uptake rates in the presence and absence of CB would give the carrier-mediated transport activity. Cushman and Wardzala (1980) prepared the plasma membrane and microsomal fractions of fat cells by differential centrifugation and subjected them to the assay for the glucose-inhibitable cytochalasin B-binding capacity. In both studies, insulin increased the number of glucose transporters in the plasma membrane with a concommitant decrease in glucose transpoters in the microsomal fraction, as compared with controls (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). It was concluded that insulin stimulated glucose transport in fat cells by facilitating translocation of glucose transporters to the plasma membrane from an intracellular storage site. Subsequent studies have shown that other factors including pH and diabetes also affect the translocation of glucose carriers (Karnieli et al., 1981, 1985; Toyoda and Kono, 1985; Armani et al., 1985).

The translocation hypothesis may be compatible with the requirement for ATP or metabolic energy and Ca^{2+} for insulin action. Haring et al. (1981) suggested that the initial lag phase of insulin action reflects an unknown process, possibly translocation that couples the binding of insulin to the activation of sugar transport. They showed that coupling was ATP dependent whereas the initial binding of insulin to its receptor and sugar transport itself were ATP independent. Ca^{2+} depletion slowed coupling and decreased the maximal

response, suggesting that Ca^{2+} may participate or act as the second messenger in the coupling process. In a model proposed by Haring et al. (1981), the insulin-occupied receptor produces a signal involving Ca^{2+} that triggers phosphorylation in the microtubular system which initiates translocation of carrier containing vesicles. Ca^{2+} could also be involved in the fusion of the vesicular membrane with the plasma membrane. Other phosphorylation-dephosphorylation modulated changes in the activity of the glucose transport protein may also depend on Ca^{2+}.

Evidence supporting a role for Ca^{2+} in the activation of sugar transport has been largely indirect. The rapid turnover time of the cytosolic pool of free Ca^{2+} and the heterogeneous cellular distribution of Ca^{2+} has made it difficult to obtain more direct evidence. The development of more sensitive techniques including intracellular fluorescent Ca^{2+} indicators (Tsien, 1981; Carvalho, 1978) for detection of transient changes in cellular Ca^{2+} distribution promises a more direct approach to defining the role of Ca^{2+} in the regulation of sugar transport.

Integration of Cellular Function and Metabolism: Role of Ca^{2+}

In tissues such as muscle and fat, where glucose transport is rate-limiting for its utilization, energy requirements vary widely in response to different cell functions including muscle contraction, synthesis and storage of metabolic substrates, and supply of substrate to anaerobic glycolysis. Although these cellular functions vary widely, they share in common the ability to stimulate the glucose transport system. It seems appropriate with respect to economy of cellular energy

that these regulators of sugar transport share at least some aspect of a common mechanism underlying transport regulation. Evidence described above suggests that Ca^{2+} may serve as a common mediator for transport regulation (Bihler, 1972; Elbrink and Bihler, 1975).

In addition to sugar transport, several other cell functions are also associated with changes in cellular Ca^{2+} distribution. These include membrane events (e.g. cell adhesion, platelet aggregation, stimulus-response coupling), contractile mechanisms (e.g. contraction of muscle myofibrils, microtubule/microfilament-associated events such as cell division), secretory mechanisms (e.g. intracellular transport and discharge of neurotransmitters, hormones from endocrine cells, and proteins and mucus from exocrine cells), activation of enzyme systems (e.g. pyruvate dehydogenase kinase, pyruvate dehydrogenase phosphatase, isocitrate dehydrogenase) and others (e.g. photoreception, bioluminescence, activation of egg at fertilization) (for review, see Case, 1980).

It may be speculated that, in a wider sense, Ca^{2+} may provide a link between cellular metabolism and cellular function. For example, in secretory cells, the rise in cytoplasmic Ca^{2+} which elicits secretion may simultaneously activate the sugar transport system, and thereby provide more energy-yielding substrate in response to increased metabolic demand. As described earlier, Millaruelo et al. (1982a) found that in response to acetylcholine stimulation of catecholamine secretion, glucose utilization and oxidation was stimulated. Hence the adrenal chromaffin cell may serve as a useful model for the study of the role of Ca^{2+} in the relation of cellular metabolism (glucose transport) and cellular function (catecholamine secretion).

E. STATEMENT OF THE PROBLEM

The transport of glucose in most animal cells is mediated by a specific and energy-independent equilibrating process. In muscle and adipose tissue, the transport step is rate-limiting for overall glucose utilization and is subject to modulation by metabolic, hormonal and pharmacological factors. In mature human erythrocytes and in the liver, glucose transport is not subject to regulation and is faster than glucose utilization (for review, see Elbrink and Bihler, 1975; Clausen, 1975).

Although glucose is the major metabolic substrate of brain and nerve (Gibbs et al., 1942), the regulatory nature of glucose transport into neuronal cells has not been defined. This is largely due to technical difficulties (Lund-Andersen, 1979). The brain consists of a heterogeneous population of cells, each type of which may differ in their sugar transport systems. The blood-brain barrier prevents the direct measurement of glucose transport into the cells, and data in vivo or in perfused brain preparations tend to reflect the transport characteristics of capillary endothelial cells rather than of neuronal and glial cells. The glucose level in the brain is difficult to measure because of the very rapid metabolism and the inability to accurately determine the brain extracellular space. Brain slices have the disadvantages of a slowly equilibrating extracellular space, cell heterogeneity, and the presence of many damaged cells. Whole peripheral nerve preparations consist of a perineurial membrane barrier in addition to a heterogeneous population of cells, which may differ in their metabolic characteristics. Synaptosomes consist of a highly specialized region of the neuron, the synapse, which may not be

representative of the rest of the neuronal plasma membrane. The use of cultured neuronal and glial cells excludes these complicating factors, but it remains to be established whether these secondary neural cell cultures are truly representative of normal cells in vivo with respect to transport and metabolism (Kalckar, 1976). Thus, currently available preparations have not been fully adequate for providing consistent data for determining the regulatory nature of glucose transport into neuronal and glial cells.

Chromaffin cells isolated from the adrenal medulla share in common with adrenergic neurons their ectodermal origin and some morphological (neurite outgrowth) and functional (amine uptake, stimulus-secretion coupling) characteristics. Primary cultures of adrenal chromaffin cells have the advantage over "primary" explant cultures of adult and fetal sympathetic tissues, in that they can be harvested in abundance and with relatively good homogeneity of cell type (Livett, 1984). Adrenomedullary cells may thus serve as a model of a homogeneous neuronal cell population of nonmalignant origin. These cells, whether freshly isolated or in short term culture may provide a useful system for the study of sugar transport regulation in at least some types of neural cells. Bovine adrenal chromaffin cells undergo changes in their morphology in culture, developing into neuron-like structures; hence, chromaffin cell cultures may also be useful to study changes in the glucose transport system during neuronal cell development.

The objectives of this study were to determine whether the adrenal chromaffin cell could serve as a model for the study of sugar transport regulation in neuronal cells; to compare the regulatory nature of glucose uptake in chromaffin cells with that in other cell types; and to determine whether the characteristics of cultured cells are comparable to those of isolated cells. The experimental approach consists of three steps:

(1) to characterize the sugar transport system in freshly isolated bovine adrenal chromaffin cells using the nonmetabolized glucose analogue, 3-O-methyl-D-glucose (3OMG);

(2) to investigate the regulatory nature of 3OMG transport in freshly isolated chromaffin cells with respect to:

   a) the effect of insulin,

   b) the effect of other factors known to stimulate sugar transport in muscle and adipose tissue (metabolic inhibiton, Na^+ pump inhibition, hyperosmolarity),

   c) the effect of secretory stimuli (acetylcholine, carbamylcholine, K^+-depolarization),

   d) and the dependence of the sugar transport system on external Ca^{2+}, effects of regulators on Ca^{2+} fluxes; and

(3) to investigate the effects of culturing and the synthetic glucocorticoid dexamethasone, which inhibits process outgrowth, on the characteristics and regulatory nature of 3OMG uptake.

SECTION II

METHODS

A. EXPERIMENTS WITH FRESHLY ISOLATED CELLS

1. Isolation of Bovine Adrenomedullary Chromaffin Cells

(i) Materials

Fresh bovine adrenal glands were obtained from the local slaughterhouse, and transported in ice cold standard medium (see below) saturated with 95%O_2:5%CO_2. The time for transit between the slaughterhouse and the lab was approximately 20-30 min.

DNase I, Type III (deoxyribonucleate 5'-oligonucleotidohydrolase EC 3.1.21.1), collagenase Type 1S (clostridiopeptidase A: EC 3.4.24.3), glutamine, carnitine, bovine serum albumin, pyruvate, and cytochalasin B were obtained from Sigma Chemical Co. (St. Louis, MO.); 50 X concentrated solution of amino acids without L-glutamine from GIBCO (Grand Island, N.Y.); and insulin from Connaught - Novo (Willowdale, Ontario). Collagenase Type III was purchased from Worthington Biochemicals (Freehold, N.J.). Radiolabelled compounds : 3-O-[methyl-^{14}C]-methyl-D-glucose (20-55 mCi/mmol), $^{45}CaCl_2$ (4-50 Ci/g Ca), L-[1-^{3}H(N)]-glucose (10-20 Ci/mmol), and [^{3}H(G)]-Inulin (100-500 mCi/g) were from New England Nuclear (Boston, MA). All other reagents were of the highest commercial quality.

The standard medium used for transporting, dissection, and perfusion of the adrenal glands was Joklik tissue culture medium, modified as described (Bihler et al., 1984). It contained 120 mM NaCl, 23.8 mM $NaHCO_3$, 9.6 mM NaH_2PO_4, 5.36 mM KCl, and 10 mg/L phenol red, 8.0 mM glutamic acid, 2.5 mM glutamine, 2.0 mM carnitine, 3.4 mM $MgCl_2$, 15 mM D-glucose, 13 amino acids at plasma concentrations (as a 50 X concentrated amino acid solution) and 0.1% bovine serum albumin, and

was nominally Ca^{2+}-free. Following equilibration of the solution with $95\%O_2:5\%CO_2$ the pH of the final solution was normally within the range of 7.0 to 7.2 (at $37^{\circ}C$). Modifications of this standard medium are noted in the text. Unless otherwise stated, all procedures were carried out at $37^{\circ}C$ and under $95\%O_2:5\%CO_2$. This and higher ($100\%O_2$) oxygen tensions are commonly used with preparations of freshly isolated adrenal chromaffin cells (Hochman and Perlman, 1976; Fenwick et al., 1978; Pocock, 1983a, b).

(ii) Experimental Procedure

The procedure for isolation of bovine adrenomedullary chromaffin cells was based on the method of Fenwick et al. (1978). Dissection of the adrenal glands was carried out at $0-4^{\circ}C$ in standard medium. The kidney-shaped, left adrenal gland was used preferably because it had a single vein. Adrenal glands were trimmed clean of fat and connective tissue, and slit along the periphery only to the surface of the medulla. The gland was then rinsed of blood by slowly flushing buffer through the central lobular vein using a 10 ml plastic syringe. While flushing buffer through the gland, the cortex was checked for surface openings due to cuts or small surface blood vessels. Surface leaks were sewn closed to ensure thorough perfusion via the central lobular vein and drainage only via the slit periphery. A glass cannula was inserted into the central lobular vein and secured with a thread. Buffer was flushed through the cannula to check for proper drainage. The cannula was attached to a Langendorff-type perfusion apparatus, modified for simultaneous perfusion of three glands.

Initially the glands were perfused with standard medium at 37°C for 5 min at a rate of 10 ml/min for each gland in an open-circuit system to wash out blood. This was followed by a 60 min closed-circuit perfusion with the standard medium containing 0.05% collagenase (Type 1S, Sigma Chemical Co. or Type III, Worthington Biochemicals), 1.33 mg/100 ml DNase I Type III (Trifaro et al., 1978), 50 uM $CaCl_2$, and 1 % albumin. Collagenase is the most widely used enzyme for isolating single chromaffin cells. DNase I is included in the digestion in order to minimize the chance of aggregation and clumping with consequent loss of cells that otherwise occurs when some DNA spills out of damaged cells during collagenase digestion. Enough Ca^{2+} was added to stimulate collagenase activity, but not enough to promote cell aggregation during the digestion process (Livett, 1984).

The medulla is more easily digested than the cortex, and after perfusion with the disaggregating enzymes, the yellowish medullary portion of the adrenal gland was flaccid and was easily dissected free of the cortex. The medullae were minced into pieces of tissue not thicker than 1 mm, pooled together and incubated with shaking for 20 min in fresh enzyme solution modified to contain 30 mM K^+ and 119 mM Na^+ (Bihler et al., 1984). During this incubation period, enzymatic disaggregation was enhanced by drawing the suspension through plastic pippettes of progressively decreasing bore.

The crude medullary suspension was filtered through 120 um nylon mesh to remove undigested tissue and cellular debris. The cells were then washed by centrifugation at low speed, which permitted separation of cellular debris and and the lighter RBC's: The cells were washed first in fresh buffer containing 2% albumin by centrifugation at

117 x g for 5 min in a Sorvall GLC-1 tabletop centrifuge. The supernatant was suctioned off, and the cells washed a second time in fresh buffer containing 1% albumin by centrifugation at 66-90 x g for 5 min. The cell suspension became progressively lighter in color.

To remove the filmy white collagen, the cells were suspended in buffer containing 1% albumin and incubated for 10 min. During this incubation period, collagen precipitated to the bottom of the tube while the cells remained in suspension. The cell suspension was then filtered through 120 um nylon mesh to remove the filmy, white collagen. The filtrate was then washed for a final time by centrifugation at 7 x g for 5 min, and suspended in incubation or culture medium.

Two different methods have been used to purify chromaffin cells from the initial digestion: 1) isopycnic density-gradient centrifugation and 2) differential plating (Livett, 1984). In the present study, the cell suspension was initially purified using a self-generating Percoll gradient. Because the cell yield was decreased considerably to amounts which were not sufficient for transport assays, this purification step was omitted.

2. Assessment of Structural and Functional Integrity

(i) Cell Yield

The cell yield was measured routinely by standard hemocytometry using a Fuchs-Rosenthal ULTRAPLANE chamber (C.A. Hausser and Son, Max Levy). At least 100 or more cells were counted under a light microscope (Wild - Herbrugg, Switzerland). The yield (cells/ml cell suspension) was calculated from the following formula:

$$\frac{\text{number of chromaffin cells} \times \text{dilution factor (25)} \times 10^4}{\text{number of 1 square mm area counted}}$$

(ii) Cell Viability

Assay methods to determine cell viability have been grouped into six broad categories: (1) survival and growth in tissue culture, (2) membrane integrity, (3) metabolite incorporation, (4) enzyme spectrum, (5) transplantation potential, and (6) structural alteration, chemical composition, and electroconductivity (Patterson, 1979). For routine purposes "dye exclusion" tests are preferred. Schrek (1936) was the first to suggest that an intact membrane was necessary for the exclusion of certain dyes. The most common procedure, also used in the present study, is that with trypan blue and visual count of the unstained "live" cells (Tennant, 1964; Sawicki et al., 1967; Patterson, 1979). An aliquot of diluted cell suspension was mixed with an equal volume of 0.4% trypan blue in the incubation medium, and the number of stained and unstained cells counted by standard hemocytometry as described above. The percentage of cells that was not stained with trypan blue was taken as a measure of cell viability.

(iii) Homogeneity

Stuart et al. (1974) developed a vital dye technique that selectively stained specific cells in leech ganglia without damaging them. The stained neurons were those which have been shown by other techniques to contain catecholamines or 5-hydroxytryptamine, substances that could be the neurotransmitter or neurohormones secreted by these cells. In this study, an aliquot of the diluted chromaffin cell suspension (see above) was mixed with an equal volume of neutral red solution (0.3 mg neutral red per ml 0.9% NaCl/5 mM Tris-HCl, pH 7.4) (Wilson and Viveros, 1981; Role and Perlman, 1980), and the number of stained cells counted by standard hemocytometry, as described above. Homogeneity was estimated from the percentage of the total number of cells stained with neutral red.

Another method of assessing chromaffin cell purity is measurement of total catecholamines, expressed per mg of cell protein. If the chromaffin cell preparation is contaminated by large-sized fibroblasts, the ratio of 200-250 nmol catecholamines/mg protein will be decreased. The catecholamine content of freshly isolated chromaffin cells has been well documented in several studies using the same method of cell isolation (without the Percoll gradient purification step) as used in the present study. The purity of chromaffin cell preparations measured in these studies (Hochman and Perlman, 1976; Fenwick et al., 1978; Pocock, 1983a, b; Kao and Schneider, 1985) was similar to the purity as measured using neutral red in the present study. Hence, it did not seem necessary to measure total catecholamine content of freshly isolated bovine adrenal chromaffin cells in the present study.

(iv) Functional Integrity

Samples of the cell suspension were taken approximately 15 min after cell isolation for determination of cellular Na^+, K^+, and ATP (Refer to Section II. A. 4 - 6).

3. 3-O-methyl-D-glucose and ^{45}Ca Uptake

(i) Materials

The incubation medium consisted of the standard medium described above with the addition of 1% bovine serum albumin and 5 mM pyruvate, but no glucose. In experiments using La^{3+}, the standard medium was buffered with 25 mM triethanolamine.HCl instead of $NaHCO_3$ and NaH_2PO_4, and gassed with 100% O_2 in order to avoid precipitation of La^{3+}. Na^+ was replaced with LiCl or mannitol in Na^+-free medium and with mannitol in low (89 mM) Na^+ medium to maintain iso-osmolarity. The incubation medium was made hyperosmolar with the addition of 100 mM mannitol. In K^+-free medium, NaCl was increased by 5.4 mM to compensate for the loss of KCl.

The nonmetabolizable glucose analogue, 3-O-methyl-D-glucose (3OMG) has been used to trace sugar transport (Csaky and Wilson, 1956) because: (1) The kinetic characteristics of 3OMG closely resembles those of glucose as shown in frog skeletal muscle (Narahara and Ozand, 1963) and isolated perfused rat heart (Morgan and Park, 1958); (2) It was generally accepted that 3OMG was not utilized by the cell and hence metabolic breakdown would not interfere with calculations of transport rate. Gatley et al. (1984) recently showed that 3OMG could undergo phosphorylation and dephosphorylation in the perfused rat heart. However 3OMG is a poor substrate for the hexokinase, and the phosphorylation of 3OMG is slow. Hence these observations may not be applicable to the present study, in which incubation with 3OMG is relatively short (2.5 min) in comparison with the longer incubation period in the perfused rat heart (>17 min). (3) The assumption that the

membrane transport of glucose is rate-limiting for glucose consumption is not necessary when using 3OMG as is the case with another commonly used glucose analogue, 2-deoxyglucose (2DG). After 2DG is transported across the cell membrane, it is phosphorylated to 2-deoxyglucose 6-phosphate which is neither metabolized further nor transported through the cell membrane. In order to fully attribute uptake of 2DG to unidirectional transport of the tracer, all 2DG that is transported must be immediately phosphorylated within the cell. For this to occur the rate of phosphorylation must be faster than the rate of membrane transport i.e. glucose transport must be rate-limiting for its utilization (Lund-Andersen, 1979). It is not known whether the membrane transport of glucose is the rate-limiting step in the utilization of glucose by adrenal chromaffin cells. Thus sugar transport was measured in adrenal chromaffin cells by following the cell/medium distribution of the nonmetabolized sugar analogue 3OMG.

The polysaccharide inulin, which equilibrates within the extracellular space but does not penetrate the cell membrane, is widely used for determination of the extracellular fluid volume (Clarkson and Toole, 1964). The suitability of radiolabelled inulin for the determination of extracellular fluid has been questioned. It was found that some commercially available lots of inulin labelled with ^{14}C or ^{3}H differed from others and could provide inconsistent and sometimes incorrect estimates of extracellular fluid volume. For example, unstable forms of labelled inulin may undergo hydrolysis to smaller molecules or monomers, which may enter the intracellular compartment causing an overestimate of the extracellular fluid volume. The suitability of a given lot of [^{3}H]-inulin can be assessed by

determining if the apparent inulin distribution space is time dependent and by comparing the values obtained with different [^{3}H]- and [^{14}C]-inulin preparations or other reliable extracellular markers (Dinda et al., 1975). On the basis of earlier experience in this laboratory, generally labelled ^{3}H-inulin was used for the determination of the extracellular fluid volume in the adrenal chromaffin cells. Several different lots of ^{3}H-[G]-inulin were used, and the extracellular space was consistent at 7.4 $\pm$ 0.2 (78)% of the total water space in the cell pellet centrifuged through the organic layer.

Morgan et al. (1964) measured the apparent volume of distribution (space) of L-glucose in perfused rat hearts. Since L-glucose is not phosphorylated by hexokinase, a volume of distribution greater than the extracellular space would indicate that L-glucose was transported across the cell membrane. However the apparent volume of distibution of L-glucose was not significantly different from that of sorbitol, an extracellular marker. It was concluded that the membrane was impermeable to L-glucose. Experiments with ^{14}C-labelled D- and L-glucose in bovine mesenteric arteries and smooth muscle from rabbit colon led to the same conclusion (Arnqvist, 1972). On the other hand the distribution of D-glucose exceeded that of L-glucose and continued to increase. These observations indicated that the membrane permeability in these smooth muscle preparations showed substrate stereospecificity for monosaccharides.

Although L-glucose is not transported by the specific facilitated transport system, L-glucose may enter the cell via passive diffusion. Tritiated L-glucose was used to determine the passive diffusion component of 3OMG transport and this value was subtracted

from the total transport, as measured by 3OMG, to yield the true value for specific facilitated transport.

(ii) Experimental Procedure

The cell suspensions were preincubated in the presence and absence of test factors for 15-30 min at 37°C under constant oxygenation in a shaking water bath. Aliquots of the cell suspension were taken for determinations of wet and dry weights of the cell pellet. These data are needed in the calculation of cell water and total water volume (See below).

The preincubation period was followed by incubation for 2.5 min (3OMG transport) or 1.5 min (^{45}Ca uptake) in the presence of ^{14}C-labelled nonmetabolized sugar analogue, 3OMG (0.25 uCi/ml, total concentration 2.0 mM) or $^{45}CaCl_2$ (0.25 uCi/ml), and G-[^{3}H]-labelled extracellular marker, inulin (2.5 uCi/ml). These incubation times were chosen on the basis of time curves for 3OMG (Figure 1) and ^{45}Ca uptake (Figure 15). The reaction was terminated by 4-fold dilution of 0.2 ml aliquots of the cell suspension with ice cold stopping solution in the presence (3OMG transport) or absence (^{45}Ca uptake) of 20 uM cytochalasin B. For Na^+ and K^+ determinations, the reaction was terminated in solution maintained at room temperature and lacking cytochalasin B. Cells were separated from the medium by centrifugation through a layer of 1:3 dibutyl phthalate:n-octyl phthalate at 12,000 x g for 2 min in an Eppendorf Model 5412 Microcentrifuge (Brinkman Instruments, Inc., Toronto, Ontario).

The layers of medium and oil were discarded, and the tube carefully wiped dry. The cells were treated with 100 ul 5%

trichloroacetic acid (TCA) and centrifuged at 12,000 x g for 30 secs to obtain a cell extract. The rest of the cell suspension was centrifuged at approximately 700 x g for 5 min and aliquots of the supernatant taken for determination of radioactivity in the medium. The aliquots of incubation medium and cell extract were added to liquid scintillation cocktail.

(iii) Analysis / Calculations

Isotopes were measured by double label liquid scintillation counting. The instrument used for analysis was a Beckman LS-250 Liquid Scintillation System (Beckman Instruments, Inc). The instrument was set up for double label counting so that one channel counted all ^{3}H (with some ^{14}C or ^{45}Ca overflow) and the other counted only ^{45}Ca or ^{14}C, excluding all ^{3}H. To obtain the true amount of ^{3}H in the ^{3}H channel, the percent overflow from the ^{14}C or ^{45}Ca channel into the ^{3}H channel was determined using ^{14}C or ^{45}Ca standards. The cpm (counts per minute) in each channel was corrected for background counts and quenching, and the results expressed for each isotope as dpm (disintegrations per minute). To correct for quenching, the dpm were calculated for each isotope by the method of simultaneous equations, using three quench curves (^{3}H, ^{14}C or ^{45}Ca, and ^{14}C or ^{45}Ca overflow into the ^{3}H channel). Efficiency for counting as determined by internal standards was around 75% for ^{14}C, 63% for ^{45}Ca, and 25% for ^{3}H.

Sugar transport and ^{45}Ca uptake were expressed as percent equilibration, i.e. the concentration in the intracellular water was expressed as a percentage of the final concentration in the medium (Bihler and Sawh, 1973). The following calculations were done to derive these values:

A. Medium dpm/ml (^{14}C or ^{3}H) =

medium dpm/vial (^{14}C or ^{3}H) x 40 (dilution factor)

B. Cell dpm/ml cell water (^{14}C or ^{3}H) =

cell dpm/vial (^{14}C or ^{3}H) x 20 (dilution factor) x CW/TWV

Cell water (CW) is the product of the wet weight (g) of the cell pellet and the fractional water content determined separately. Total water volume (TWV) is the sum of cell water and 100 ul of 5% TCA added for deproteinization (see above). For determination of water content, aliquots of the cell suspension were dried in vacuo to constant weight. From the wet weight (X), dry weight (Y), and loss in weight (X - Y) the fraction of cell water was determined: (X - Y) / Y, and used as the fraction of water in the total cell weight.

C. The counts were expressed as a percentage of the concentration in the medium:

1. Virtual Distribution Space (VS) =

[Cell dpm/ml (^{14}C) / medium dpm/ml (^{14}C)] x 100

2. Extracellular Space (ECS) =

[Cell dpm/ml (^{3}H) / medium dpm/ml (^{3}H)] x 100

3. % equilibration of ^{14}C in the intracellular water space =

$$\frac{(VS - ECS)}{(1 - ECS)}$$

For the ^{45}Ca uptake experiments, dpm/ml of ^{45}Ca in the cells and medium were calculated instead of those for ^{14}C.

Data were presented as rates (nanomol/mg protein/min) of 3OMG or ^{45}Ca uptake =

$$\frac{\text{percent equilibration} \times \text{substrate concentration in medium (mM)} \times 1000}{\text{incubation time (min)} \times \text{mg protein/ml intracellular water}}$$

As there was a fairly large variability in basal transport rates between experiments performed on separate pools of cells (Table 1), the data were normalized in some instances by expressing rates of sugar transport as percentages of the control value in the same pool of cells.

4. ^{45}Ca Efflux

(i) Materials

The incubation medium was similar to that used in 3OMG transport and ^{45}Ca uptake experiments [II.A.3.i]. All incubations were carried out at 37^{o}C under constant oxygenation.

(ii) Experimental Procedures

The procedure for measurement of ^{45}Ca efflux from freshly isolated bovine adrenal chromaffin cells was based on the method of Pocock (1983b). The cells were incubated in standard medium containing 1.25 mM Ca^{2+} with the addition of 20 uCi/ml $^{45}CaCl_2$ for 75 min in a shaking water bath at 37^{o}. The cells were then washed 3 times by centrifugation at 117 x g for 3 min in ice cold medium. After washing, the cells were suspended in Ca^{2+}-containing or Ca^{2+}-free medium adjusted for treatment or control conditions, and incubated in a shaking water bath. Aliquots (200 ul) of the cell suspension were obtained at 0, 10, 20, 30, 40, and 50 min from the start of the incubation period. ^{45}Ca efflux was terminated and the cells treated and analysed for radioactivity as described above [Section II.A.3. ii-iii].

(iii) Calculations

Data were expressed as cpm of ^{45}Ca per ml of tissue water =

$$\frac{CW \times \text{dilution factor } (20) \times {}^{45}Ca \text{ cpm}}{TWV}$$

Results were presented as the percent of the value obtained at time = 0 min i.e. the start of the incubation period.

5. Na^+ and K^+ Determinations

Aliquots of the TCA extract were taken for the determination of Na^+ and K^+ ion contents. Na^+ and K^+ ion contents were determined by emission flame photometry using the lithium internal standard procedure. The instrument was a KLiNa Flame Photometer (Beckman Instruments, Fullerton, CA) and was standardized with a solution containing 15 meq/ml of lithium and known concentrations of Na^+ and K^+. The Na^+ and K^+ values were corrected for percent water content of the cells, volume of TCA used for extraction, and extracellular space (ECS). Results were expressed as millimoles Na^+ or K^+ per liter intracellular water =

$$\frac{\text{Cell content (mmol)} - \text{ECS content (mmol)}}{\text{Intracellular Space (ICS)}}$$

where:

(1) Content in total cell water = Tissue Reading (mmol/L) x CW/TWV

(2) Content in extracellular water = Medium Reading (mmol/L) x ECS

(3) ICS = 1 - ECS

6. ATP determination

(i) Preparation of Samples

Aliquots (0.2 ml) of the cell suspension were added to 1.0 ml glycine buffer, pH 9.2, heated to 95°C. The mixture was heated by immersion in a boiling water bath for 10 min, cooled for 10 min on ice, and centrifuged at 12,000 x g for 2 min. In this way, cellular enzymes were inactivated, and ATP was extracted and remained protected from degradation by the high pH of the buffer (Bihler and Jeanrenaud, 1970). ATP in the alkaline glycine extract was determined by the luciferin-luciferase bioluminescence technique.

(ii) Assay Principle

Luciferin, a heat-stable heterocyclic phenol, and luciferase, a heat-labile enzyme, have both been extracted from fireflies and crystallized. In the first step, luciferin (LH_2) and ATP react to form luciferyl adenylate (LH_2-AMP), which remains tightly bound on the catalytic site of luciferase (E):

$$LH_2 + ATP + E \longrightarrow E\text{-}LH_2\text{-}AMP + PPi$$

When this form of the enzyme is exposed to molecular oxygen, the enzyme-bound luciferyl adenylate is oxidized to yield oxyluciferin (L), which emits light on returning to the ground state:

$$E\text{-}LH_2\text{-}AMP + O_2 \longrightarrow L + H_2O + \text{bioluminescence}$$

One quantum of light is emitted for each molecule of luciferin oxidized. The luciferin-luciferase reaction, followed in a recording photometer, is often used as a highly sensitive quantitative assay for ATP. The quantum yield, defined as the ratio of the number of emitted photons and the number of converted ATP molecules is almost 100%. The

maximum of the emitted light is at 562 nm with a broad peak between 500 and 600 nm. Under the assay conditions, the rate of the luciferase reaction is slow: reduction of ATP concentration is 0.5% per min. Thus an almost constant light signal can be produced at a defined ATP concentration. The intensity of the emitted light is directly proportional to the ATP concentration (McElroy and Green, 1956).

(iii) Assay Procedure

The reagent solution (type CLS - Boehringer - Mannheim) consisted of 40 mM HEPES buffer, pH 7.75, 1.6 ug/ml luciferase from Photinus pyralis, 0.70 mM D-luciferin, 20 mM $MgCl_2$, 4 mM EDTA, 0.36 mM dithiothreitol and 0.3 mM AMP. ATP standards were prepared in HEPES buffer, pH 7.75, using the disodium salt of ATP (mol. wt. 605.2; absorption coefficient: E = 15 L $mmol^{-1}$ cm^{-1}) (Boehringer - Mannheim), and ranged from 1.32 x 10^{-12}M to 1.32 x 10^{-6}M ATP. The glycine extracts (0.1 ml) of the samples were diluted 100-fold in HEPES buffer, pH 7.75, such that the final dilution factor was 600.

The instrument used to measure the reaction was a Model 1250 luminometer (LKB Wallac, Turku, Finland). The sample or standard (0.2 ml) was added to a plastic cuvette. Addition of an equivalent volume (0.2 ml) of the reagent solution initiated the reaction. The concentration (M) of ATP in each sample was determined from the standard ATP curve, a semilogarithmic plot of ATP concentration (M) as a function of voltage. This value was corrected for dilution (600) and divided by the dry weight.

ATP content of each sample was expressed as umol/g dry weight =

$$\frac{\text{ATP (mol/L)} \times 600 \times 1000}{\text{dry weight (g)}}$$

7. Protein Estimation

The protein contents of cell pellets (from isolated cell experiments) and of cells plated in multiwells (from cell culture experiments) were estimated by the widely used method of Lowry et al. (1951).

The reagents consisted of 1N NaOH, alkaline copper solution (50 ml 2% Na_2CO_3, 0.5 ml 1% copper sulfate, 0.5 ml 2% Na K Tartrate) and Folin phenol reagent diluted 1:2 with distilled water. Standards were prepared in 0.1N NaOH using crystalline bovine serum albumin (Sigma), and ranged from 0.01 to 0.50 g/L. Samples were diluted to 500 ul with 0.1N NaOH and heated in a water bath at approximately 75^{o}C for 15 min.

Standards and samples were place into an automatic sampling module of a Technicon Autoanalyzer that delivered equal volumes of the sample or standard via a proportioning pump to a colorimeter. The optical density, which was measured by the colorimeter, was recorded on a chart recorder. From the peak heights of the standards, the optical density of each sample was determined and converted to mg protein using a Polynominal Regression computer program.

8. 3-O-methyl-D-glucose Uptake in Adrenomedullary Chromaffin Slices

Fresh bovine adrenal glands were obtained from the local slaughterhouse and transported within half an hour to the laboratory in standard medium saturated with 95%O_2:5%CO_2. The dissection was carried out at 0-4^{o}C in fresh buffer saturated with 95%O_2:5%CO_2. Adrenal glands were trimmed clean of fat and connective tissue and cross-sectioned into several slabs. Each slab was dissected free of peripheral cortical tissue to yield a fairly homogeneous section of medullary tissue. Sections which showed infiltration by cortical tissue were discarded. Medullary sections were then cut into 0.3 mm thick slices using a Stadie-Riggs tissue slicer. A tissue thickness of 0.3 mm or less was required for adequate tissue oxygenation as determined by Warburg (1923). The entire dissection lasted approximately 15 min.

Three slices were distributed randomly to each control or treatment flask and preincubated for 20-30 min at 37^{o}C with gentle shaking in 4.0 ml of appropriate buffer supplemented with 10 mM D-glucose and saturated with 95%O_2:5%CO_2. Preincubation was followed by a 5 or 10 min incubation period under the same conditions but with the addition of ^{14}C-labelled 3-O-methyl-D-glucose (0.0625 uCi/ml, total concentration 5.0 mM), and tracer amounts of ^{3}H-[G]-labelled inulin (0.3125 uCi/ml) and replacing 10 mM D-glucose with 5 mM pyruvate. After incubation, the adrenomedullary slices were blotted, weighed and boiled for 30 min in 2.0 ml of distilled water to extract sugar and ions. The boiled mixture was cooled on ice, deproteinized with 0.05 ml 100% TCA, centrifuged at 1500 x g for 10 min on a clinical centrifuge (International Equipment Co., Needham Hts., Mass.), and the supernatant decanted for analysis. Aliquots of the supernatant were evaporated and

a constant volume of water (0.05 ml) and scintillation solution (10.0 ml) added. To avoid catecholamine oxidation which results in colouring and consequent quenching of scintillation, the tissue extract was evaporated to dryness under 100%N_2. Incubation media from treatment and control flasks were centrifuged at 1500 x g for 10 min, and 0.05 ml of the supernatant was added to 10 ml of scintillation medium. Radioactivity was assayed by double label liquid scintillation spectrometry as described above. The results were expressed as percent equilibration and presented as rates (nmol/mg protein/min) of sugar transport, as described above.

B. EXPERIMENTS WITH CULTURED CELLS

1. Culture of Bovine Adrenomedullary Chromaffin Cells

(i) Sources of Chemicals

Dulbecco's Modified Eagle's Medium (with L-glutamine, 1000 g D-glucose/L, and sodium pyruvate and lacking sodium bicarbonate), mycoplasma-tested and virus-screened fetal bovine serum, penicillin, streptomycin, and tissue culture dishes were obtained from GIBCO (Grand Island, NY). Cyto-1-beta-D-arabinofuranoside (cytosar), 5'-fluorodeoxyuridine (FDU), HEPES, tetracycline, gentamycin, mycostatin, and collagen were from Sigma Chemical Co. (St. Louis, MO). Dexamethasone sodium phosphate (HEAXADROL injection) was obtained from Organon Canada Ltd. (Toronto, Ontario).

(ii) Tissue Culture Medium

Dulbecco's Modified Eagle's Medium (DMEM), a synthetic medium developed for mammalian cell lines, is made up of a balanced salt solution containing 15 amino acids, 9 vitamins, glucose, phenol red, and antibiotics (Smith et al., 1960; Dulbecco and Freeman, 1959; Morton, 1970). The commercially available DMEM (GIBCO) was supplemented with 10% fetal calf serum, 5 mM glucose, 15 mM N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid (HEPES), penicillin (100 ug/ml), streptomycin (100 ug/ml), tetracycline (5 ug/ml), mycostatin (25 U/ml), and gentamycin (10 ug/ml), 10^{-5}M 5-fluorodeoxyuridine (FDU) and 10^{-5}M cytosine arabinoside (Cytosar) (Trifaro and Lee, 1980). The pH of the complete DMEM culture medium was normally within the range of 7.0-7.2 at 37^{o}C.

Serum was added to the culture medium to enhance adhesion of the cells to the culture surface. 10% fetal calf serum contains approximately 2-3 ug/ml fibronectin (Hayman and Ruoslahti, 1979), which is an attachment glycoprotein secreted from certain cells (Grinnell and Feld, 1979; Hughes et al., 1979; Hayman and Ruoslahti, 1979). The fibronectin is adsorbed on the culture surface and subsequently incorporated into the extracellular matrix of the spread cells (Hayman and Ruoslahti, 1979). HEPES buffer was used because it has a pKa of 7.31 at 37^{o}C and therefore its most effective buffering range is around the physiological pH.

The penicillins act by interfering with the synthesis and cross-linkage of mucopeptides essential for the formation and integrity of bacterial cell walls; therefore they affect growing microbial cells but do not attack bacteria or intracellular organisms that are dormant. Penicillins are bactericidal against Gram-positive cocci and bacilli, Gram-negative cocci, and spirochetes. They are usually added as sodium penicillin G to culture media. A concentration of 100 ug/ml is completely harmless to all animal cell types and is inhibitory to the vast majority of bacteria. Streptomycin, an aminoglycoside antibiotic, acts by inhibiting protein synthesis through inhibition of the function of the 30S unit of bacterial ribosomes. Streptomycin sulphate (100 ug/ml) is useful against Gram-positive and Gram-negative bacteria. Tetracyclines inhibit the metabolism of microbial cells by blocking the attachment of aminoacyl transfer RNA to ribosomes, which interferes with protein synthesis. Tetracyclines (10 ug/ml) are bacteriostatic in their action against a broad spectrum of bacteria, including mycoplasma. Fungal and yeast growth may be inhibited by the use of

mycostatin at a concentration of 50 ug/ml. Another aminoglycoside antibiotic, gentamycin (200 ug/ml) is useful in controlling Gram-positive and Gram-negative bacteria and mycoplasma contamination in tissue culture (Paul, 1975; Perlman, 1979). As shown in Section II.B.1.iv., higher concentrations of these antibiotics were added to the transporting and perfusion media to sterilize possibly contaminated tissues prior to explantation (Paul, 1975).

Estridge and Bunge (1978) studied the effects of 1-beta-D arabinofuranosylcytosine (ara-C) and 5-fluoro-2'-deoxyuridine (FUdR) on dorsal root ganglia and superior cervical ganglia from perinatal rat pups. The actions of these antimetabolites were directed mainly at fibroblastic cells and Schwann cells. Ara-C gave the most reproducible killing of all types of non-neuronal cells, and FUdR served to keep the non-neuronal cells from resuming proliferation after ara-C withdrawal. The elimination of the supporting cell types from sensory ganglia was more difficult, perhaps due to the potent signal for Schwann cell proliferation present in the sensory axon. The drugs used are both S-phase (period of DNA synthesis in cell cycle) cytotoxic agents. FUdR is an inhibitor of thymidylate synthetase, and ara-C is known to act at a number of loci during DNA synthesis, including inhibition of DNA polymerase II the replicative polymerase from hepatic cell nuclei, inhibition of the polymerase responsible for DNA repair and interference with RNA metabolism. The exact molecular mechanism responsible for cell killing is not fully understood. The concentration of the antimetabolites (10^{-5}M) did not appear to affect neuronal protein synthesis (Estridge and Bunge, 1978). In this study, 5-fluorodeoxyuridine and cytosine arabinoside at 10^{-5}M each were added to prevent fibroblast proliferation (Trifaro and Lee, 1980).

In experiments using dexamethasone, appropriate aliquots of the presterilized dexamethasone sodium phosphate solution were added using a sterile syringe to the DMEM culture medium. The dexamethasone-treated chromaffin cell cultures were plated and fed as described below, but with culture medium containing dexamethasone.

(iii) Preparation of Collagen - Coated Tissue Culture Dishes

The connective tissue protein, collagen has proved to be a valuable cell culture substrate (Ehrmann and Gey, 1956). In the present study, the dishes were coated with collagen to improve adhesion of the cells to the plating surface and hence reduce the loss of cells during subsequent feedings. Chromaffin cells plated on dishes, which were not coated with collagen, undergo the same morphological changes as cells plated on collagen-coated culture dishes. Thus, collagen does not appear to influence the morphological changes of the chromaffin cells in culture. The procedure for coating the culture dishes with collagen is based on the method of Bornstein (1958), and was done under sterile conditions in a laminar air flow hood, LABGARD Model #NU-408 FM-400 (NuAire, Inc., Plymouth, MN).

The collagen solution consisted of 50 mg/L collagen Type VII (acid soluble from rat tail, Sigma) and 1 ml/L concentrated glacial acetic acid, diluted with glass distilled water and filtered through a 0.20 micron sterilizing filter unit (Nalgene). The collagen solution was coated on 35 x 10 mm Nunc plastic petri dishes (2 drops/dish) or on 24-well Nunc multidishes (1 drop/well), and was fixed onto the plastic surface of the tissue culture dishes by exposure to ammonia vapors for 2 min. These ammonia vapors were produced in a dessicator containing

100 g NaOH pellets, 10 g NH_4Cl, and gauze soaked with NH_4OH, over non-indicating Drierite. The ammonia vapors were then removed by washing with 2 ml (petri dishes) or 1 ml (multiwells) of sterile 0.002% phenol red solution until all traces of alkalinity indicated by the pink colour of the indicator in the wash disappeared. The dishes were finally washed with sterile glass distilled water and exposed to ultraviolet light for at least 2 hr before use for further sterilization.

(iv) Isolation of Chromaffin Cells

Bovine adrenal chromaffin cells were isolated as described in Section II. A. 1. In experiments where cells were isolated for the purpose of culture, antibiotics and antifungal agents were routinely added to the standard medium described in Section II. A. 1. ii. These antibiotics included penicillin (100-200 ug/ml), streptomycin (50-100 ug/ml), tetracycline (5-40 ug/ml), mycostatin (25 U/ml), and gentamycin (10 ug/ml). The cells were suspended in complete Dulbecco's Modified Eagle's Medium (DMEM) at a concentration of 0.5 X 10^6 cells/ml.

(v) Plating Cells, Maintenance of Cell Cultures

Bovine adrenal chromaffin cells, suspended in complete DMEM culture medium were plated on 35 X 10 mm plastic, collagen-coated petri dishes at a density of 10^6 cells per dish. These were used generally for inspection of morphology by light microscopy. For sugar transport experiments, chromaffin cells were plated on plastic, collagen-coated 24-well multidishes at a density of 5 X 10^5 cells per well. The tissue culture dishes were incubated at 37^oC in a water-jacketed,

CO_2-incubator (SHEL-LAB Model 200, Sheldon Manufacturing Inc., Portland, Oregon) under a CO_2 + air (5:95) atmosphere. Most culture media are designed for use with 5% CO_2, which is close to the value found in body fluids. DMEM was originally formulated to be used with 10% CO_2, but 5% CO_2 was found satisfactory with DMEM in culturing adrenal chromaffin cells, indicating that chromaffin cells have a high glycolytic activity and produce sufficient CO_2 by themselves (Livett, 1984). The culture medium was changed twice a day for the first 2-3 days in culture and then once a day for the rest of the culture period. In the initial stages of culturing, the chromaffin cells appeared to be at a phase of rapid growth and extensive morphological change. It is possible that this active development process was associated with rapid consumption of nutrients and accumulation of waste products. Frequent change of the culture medium in the first few days of culturing appeared to prevent the onset of contamination. Adrenal chromaffin cells were maintained in culture for 5 to 7 days.

At the time of feeding, cell cultures were also inspected, using an inverted research microscope (Olympus Model IMT-2). The microscope was equipped for photomicrography with a built-in 2.5 FK photo eyepiece and OM system bayonet mount for 35 mm camera back (OM light path). Photographs were taken using a 45-LBD (light balancing daylight)-2N filter specified for daylight colour film, a lamp voltage of 8V and Kodacolor VR film (ASA 200).

2. 3-O-Methyl-D-Glucose Transport

(i) Materials

The incubation medium and radioisotopes, which were used, are described in Section II.A.3.i.

(ii) Experimental Procedure

The multidishes were removed from the incubator and placed in a water bath at 37°C. The DMEM culture medium was suctioned off, the appropriate (control or treatment) incubation medium was added, and the cells were preincubated for 15 min at 37°C. The medium was removed, incubation medium containing the radioisotopes (^{14}C-labelled 3OMG, 0.25 uCi/ml; ^{3}H-[G]-labelled L-glucose, 2.5 uCi/ml) and varying concentrations (1-25 mM) of unlabelled sugar was added, and the cells incubated for 0.25 min (15 sec). During the incubation period, the incubation medium in the well was drawn continuously in and out of a plastic pipette tip using an air displacement pipettor (Gilson Pipettman). The reaction was terminated, and the cells treated as described above (Section A.II.B.ii), except that the cell pellet was dissolved in 0.10 ml 1N NaOH instead of being treated with 5% TCA. Aliquots of the extract were taken for protein determinations, and the rest of the extract centrifuged at 12,000 x g for 30 sec, and aliquots (50 ul) taken for liquid scintillation counting.

(iii) Analysis / Calculations

Isotopes were measured by double label liquid scintillation counting, as described in Section II. A. 3. iii. Tissue cpm (^{14}C or ^{3}H) was corrected for background, dilution and mg protein/well to give

tissue dpm (^{14}C or ^{3}H)/mg protein. Mediun cpm (^{14}C or ^{3}H) was corrected for dilution to give medium dpm (^{14}C or ^{3}H)/ml of medium. The ratio of tissue dpm/mg protein to medium dpm/ml medium represented the percent equilibration of ^{14}C or ^{3}H.

1) Tissue dpm (^{14}C or ^{3}H)/mg protein =

$$\frac{\text{Tissue dpm } (^{14}C \text{ or } ^{3}H) \times \text{dilution}}{\text{mg protein/well}}$$

2) Medium dpm (^{14}C or ^{3}H)/ml medium =

$$\text{Medium dpm } (^{14}C \text{ or } ^{3}H) \times \text{dilution}$$

3) Percent Equilibration of ^{14}C or ^{3}H =

$$\frac{\text{Tissue dpm } (^{14}C \text{ or } ^{3}H)/\text{mg protein}}{\text{Medium dpm } (^{14}C \text{ or } ^{3}H)/\text{ml medium}}$$

The difference between the equilibration values for ^{14}C and ^{3}H represented the specific transport (ST) of 3OMG. The results were presented as rates of 3OMG uptake (nmol/mg protein/min) =

$$\frac{\text{ST(ml/mg protein)} \times \text{substrate concentration (umol/ml)} \times 1000}{\text{incubation time (min)}}$$

C. STATISTICAL EVALUATION

The results are given as the mean $\pm$ standard error of the mean (S.E.) and the following numbers in brackets indicate the number of separate experiments. In single representative experiments, the results are given as the mean $\pm$ standard deviation (S.D.) instead of S.E. Statistical evaluation was by two-tailed Student's t-test (Goldstein, 1967).

SECTION III

RESULTS AND DISCUSSION

A. FRESHLY ISOLATED BOVINE ADRENOMEDULLARY CHROMAFFIN CELLS

1. Structural and Functional Integrity

The structural and functional integrity of isolated bovine adrenal chromaffin cells was assessed by measuring the following parameters. The average yield was 16.1 ± 1.4 (55) million cells per preparation or 10^6 cells/ml or 10^7 cells/g wet weight. Intact adrenal chromaffin cells appeared spherical in shape. 97.0 ± 0.6 (16) % of the cells were viable, and 82.6 ± 1.8 (14) % of the total number of cells were chromaffin cells. The contaminating cells consisted of erythrocytes, fibroblasts and adrenal cortical cells. The glucose transport systems of erythrocytes (Pletscher et al., 1955) and of fibroblasts under normal conditions (i.e. cells are fed, serum present) (Shaw and Amos, 1973) have been shown to be insensitive to physiological concentrations of insulin. There is no information on adrenal cortical cells but it is unlikely that the remaining very small percentage of these cells could be responsible for the insulin sensitivity observed in the present study, as described below (Figures 6 to 10, Table 3).

One milliliter of the final cell suspension had a wet weight of 70.8 ± 3 (113) mg, dry weight of 14.2 ± 2 (12) mg, and protein content of 13.5 ± 0.9 (7) mg. The cells maintained significant Na^+ and K^+ gradients. Intracellular Na^+ content was 46 ± 3.0 (14) mM and K^+ content was 90.6 ± 8.5 (13) mM. The high Na^+ content is in agreement with the observations that adrenal chromaffin cells appear to have significant resting membrane permeability to Na^+ ions (Brandt et al., 1976).

ATP levels in isolated bovine adrenal chromaffin cells were 213.2 $\pm$ 18.4 (9) umol/g dry weight, which is approximately 38 umol/g wet weight. The high ATP levels in chromaffin cells may in part be attributed to the reserves of ATP co-stored with catecholamines within the chromaffin granules (Livett, 1984). The ATP content of bovine adrenomedullary tissue was given as 14 umol/g wet weight (Falck et al., 1956) and of adrenal chromaffin granules of 100.8 (Njus et al., 1981) or 54 (Blaschko et al., 1956) umol/g wet weight. The older ATP determinations (Blaschko et al., 1956; Falck et al., 1956) are likely to underestimate the true ATP content because of methodological inadequacies.

The ATP content of adrenal medullary tissue preparations was found to be much greater than of other cell types or organelles, including rat heart muscle sarcosomes (3.4), rat liver mitochondria (4.1), rabbit striated muscle (whole) (5.4), rabbit blood platelets (8.3) and adult rat myocytes (4.5), where numbers in brackets represent ATP content in umol/g wet weight (for review, Blaschko et al., 1956; Bihler et al., 1985).

Thus the modified method for the isolation of bovine adrenal chromaffin cells used in this study produced large yields of cells with high purity, good viability and functional integrity.

2. Characteristics of 3OMG Transport

(a) Time Dependence

The time course of 3OMG equilibration was determined in isolated bovine adrenal chromaffin cells incubated with 2.0 mM 3OMG for up to 10 min. As seen in Figure 1, the time curve consisted of an initial linear portion followed by a progressively decreasing rate, typical for an equilibrating process. On the basis of these results, a standard incubation period of 2.5 min was chosen, representing a point near the top of the linear portion of the curve, so that the equilibration values presented here reflect initial unidirectional influx rates.

If the incubation period is too short, differences in equilibration between control and treatment will be negligible, because of the small degrees of equilibration reached. If the incubation period is too long, transport will no longer be unidirectional. After still longer incubation times, net transport rates will be negligible as a state close to equilibrium will be reached.

For comparison, the cell/medium distribution of ^{3}H-L-glucose did not vary with duration of incubation, and was 7.0 $\pm$ 0.6 (23) % of the total water space of the cell pellet, not significantly different from the extracellular space, determined with inulin. This suggests that the passive diffusion component of total sugar uptake was negligible. In other neural preparations, the proportion of sugar influx via passive diffusion also appeared to be negligible in vivo (some 5% or less) and 10-15% in vitro (Bachelard, 1983).

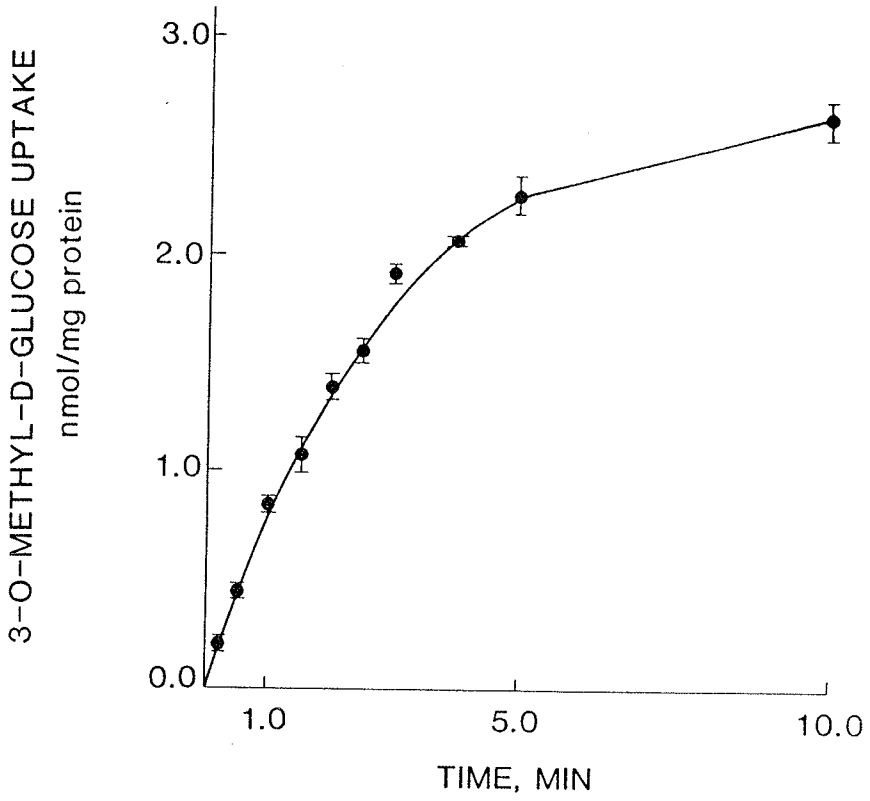


FIG. 1. Time-course of uptake of 3OMG. Cell suspensions (0.3 ml, 10^6 cells/ml) were incubated with 2 mM 3OMG as described in METHODS for various periods of time. Points represent the mean $\pm$ S.E. for 4-5 separate experiments, each done in triplicate.

(b) Concentration Dependence

Saturation Kinetics

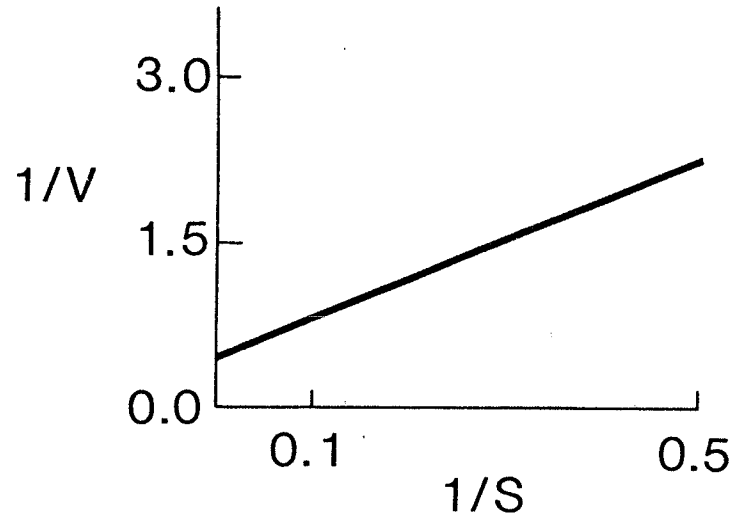
Mediated or facilitated membrane transport processes exhibit saturation kinetics i.e. the transport system becomes saturated with the substance transported just as enzymes become saturated with their substrates. Plots of the initial rate of a mediated transport process against the substrate concentration usually show a hyperbolic curve approaching a maximum at which the rate is zero order with respect to substrate concentration, similar to the Michaelis-Menten relationship of enzyme kinetics. Such behaviour suggests that the membrane transport system contains a limited number of specific sites to which the substrate must bind reversibly in order to be transported across the membrane (Lehninger, 1975).

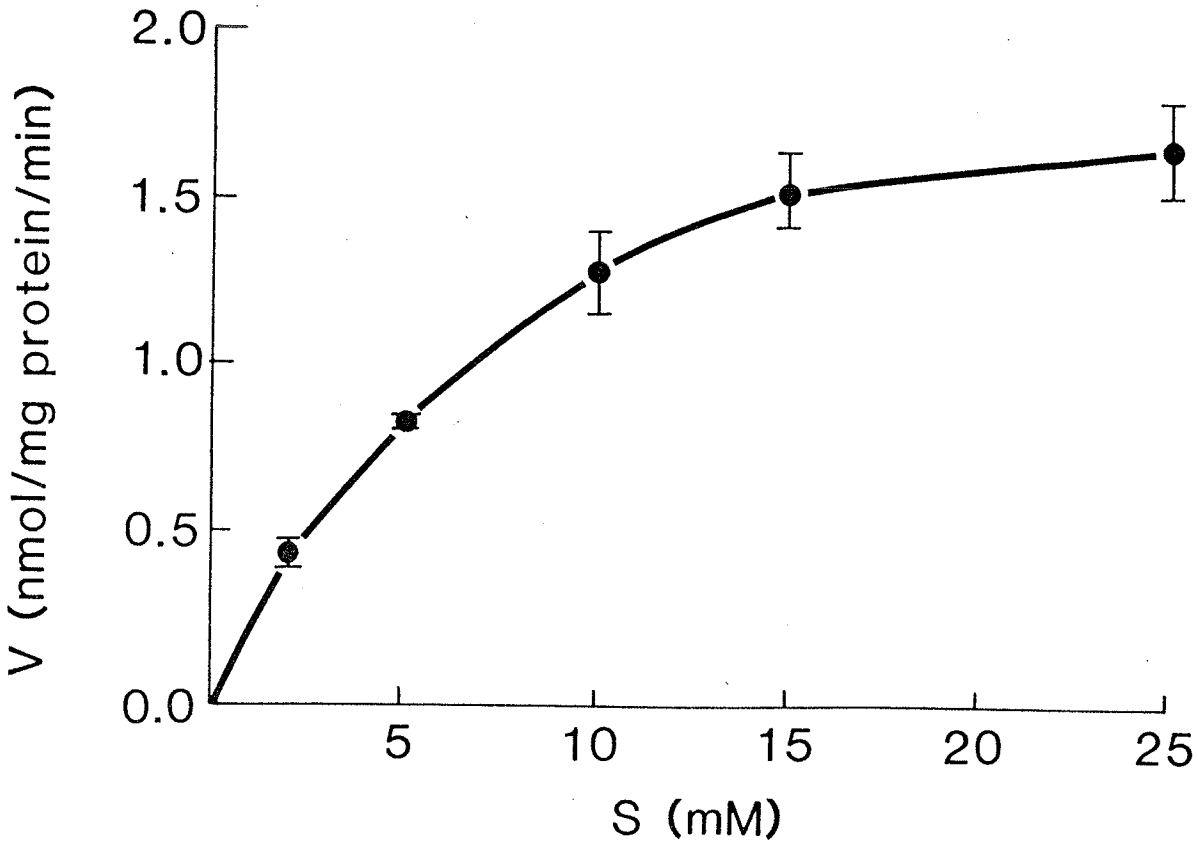
Figure 2 illustrates the concentration dependence of 3OMG uptake in a representative experiment. The plot of the rate of 3OMG uptake as a function of 3OMG concentration yields a hyperbolic curve, which is characteristic for glucose transport by facilitated diffusion (Widdas, 1952). Thus, as in other tissues of neural origin (Lund-Andersen, 1979; Baker and Carruthers, 1984), glucose uptake into adrenal chromaffin cells is mediated by a saturable process.

Kinetic Parameters

Saturation (Michaelis-Menten) kinetics are characterized by two constants, Vmax and Km, which have been termed the capacity and affinity factors, respectively. Vmax, the maximal velocity is a function of the number of carriers present and their mobility i.e. their rate of reorientation or translocation through the cell membrane.

FIG. 2. Concentration curve of 3OMG uptake. Cells were incubated as described in METHODS with various concentrations of 3OMG. Points represent the mean ± S.D. of triplicate samples from one representative experiment. The inset is a double reciprocal plot of the same data calculated by linear regression.


3.0
1.5
0.0
1/V
0.1
0.5
1/S


2.0
1.5
1.0
0.5
0.0
V (nmol/mg protein/min)
5
10
15
20
25
S (mM)

Km describes the strength of interaction between the substrate and the transport system's reactive site (carrier) for each sugar, and is the substrate concentration at which transport proceeds at half maximal velocity (Wilbrandt and Rosenberg, 1961).

The kinetic parameters can be calculated from the Michaelis-Menten equation, the rate equation for reactions catalyzed by enzymes (or carriers) having a single substrate:

$$V_o = V_{max} \frac{S}{Km + S}$$

where V_o = initial rate, V_{max} = maximum initial velocity, S = substrate concentration and Km is the Michaelis-Menten constant. The Michaelis-Menten equation relates the initial velocity and the initial substrate concentration through the Michaelis-Menten constant.

The Michaelis-Menten relationship can be algebraically transformed into other forms that are more useful in plotting experimental data. One common transformation is derived simply by taking the reciprocal of both sides of the Michaelis-Menten equation:

$$\frac{1}{Vo} = \frac{Km}{Vmax}\frac{1}{S} + \frac{1}{Vmax}$$

to obtain the Lineweaver-Burk equation. When $1/V_o$ is plotted as a function of 1/S, a straight line is obtained. This line will have a slope of Km/V_{max}, an intercept of $1/V_{max}$ on the y-axis and -1/Km on the x-axis. Such a double-reciprocal plot has the advantage of allowing a more accurate determination of Vmax, which can only be approximated as a limiting value at infinite substrate concentration from a simple plot of V_o vs [S]. The double-reciprocal plot can also give valuable information on enzyme or transport inhibition (Lehninger, 1975)

Reciprocals of substrate concentrations and of the mean uptake rates of data in Figure 2 were fitted to a linear regression equation to obtain the values for kinetic constants. The straight line double reciprocal plot (inset, Figure 2) is consistent with Michaelis-Menten kinetics. The kinetic constants for this experiment were Vmax = 2.3 nmol/mg protein/min and Km = 8.2 mM.

Glucose uptake within the brain may be mediated by two distinct transport systems, which differ mainly in their affinities for glucose molecules; one is a low-affinity process of Km 6-8 mM and the other shows a much higher affinity, with a Km of 0.2-0.3 mM. Both types occur in slices and in mixed cell suspensions, so both may occur in vivo. Glial cell preparations do not show the presence of the high affinity system, whereas in synaptosomes, the low-affinity system is not detected. Current data are insufficient for analysis of the glucose transport system in bulk isolated neurons or cultured neuroblastoma, but some evidence suggests that glucose is transported into neurons more readily and rapidly than into glial cells (Bachelard, 1983). There are few qualitative, much less quantitative studies of glucose transport in peripheral nerve. In squid giant axon, it was shown that the glucose transport system had an apparent Km of 3.6 mM (Baker and Carruthers, 1984). The Km of 8.2 mM of sugar transport in adrenal chromaffin cells (Figure 2) is consistent with the range of affinities measured in most cells, including peripheral nerve and glial cells, but is greater than that of the high affinity system described in neuronal membranes in the brain (Bachelard, 1983).

Separate experiments (8 adrenal glands per experiment) testing the relation between transport rate and substrate concentration were grouped together (24-32 adrenal glands per group, see Table 1) according to the magnitude of the 3OMG uptake rates, and the groups labelled A, B and C. Although all three groups showed saturability and gave straight line double-reciprocal plots, there was a marked difference in basal 3OMG uptake rates (Table 1) resulting in a three-fold variation between the groups: Vmax for basal uptake rates in groups A, B, and C was 0.69, 1.10 and 2.24 nmol/mg protein/min respectively, whereas Km was relatively constant and ranged between 5-8 mM. It is unlikely that this variability was due to differences in the destruction of sugar transporters, since cells from all groups were subject to the same degree of mechanical and enzymatic disaggregation during cell isolation.

This variability may be related to the characteristics of the adrenal glands. As discussed by Livett (1984), the consistency (and "disaggregability") of the adrenal gland and hence characteristics of the cells are influenced by the breed of the cattle, age and size of the animal and, in colder climates, by seasonal changes. Larger sized glands showed greater infiltration of the medullary portion by cortical tissue than smaller adrenal glands. A clean dissection of medulla from cortex was more difficult to achieve with the larger glands. Thus, it is possible that cell suspensions made from larger glands may have a greater percentage of nonchromaffin cells (i.e. cortial cells). However, the measurement for purity (82.6 $\pm$ 1.8%, n = 14) was not highly variable, suggesting that differences between the three groups cannot be completely attributed to variability in the purity of cell

TABLE 1

VARIATION IN BASAL 3-O-METHYL-D-GLUCOSE UPTAKE RATES IN DIFFERENT BATCHES OF BOVINE ADRENAL CHROMAFFIN CELLS

Cells were incubated as described in METHODS with various concentrations of 3OMG. Numbers in brackets indicate the number of separate experiments, each done in duplicate. Vmax is given as nmol/mg protein/min and Km as mM.

S (mM)	V (nmol/mg protein/min)		
	GROUP A	GROUP B	GROUP C
1	0.08±0.004(3)	0.18±0.01(4)	0.33±0.01(3)
2	0.12±0.01(3)	0.30±0.01(4)	0.53±0.01(3)
5	0.26±0.04(3)	0.53±0.01(3)	1.04±0.01(3)
10	0.45±0.02(3)	0.77±0.01(3)	1.55±0.01(3)
Vmax	0.69	1.10	2.24
Km	8.2	5.2	5.9

preparations. It may also be argued that the three distinct groupings may be related to differences in cell viability between groups. However, the cell viability (= 97.0 $\pm$ 0.6%, n = 16) also was not highly variable.

It was suggested that the differences between groups may be related to differences in the activity of the glucose transport system under basal conditions. For example, the higher values of 3OMG uptake under basal conditions may result from activation of basal transport by unknown stimulatory factors.

(c) Chemical Specificity, Competitive Inhibition, and Countertransport

Another criterion of mediated transport is specificity for the substance transported. For example, the amino acid transport systems of animal cell membranes are much more active with L-amino acids than the D-isomers. From such findings it has been postulated that mediated transport systems in membranes contain a binding site complementary to the substance transported, resembling in its specificity the active site of enzyme molecules (Lehninger, 1975)

The glucose transport system in human erythrocytes (LeFevre and Marshall, 1958) and rat cardiac and skeletal muscle (Park et al., 1959; Battaglia and Randle, 1960; Battaglia et al., 1960) was shown to be specific for D-sugars. For maximal activity, the transported sugar must be in the C-1 conformation where all the hydroxyl groups are in equatorial orientation. It has been postulated that the glucose carrier binds the sugar at three equatorial hydroxyl groups (C1, C4, C6). D-glucose, 3-O-methyl-D-glucose, 2-deoxyglucose, D-xylose, D-mannose and D-galactose competitively inhibit the transport of each other, suggesting that they are all transported by the same carrier system.

The effect of D-glucose on the concentration dependence of 3OMG uptake was examined (Table 2, Figure 3). D-glucose (10 mM) significantly decreased the rate of 3OMG uptake at all substrate concentrations tested. The data in Figure 3 and Table 2 shows that 10 mM D-glucose did not affect Vmax but increased the Km for 3OMG uptake more than 2-fold, from 7.4 mM in the absence of D-glucose to 19.0 mM in the presence of D-glucose. Thus inhibition of 3OMG uptake by D-glucose is of the competitive type.

As described above, facilitated transport is a process involving interaction of the substrate with a limited number of specific binding and transport sites. A key test to distinguish facilitated diffusion from simple diffusion is demonstration of countertransport (Wilbrandt and Rosenberg, 1961). Countertransport also demonstrates the "sidedness" of sugar transport i.e. influx and efflux are two distinct processes. In other words, the Michaelis-Menten equation expresses influx as a function of binding to the carrier on the outside of the cell membrane, and efflux is a function of binding to the carrier on the inner plasma membrane. As shown in Figure 4, the cells were incubated with 3OMG (0.1 mM) until full equilibration was nearly reached. In other words, influx was almost balanced by efflux such that minimal net inward transport occured. A comparatively high concentration of a competing sugar (10 mM D-glucose) was added, and caused a net efflux of 3OMG from the cells, against its concentration gradient. This is explained by competition for influx only, as there is initially no D-glucose within the cells. Because glucose is rapidly metabolized within the cells, its intracellular concentration remains

TABLE 2

CONCENTRATION DEPENDENCE OF 3-O-METHYL-D-GLUCOSE UPTAKE IN THE PRESENCE AND ABSENCE OF D-GLUCOSE

Cells were incubated with various concentrations of 30MG in the presence and absence of 10 mM D-glucose. Numbers in brackets represent the number of separate experiments, each done in triplicate. Vmax is given as nmol/mg protein/min and Km as mM.

S (mM)	V (nmol/mg protein/min)	
	- D-GLUCOSE	+ D-GLUCOSE
1	0.26 ± 0.04 (4)	0.11 ± 0.01 (4)
2	0.46 ± 0.07 (4)	0.22 ± 0.03 (4)
3.5	0.71 ± 0.10 (4)	0.33 ± 0.02 (4)
5	0.84 ± 0.13 (4)	0.44 ± 0.04 (4)
10	1.25 ± 0.20 (4)	0.74 ± 0.09 (4)
Km	7.4	19.0
Vmax	2.19	2.21

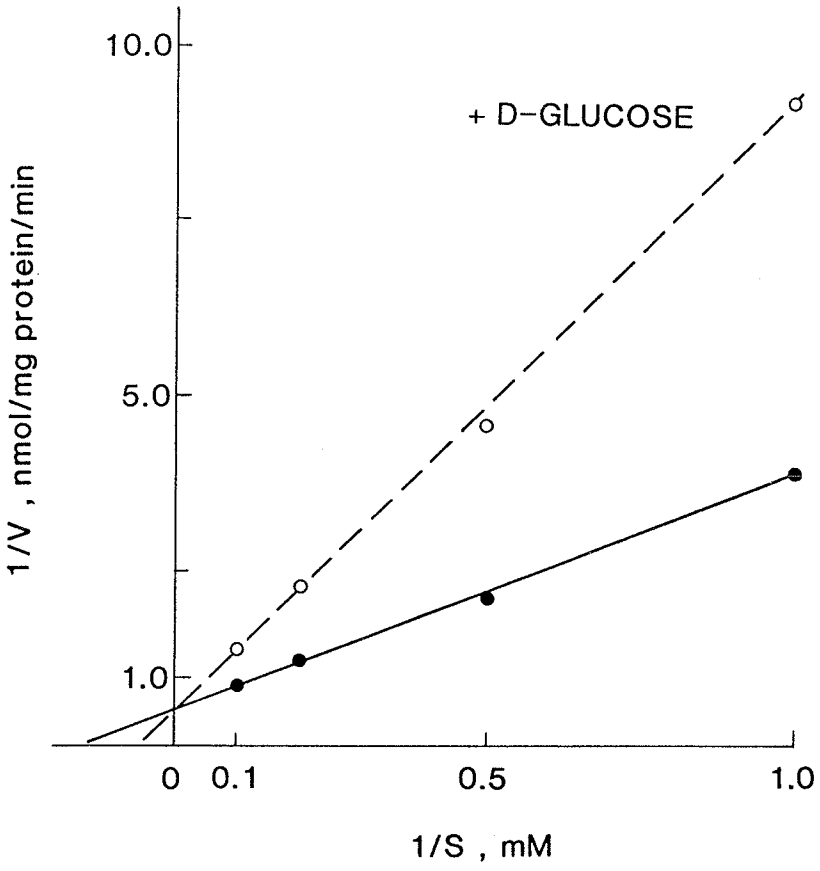


FIG. 3. Double-reciprocal plot of 3OMG uptake in the presence (0---0) and absence (●——●) of 10 mM D-glucose. Experimental conditions as described in Figure 2. Points represent the mean ± S.E. of 4 separate experiments, each done in triplicate.

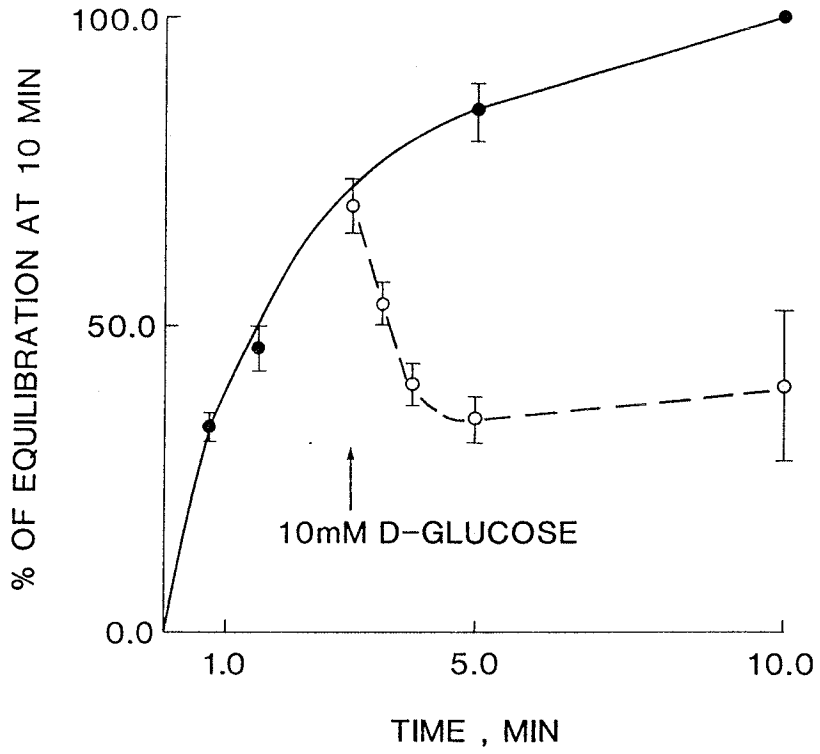


FIG. 4. Countertransport of 3OMG induced by 10 mM D-glucose. Cells were incubated for various periods of time with 0.1 mM 3OMG (●——●). After 3 min, 10 mM D-glucose was added (0--0). Data were corrected for nonspecific uptake determined with 0.1 mM L-glucose in the same cell sample. Points represent the mean ± S.E. of 4-5 separate experiments.

low, and 3OMG distribution reaches a new, lower equilibrium. Because simple diffusion cannot take place against a concentration gradient, 3OMG transport must be carrier-mediated.

(d) Effects of Phloridzin, Phloretin, and Cytochalasin B

Another characteristic of mediated transport is that it can be inhibited quite specifically. Some biological transport systems can be inhibited by substances structurally related to the substrate, which compete with the substrate for its specific binding site. Other transport systems may be inhibited noncompetitively by reagents capable of blocking or altering specific functional groups in protein molecules such as N-ethylmaleimide, a sulfhydryl group blocking agent or 2,4-dinitrofluorobenzene, which blocks free amino groups (Lehninger, 1975).

Phloretin is a potent competitive inhibitor of facilitated diffusion of glucose, whereas phloridzin is more potent in intestinal and renal active hexose transport systems (Stein, 1967). Cytochalasin B (CB) was first shown to inhibit the glucose transport system by Kletzien and Perdue (1973). It is now widely accepted that CB is a specific inhibitor of glucose tansport by facilitated diffusion in most cell types. Taylor and Gagneja (1975) developed a molecular model, which describes the spatial distribution of four oxygen atoms in the C-1 chair conformation of beta-D-glucopyranose. They suggested that these oxygen atoms may be involved in hydrogen bonding of glucose to the transport protein. It was shown that the molecular structure of CB overlaps with that of glucose and other specific inhibitors of glucose transport (e.g. phloretin) largely with respect to the four oxygen

atoms involved in binding. This supports the conclusion from kinetic data that the inhibitory effect of CB was competitive in nature.

The effects of these three specific inhibitors on basal 3OMG uptake were investigated (Figure 5). Basal sugar transport was significantly inhibited by 0.1 mM ($P<0.005$) and 0.5 mM ($P<0.001$) phloridzin, and 0.05 mM ($P<0.005$), 0.1 mM ($P<0.001$), and 0.5 mM ($P<0.001$) phloretin. Thus phloretin caused a greater degree of inhibition than phloridzin. CB (2 uM) also significantly ($P<0.001$) inhibited basal uptake. At a higher concentration of 20 uM (as used in the stopping solution) CB caused a 90.2% and 91.4% inhibition of basal and insulin-stimulated 3OMG uptake, respectively. At a concentration of 100 uM, which is the Ki for CB inhibition of 3OMG transport in squid giant axon (Baker and Carruthers, 1984), CB inhibited 3OMG uptake by 100 ± 1 (4)%. This, together with the data on L-glucose distribution (see above), suggests that the CB-insensitive or passive diffusion component of sugar transport is negligible in freshly isolated bovine adrenal chromaffin cells. The selective inhibition of 3OMG uptake by specific inhibitors of carrier-mediated transport, including phloretin and cytochalasin B (Figure 5), provides further evidence for a specific facilitated diffusion mechanism of glucose transport in chromaffin cells.

In summary, the uptake of 3OMG in adrenal chromaffin cells was characterized by saturability, competitive inhibition, countertransport, and inhibition by phloretin and cytochalasin B. These same features have been reported for sugar transport in other preparations of neural origin, including glioma and neuroblastoma

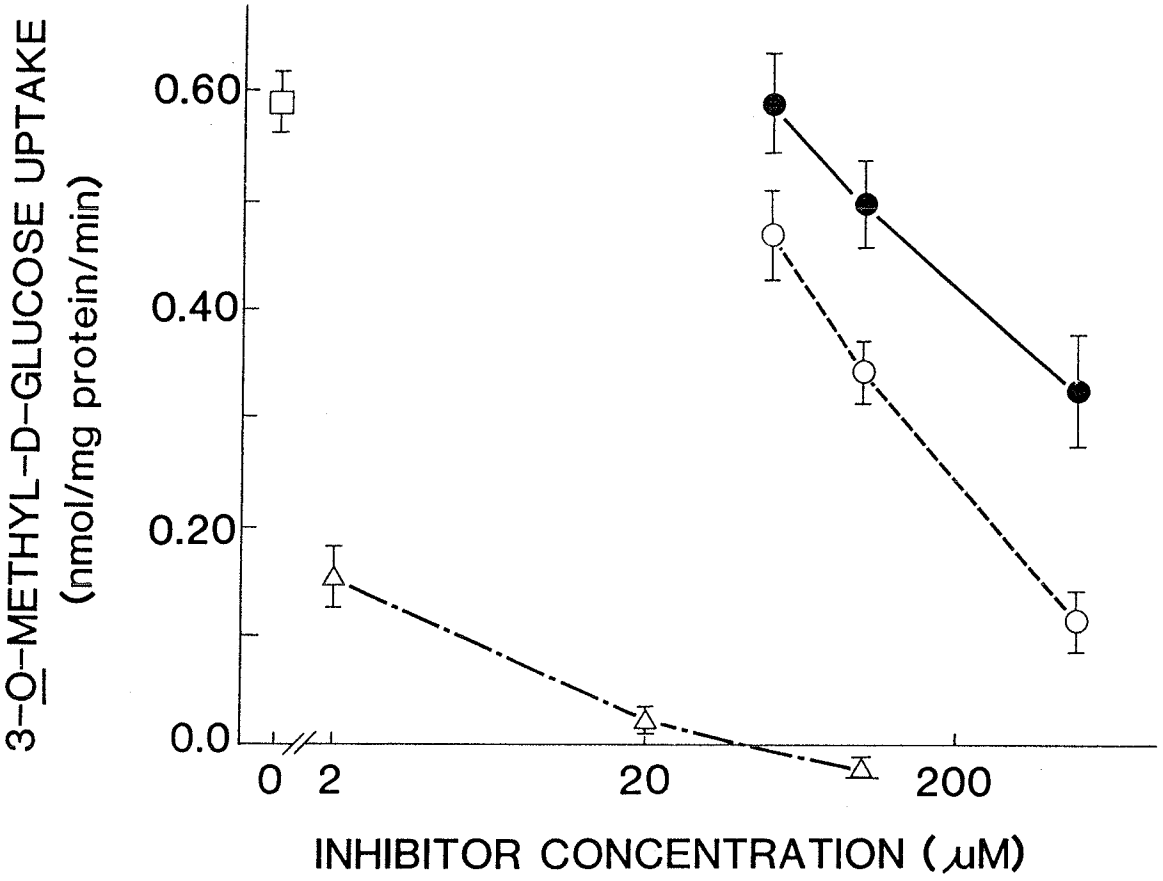


FIG. 5. Effects of phloridzin (●——●), phloretin (O---O), and cytochalasin B (Δ-·-Δ) on 3OMG uptake. Cells were incubated with 2 mM 3OMG as described in METHODS. Uptake in the absence of any inhibitor (□) was 0.59 ± 0.03 (9) nmol/mg protein/min. Points represent the mean ± S.E. of 4-5 separate experiments, each done in triplicate.

cells, synaptosomes, brain slices, whole perfused brain and peripheral nerve (Lund-Andersen, 1979; Baker and Carruthers, 1984) and most other cell types of vertebrates. After determining that sugar transport in adrenal chromaffin cells was mediated by a facilitated diffusion mechanism, it was then investigated whether the membrane transport step is subject to modulation as in muscle or is unregulated as in erythrocytes. The effects of insulin, hyperosmolarity and other factors, shown to stimulate sugar transport in muscle and adipose tissue, were examined on 3OMG uptake in adrenal chromaffin cells.

3. Effect of Insulin

Based on observations on galactose (hexose utilized only in liver) distribution in eviscerated, nephrectomized dogs and rats, Levine et al. (1949) hypothesized that insulin acted upon the cell membranes of certain tissues (skeletal muscle, etc.) to facilitate the transfer of some monosaccharides from the extracellular fluid into the cell. It is now widely accepted that one of the primary actions of the hormone insulin is to stimulate the membrane transport of glucose in some cells such as muscle and adipose tissue. Insulin has several other actions: (i) in adipose tissue, insulin increases fatty acid and glycerol phosphate synthesis, increases triglyceride deposition, activates lipoprotein lipase, and inhibits the hormone sensitive lipase; (ii) in the liver, insulin increases glycogen, protein, and lipid synthesis, decreases levels of cyclic AMP, decreases ketogenesis, and decreases glucose output (due to decreased gluconeogenesis and increased glycogen synthesis); (iii) in muscle, insulin increases glycogen and protein synthesis, increases the uptake of amino acids, and decreases protein catabolism; (iv) other actions of insulin include stimulation of the Na^+ pump and hyperpolarization of the cell membrane. Thus insulin, via its many actions, shifts the intracellular metabolism of glucose and other energy substrates (proteins, lipids) toward the synthesis of energy stores.

In a number of studies, insulin was shown to stimulate glucose transport in the nervous system in vivo in man (Gottstein et al., 1965; Gottstein and Held, 1967; Butterfield et al., 1966; Hertz et al., 1982) and rat (Daniel et al., 1977) and in vitro in rat spinal cord, isolated

cerebellum, cortical slices (Rafaelsen, 1961a, 1961b; Prasannan, 1972), rabbit sciatic nerve (Field and Adams, 1964) and C-6 astrocytoma cells (Passonneau, 1976). However some studies have reported no effect of insulin (Crone, 1965; Buschiazzo et al., 1970; Betz et al., 1972; Daniel et al., 1975; Sloviter and Yamada, 1971). Most of these negative findings were made in whole brain preparations or in vivo and hence may actually reflect the insensitivity to insulin at the level of the capillary endothelial cells (Goldstein et al., 1977; Mrsulja et al., 1976), which make up the blood brain barrier. In addition, currently available in vivo and in vitro neural preparations are encumbered with technical difficulties that make the qualitative and quantitative analysis of transport regulation by insulin and other factors exceedingly difficult. The effect of insulin on sugar transport into neuronal cells was investigated using adrenal chromaffin cells as a model of a homogeneous neuronal cell population of nonmalignant origin.

Qualitative Assessment

Figure 6 shows the effect of insulin (0-100 mU/ml) on 3OMG uptake in isolated adrenal chromaffin cells in the presence of 1.25 mM Ca^{2+}. Basal transport at 0.46 $\pm$ 0.01 (7) nmol/mg protein/min was significantly increased ($P<0.001$) to 0.55 $\pm$ 0.01 (4), 0.59 $\pm$ 0.02 (12), 0.57 $\pm$ 0.01 (4), and 0.56 $\pm$ 0.03 (4) nmol/mg protein/min with insulin doses of 0.1, 1.0, 32, and 100 mU/ml, representing a stimulation of 18-27% of the control.

Figure 7 shows the effect of insulin on 3OMG uptake in adrenomedullary slices. When the cortical tissue was carefully removed from medullary tissue, a stimulatory effect of insulin on 3OMG uptake

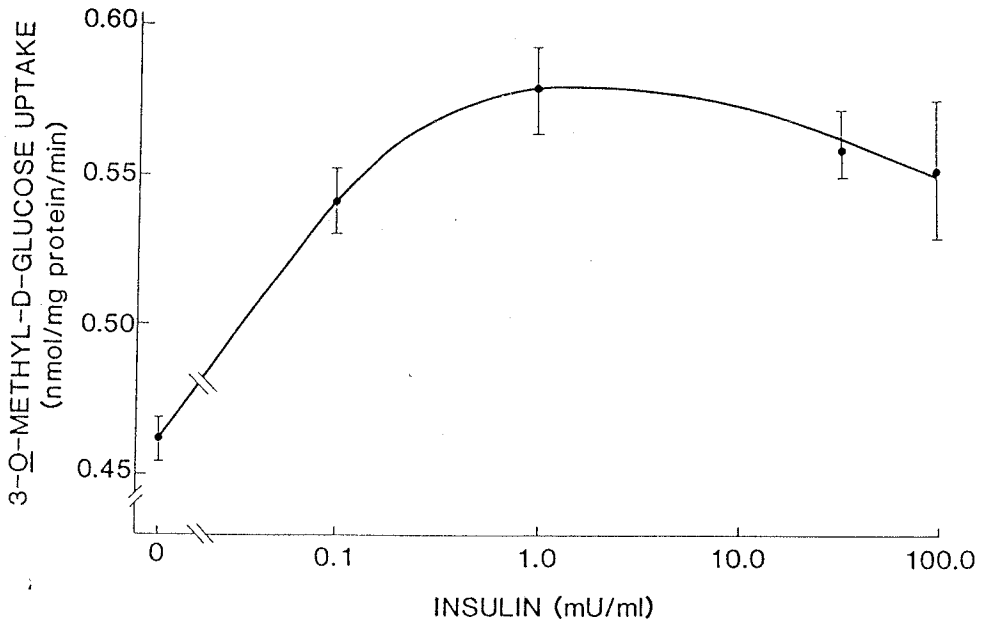


FIG. 6. Effect of insulin on 3OMG uptake in isolated adrenal chromaffin cells in the presence of external Ca^{2+} (1.25 mM). Cells were preincubated with varying concentrations (0-100 mU/ml) of insulin, and this was followed by incubation with 2 mM 3OMG and radioisotopes, as described in METHODS. Each point represents the mean $\pm$ S.E. of 1-3 separate experiments, each done in quadruplicate.

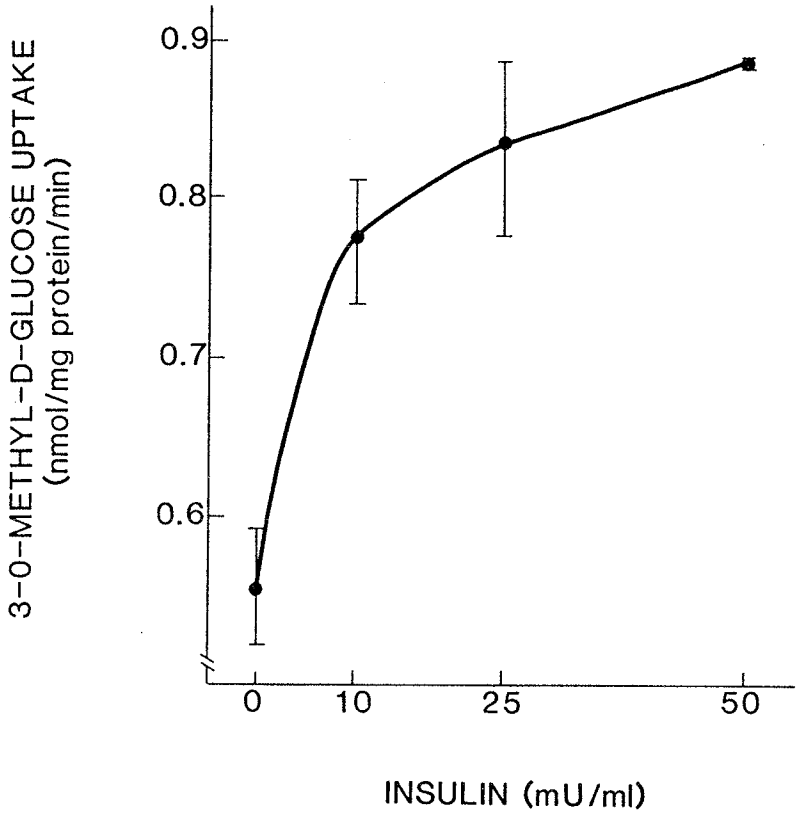


FIG. 7. Effect of insulin on 3OMG uptake in adrenomedullary slices in the presence of external Ca^{2+} (1.25 mM). Tissue slices were preincubated with various concentrations (0-50 mU/ml) of insulin, and this was followed by incubation with 5 mM 3OMG and radioisotopes, as described in METHODS. Each point represents the mean $\pm$ S.E. of 1-2 separate experiments, each done in triplicate.

was observed. Basal transport was significantly ($P < 0.001$) increased from 0.57 ± 0.03 (6) nmol/mg protein/min to 0.79 ± 0.02 (3), 0.83 ± 0.05 (6), and 0.88 ± 0.001 (3) nmol/mg protein/min with 10, 25, and 50 mU/ml insulin, respectively, representing an increase from 38-54% of control.

Thus it is clear that insulin stimulates sugar transport in adrenal chromaffin cells, but the maximal response differs between the dispersed adrenal chromaffin cells and the adrenomedullary slices. Since neither preparation is truly representative of ideal physiological conditions, it is not known which value for maximal response to insulin approaches the insulin response in vivo. It may be argued that freshly isolated cells have a lower insulin response because the number of insulin receptors may be decreased by mechanical and/or enzymatic disaggregation. However, when the insulin dose-response curves of freshly isolated cells and adrenomedullary slices are compared, the maximal rate of 3OMG uptake is considerably decreased in freshly isolated cells, but the position of the curve does not appear to be altered. This would suggest that the lower insulin response of freshly isolated cells may be related to decreased insulin responsiveness at the level of the sugar transport system rather than to altered sensitivity, which would be a function of the number of insulin receptors. Thus, the differences in insulin response between freshly isolated chromaffin cells and adrenomedullary slices cannot be completely attributed to differences in the number of insulin receptors.

Kinetic Analysis and Ca^{2+} Dependence

Because of the variation in basal 3OMG uptake described above (Table 1), it was necessary to test the stimulatory effects of insulin and its Ca^{2+} dependence within the same experiment. The rates of 3OMG uptake were determined at various substrate concentrations in the presence and absence of 1.25 mM Ca^{2+} and 50 mU/ml insulin. Figure 8 shows the effect of insulin on 3OMG uptake in the presence and absence of extracellular Ca^{2+} in experiments from group A (Table 1). Insulin significantly ($P < 0.01$) stimulated uptake from 0.12 $\pm$ 0.01 (6) nmol/mg protein/min to 0.15 $\pm$ 0.01 (6) nmol/mg protein/min, from 0.26 $\pm$ 0.04 (6) nmol/mg protein/min to 0.32 $\pm$ 0.02 (6) nmol/mg protein/min, and from 0.44 $\pm$ 0.02 (6) nmol/mg protein/min to 0.51 $\pm$ 0.04 (5) nmol/mg protein/min at 3OMG concentrations of 2, 5, and 10 mM, respectively.

Table 3 summarizes the effect of insulin in the presence and absence of Ca^{2+} on the kinetic constants of the three groups of experiments shown in Table 1. Insulin increased Vmax 1.8-fold and Km 1.7-fold in group A but had no effect on the kinetic constants of groups B and C, which have higher basal transport rates than group A (see Table 1). It is possible that enzymatic and/or mechanical disaggregation may affect the integrity of the cell membrane and thereby cause a decrease in the number of sugar transporters and insulin receptors. It may then be argued that the differences in the destruction of sugar transporters and/or insulin receptors may account for the variability in maximal rates of 3OMG uptake and insulin response between the three groups A, B and C. Assuming that the maximal rate of sugar transport is a function of the number of active sugar carriers in the membrane and the insulin response is a function of the

FIG. 8. Double-reciprocal plots of 3OMG uptake in the presence of 1.25 mM Ca^{2+} (●——●), with Ca^{2+} and 50 mU/ml insulin (O——O), in nominally Ca^{2+}-free medium (●---●), and in Ca^{2+}-free medium with insulin (O---O). Cells were incubated in various concentrations of 3OMG in the presence and absence of 50 mU/ml insulin and 1.25 mM Ca^{2+}. Reciprocals of the substrate concentration and of mean uptake rates from group C data in Table 1 were fitted to a linear regression equation to obtain double-reciprocal plots.

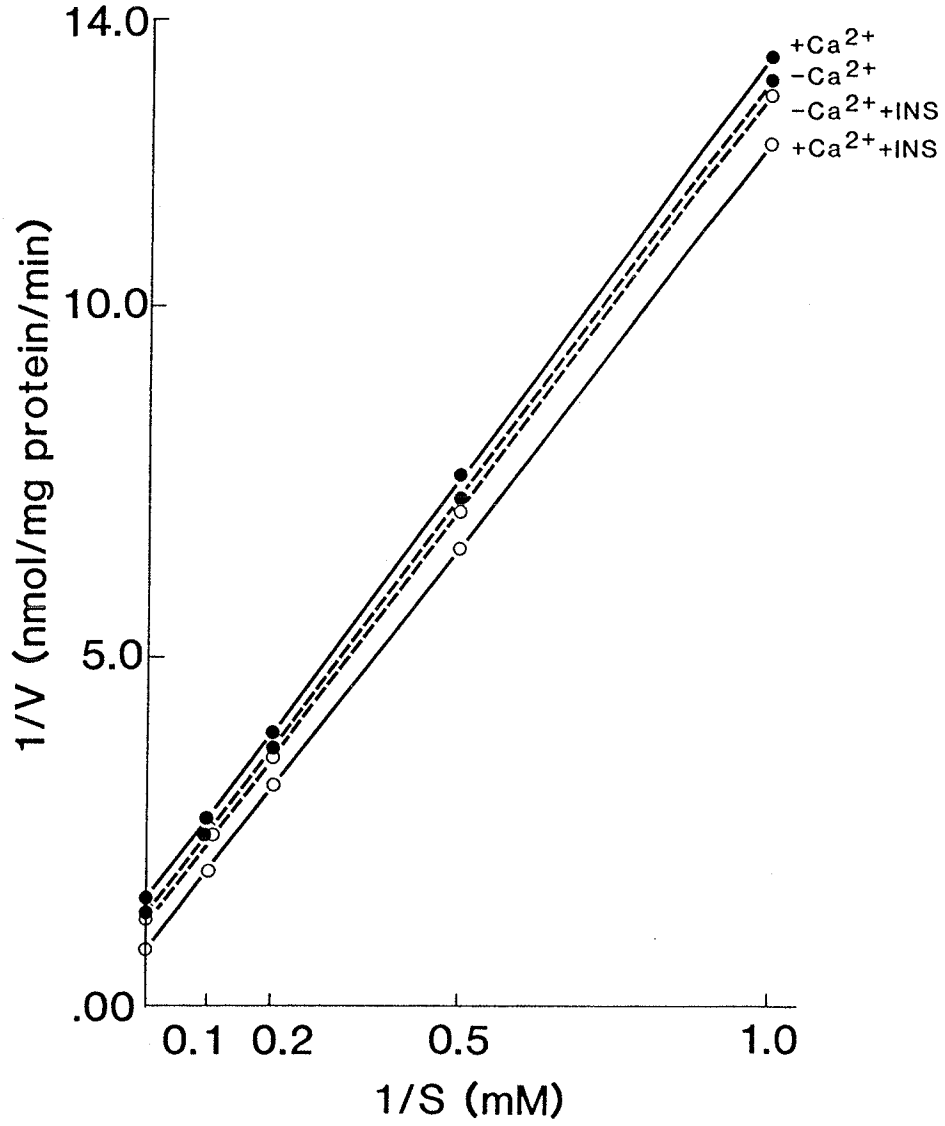

14.0
10.0
5.0
.00
1/V (nmol/mg protein/min)
0.1
0.2
0.5
1.0
1/S (mM)
+Ca2+
-Ca2+
-Ca2++INS
+Ca2++INS

TABLE 3

EFFECT OF 50 mU/ml INSULIN (INS) ON KINETIC CONSTANTS OF 3OMG UPTAKE IN THE PRESENCE AND ABSENCE OF EXTRACELLULAR Ca^{2+} (1.25 mM)

Cells from groups A, B, and C (Table 1) were incubated with various concentrations of 3OMG in the presence and absence of 1.25 mM Ca^{2+} and 50 mU/ml insulin. Reciprocals of substrate concentrations and of mean transport rates in each group were fitted to a linear regression equation to obtain the values for kinetic constants. Vmax is given as nmol/mg protein/min and Km as mM.

	+ Ca^{2+}	+ Ca^{2+} + INS	- Ca^{2+}	- Ca^{2+} + INS
GROUP A				
Vmax	0.69	1.25	0.78	0.78
Km	8.2	12.8	9.3	9.1
GROUP B				
Vmax	1.10	1.02	1.21	1.19
Km	5.2	4.2	5.8	5.7
GROUP C				
Vmax	1.67	2.11	2.61	2.64
Km	5.9	5.5	7.4	8.5

number of insulin receptors, it would follow that cells with the greatest damage would have the lowest maximal rates of 3OMG uptake and the lowest insulin response.

However, the results of the present study are not consistent with this explanation. As shown in Table 3, group A exhibited the lowest maximal rate of 3OMG uptake but the highest insulin response of the three groups. On the other hand, group C showed the highest maximal transport rate and a negligible insulin response. Hence, the variability between the groups shown in Table 3 cannot be fully explained in terms of destruction of sugar transporters and/or insulin receptors. In addition, since cells of each group were subject to the same degree of enzymatic and/or mechanical disaggregation, variability between groups cannot be due to variations in experimental procedure. Alternatively, variability in insulin effects between groups may be related to differences in the maximal rates of 3OMG uptake under basal conditions. As suggested above, the high basal transport rates may be due to unknown stimulatory effects. Hence, if stimulation already occurs and the maximal response is achieved, other activators of sugar transport such as insulin may not have a further effect.

Table 3 and Figure 8 also show that omission of extracellular Ca^{2+} did not significantly affect the parameters of basal 3OMG uptake in any of the groups. In the absence of external Ca^{2+}, insulin did not significantly alter 3OMG transport and kinetic constants, suggesting that the stimulatory effect of insulin appears to depend on the presence of external Ca^{2+}.

The insulin stimulated rise in carrier mediated glucose transport is characterized by an increase in Vmax as shown in muscle and adipose tissue (Clausen, 1975). In addition, some studies reported (e.g. in isolated perfused rat heart) that insulin increased Km. Fisher and Gilbert (1970) suggested that this effect was a theoretically predictable consequence of the rise in Vmax. Consistent with this hypothesis, they showed that the Km and Vmax of D-xylose and L-arabinose (transported by the same carrier) uptake were increased in a similar manner by the same concentration of insulin. Thus, sugar transport in adrenal chromaffin cells resembles qualitatively sugar transport in muscle and adipose tissue.

However, there may be quantitative differences between nervous tissue and muscle. The sensitivity and maximal response of the sugar transport system to insulin appears to be of lower magnitude in the chromaffin cell than in cardiac and skeletal muscle and closer to that (26.4%) in smooth muscle (Bihler et al., 1977). In the present study, the magnitude of the response, ranged from 15-30% in isolated adrenal chromaffin cells (Figure 6) and 38-54% in adrenomedullary slices (Figure 7). This is within the range of values obtained in other neural tissue preparations: insulin was shown to stimulate glucose transport by 20-50% in vivo (BBB transport) in man (Hertz and Paulson, 1983), 53% in whole peripheral nerve (Field and Adams, 1964), 40-80% in rat spinal cord (Rafaelsen, 1961a) and 27-36% in rat cortical slices (Prasannan, 1972). It was suggested (Hertz and Paulson, 1983; Rafaelsen, 1961b) that the weak insulin response in neural tissue preparations may be related to the elevated basal transport rate, which may reflect activation by unknown factors. Under these conditions, if basal sugar

transport is submaximally or maximally stimulated, insulin will have little or no effect.

Thus, in addition to sharing similarities in the affinities of their sugar transport systems, adrenal chromaffin cells resemble peripheral nerve and glial cells in their sensitivity to insulin. Hence, adrenal chromaffin cells may provide a useful model for the study of sugar transport regulation in at least some but not all types of neural cells.

4. Role of Ca^{+}

(a) Insulin

Clausen et al. (1974) first suggested that Ca^{2+} may be involved in the activation of sugar transport by insulin. The observations that insulin increased the rate coefficient of ^{45}Ca release from preloaded fat tissue (Clausen and Martin, 1977) and of hyperosmolarity-induced tension in rat soleus muscle (Clausen et al., 1974) suggest that the hormone may induce a rise in the cytosolic concentration of free Ca^{2+} ions. In developing muscle cells from chick embryo breast muscle (in culture), insulin was also found to increase Ca^{2+} uptake, the mitochondrial Ca^{2+} pool and the apparent rate constant for Ca^{2+} efflux (Schudt et al., 1976).

In cardiac and skeletal muscle, insulin stimulation of sugar transport was depressed in the absence of extracellular Ca^{2+} or in the presence of the Ca^{2+} chelator EGTA, in the presence of heavy metal ions, or the Ca^{2+} channel blocker methoxyverapamil (D600) (Bihler, 1980). In rat hemidiaphragm, insulin increased ^{45}Ca uptake and 3OMG transport in parallel, and in Ca^{2+}-free medium stimulated the efflux of both 3OMG and ^{45}Ca from preloaded rat soleus muscle (Bigornia and Bihler, 1985). A parallel relationship between insulin stimulation of sugar transport and sarcolemmal Ca^{2+} fluxes has not always been demonstrated (Czech, 1980).

In light of the above findings it was of interest to assess the role of Ca^{2+} in the stimulation of sugar transport by insulin in adrenal chromaffin cells. Because of the variation in basal 3OMG uptake rates between cell batches (Table 1), data are presented as percentages

of the control, and the control rate is given in nmol/mg protein/min. Specifically, the effect of insulin on the uptake of 2 mM 3OMG was examined in the presence and absence of extracellular Ca^{2+}, the Ca^{2+} channel blocker methoxyverapamil and the Ca^{2+}-antagonistic ion La^{3+}.

Methoxyverapamil (commonly known as D600) belongs to a group of drugs termed Ca^{2+} antagonists. The site of action appears to be the voltage-activated Ca^{2+} ion selective slow channel, and hence they would more appropriately be called slow channel inhibitors. Using the patch clamp technique, Lee and Tsien (1983) showed that D600 strongly inhibited both outward and inward currents through Ca^{2+} channels, but had no effect on activation gating. D600 was also shown to be use- or frequency-dependent in its blockade i.e. drug inhibition was accentuated by repetitive membrane depolarization. Both activated open and inactivated Ca^{2+} channels are blocked by D600 (Lee and Tsien, 1983). Reduction of the slow Ca^{2+} current by Ca^{2+} channel blockers results in their negative inotropic effect on cardiac muscle. The specificity of these drugs is not absolute, and they may also affect other pathways which are not voltage-sensitive. D600 is lipophilic and can readily enter the cell and possibly affect intracellular Ca^{2+} transport sites at the mitochondria, inner face of the sarcolemma and sarcoplasmic reticulum (Nayler and Poole-Wilson, 1981).

Figure 9 shows that in $NaHCO_3$-buffered medium, insulin (50 mU/ml) significantly increased ($P<0.001$) the basal uptake of 2 mM 3OMG to 36% of control. Omission of external Ca^{2+} did not alter basal transport but abolished the stimulatory effect of insulin. D600 stimulated basal uptake but depressed insulin-stimulated transport by

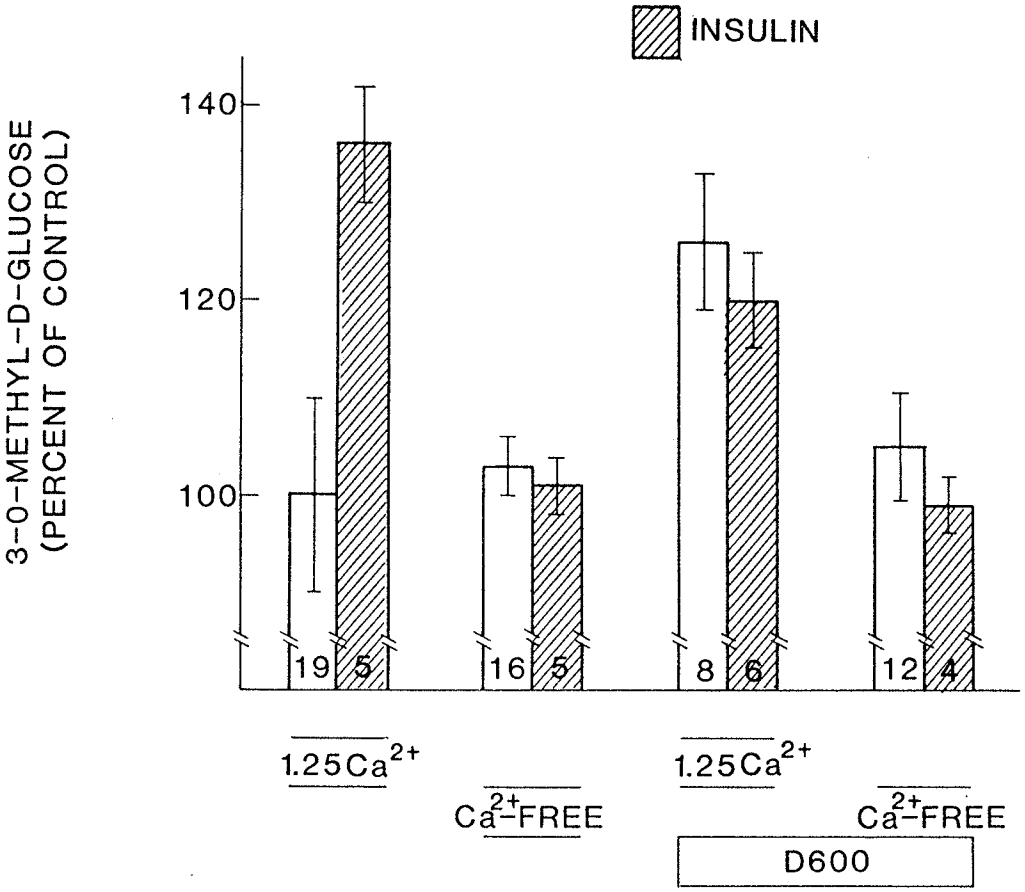


FIG. 9. Effect of insulin (50 mU/ml) on 3OMG uptake in the presence and absence of extracellular Ca^{2+} and the Ca^{2+} channel blocker methoxyverapamil (D600). Cells were incubated with 2 mM 3OMG as described in METHODS. The control uptake of 3OMG was 0.42 $\pm$ 0.04 (19) nmol/mg protein/min. Numbers in brackets denote the number of separate experiments, each done in triplicate.

16% in Ca^{2+}-containing medium. D600 had no significant effect on basal and insulin-stimulated 3OMG transport in nominally Ca^{2+}-free medium.

In rat atria the stimulatory effects of insulin, hyperosmolarity, and Na^{+} pump inhibition were inhibited by omission of Ca^{2+} and by D600 (Bihler and Sawh, 1980). Similarly, D600 was also shown to reduce transport stimulated by insulin in adrenal chromaffin cells (Figure 9). These observations suggest that the stimulatory effect of insulin depends on the presence and perhaps influx of external Ca^{2+}. In guinea pig atria D600 increased sugar transport under basal conditions in the presence and absence of external Ca^{2+}. This effect may be unrelated to alterations in Ca^{2+} influx and may reflect a displacement of Ca^{2+} from sites concerned with membrane stability (Bihler and Sawh, 1980). The observation that D600 significantly increased basal 3OMG uptake (Figure 9) in isolated bovine adrenal chromaffin cells is consistent with these findings. The stimulation of basal transport but inhibition of activated transport has also been observed with the benzodiazepine chlordiazepoxide (Bihler and Sawh, 1978) and the Ca^{2+} ionophore A23187 (Bihler et al., 1980b), compounds which have the ability to affect membrane stability.

Lanthanide ions are known to compete with Ca^{2+} for superficial binding sites (Langer et al., 1974) and to interfere with Ca^{2+} influx (Kolhardt et al., 1973; Nayler and Anderson, 1965; Sabbatini-Smith and Holland, 1969; Weiss, 1974). In experiments using La^{3+}, the standard medium was buffered with 25 mM triethanolamine.HCl instead of $NaHCO_3$ and NaH_2PO_4 and gassed with 100% O_2 instead of 95%O_2:5%CO_2 in order to avoid precipitation of La^{3+}. Figure 10 shows the effect of insulin on

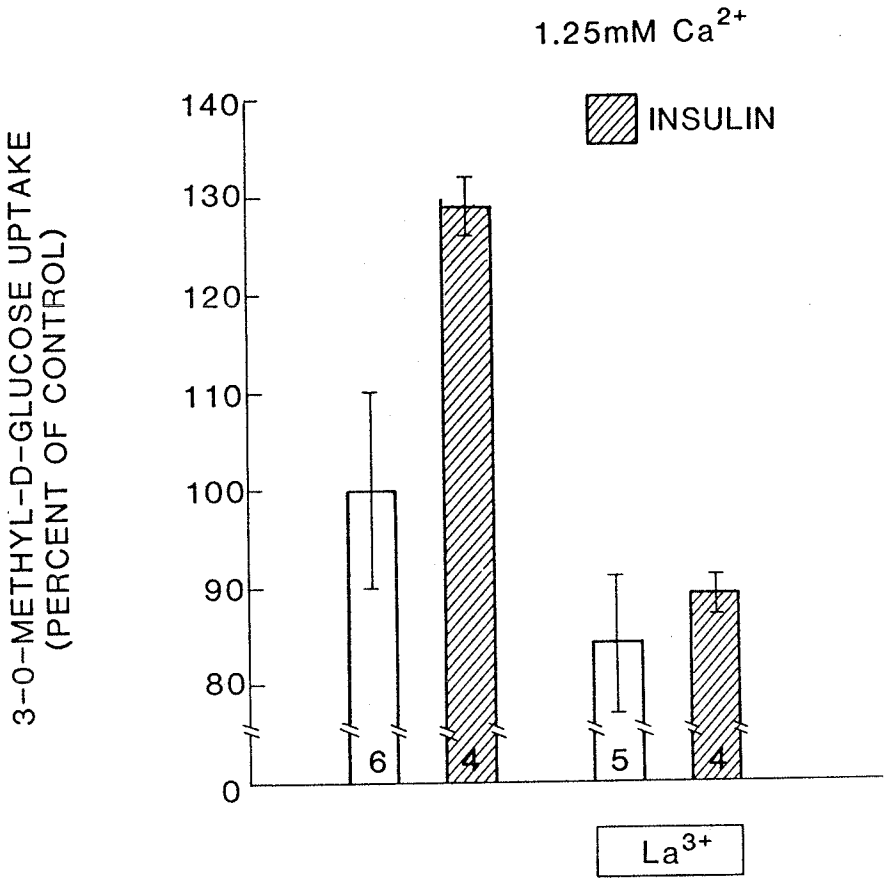


FIG. 10. Effect of 1.0 mM La^{3+} on basal and insulin-stimulated 3OMG uptake in the presence of extracellular Ca^{2+}. Cells were incubated with 1 mM La^{3+} as described in METHODS. Experiments using La^{3+} were done in standard medium buffered with 25 mM triethanolamine replacing 9.6 mM NaH_2PO_4 and 23.8 mM $NaHCO_3$. The control uptake of 3OMG was 0.63 $\pm$ 0.06 (6) nanomol/mg protein/min. Numbers in brackets denote the number of separate experiments, each done in quadruplicate.

basal transport in adrenal chromaffin cells in the presence and absence of La^{3+}. In medium buffered with triethanolamine, 50 mU/ml insulin stimulated 3OMG uptake by 30% of control. La^{3+} decreased basal transport by approximately 16% and insulin-stimulated transport by 40%. Thus La^{3+} appears to inhibit insulin-stimulated transport more than basal uptake.

In skeletal muscle (Bihler, 1972) and atrial muscle (Bihler et al., 1980a), La^{3+} (1 mM) strongly inhibited transport in the presence of insulin but did not affect basal transport. The inhibitory effects of La^{3+} may be partially overcome by doubling the external Ca^{2+} concentration. It should also be noted that low (0.1 mM) concentrations of La^{3+} stimulate sugar transport in atrial muscle, but have no significant effect on insulin-stimulated transport. Thus La^{3+} may behave as a partial agonist with some Ca^{2+}-like characteristics i.e. compete with Ca^{2+} for binding to specific sites. These Ca^{2+}-like effects are enhanced in Ca^{2+}-free medium (Bihler et al., 1980a). In the present study, the effect of 1 mM La^{3+} to depress the stimulatory effects of insulin in adrenal chromaffin cells is consistent with observations in muscle. The high basal uptake rate may indicate that the transport system is stimulated, and the effect of La^{3+} to reduce basal transport may actually reflect inhibition of the stimulatory effects by unknown factors.

The above data show that the stimulatory effect of insulin is abolished with the omission of extracellular Ca^{2+}, and depressed in the presence of the Ca^{2+} channel blocker D600 and the Ca^{2+}-antagonistic ion La^{3+}. These observations indicate that the stimulatory effect of

insulin depends on the presence and perhaps influx of extracellular Ca^{2+} and provide additional indirect evidence supporting a role for Ca^{2+} in the activation of sugar transport by insulin.

Current studies are directed towards the identification of specific Ca^{2+} pools or Ca^{2+}-binding sites which may be involved in insulin stimulation of glucose transport. Rosic et al. (1985) investigated the role of Ca^{2+} in insulin action in differentiated, cultured BC3H-1 myocytes. Although insulin-stimulated 2-deoxyglucose (2DG) uptake was not altered by the omission of extracellular Ca^{2+}, it was found to be dependent on the clamped average intracellular free Ca^{2+}. Intracellular free Ca^{2+} was clamped between 0-1.36 mM using 5 uM A23187 and varying concentrations of Ca^{2+} in the medium (fixed with Ca^{2+}-EGTA buffers). Insulin-stimulated glucose transport was also reduced in cells preloaded with Quin 2, a Ca^{2+} indicator dye used here as a Ca^{2+}-chelating agent and in the presence of compound 48/80, known as a specific calmodulin inhibitor. These results suggest that insulin activation of sugar transport depends on the availability of intracellular Ca^{2+} and may involve a calmodulin-dependent mechanism.

As discussed previously, more direct evidence may be obtained with the use of more sensitive techniques including fluorescent Ca^{2+} indicators for the detection of transient changes in cellular Ca^{2+} distribution. Mojsilovic et al. (1985) showed that insulin stimulated ^{45}Ca efflux from BC3H-1 muscle cells. In order to determine which Ca^{2+} pool was affected, they examined the effect of insulin on the signals produced by the fluorescent probe chlortetracycline, that forms highly fluorescent complexes with membrane bound Ca^{2+}, and the Ca^{2+} indicator,

Quin 2, which measures the intracellular free Ca^{2+}. Signals produced by either Quin 2 or chlortetracycline were not altered by insulin. The effect of insulin on ^{45}Ca efflux was also examined in the presence of TMB-8 [8-(N,N-diethylamino)-octyl 3,4,5-trimethoxybenzoate], an intracellular Ca^{2+} antagonist: TMB-8 was shown to inhibit the resting cellular Ca^{2+} influx and resting ^{45}Ca efflux in the guinea pig ileum preparation. It had no significant effect on the uptake of ^{45}Ca by sarcoplasmic reticulum preparation of skeletal muscle, but significantly inhibited the caffeine (20 mM)-induced release of ^{45}Ca from this preparation (Chiou and Malagodi, 1975). In the study by Mojsilovic et al. (1985), insulin-stimulated ^{45}Ca efflux was reduced in the presence of TMB-8. It was suggested that a TMB-8 accessible intracellular Ca^{2+} pool undetected by Quin 2 determinations, may contribute to the rise in ^{45}Ca efflux in the presence of insulin.

(b) Hyperosmolarity

Kuzuya et al. (1965) first showed that hyperosmolar solutions consisting of 200 mM sucrose, 100 mM NaCl or 200 mM mannitol added to 145 mM Na^+ medium stimulated glucose uptake in rat epididymal adipose tissue and diaphragm. The stimulatory effect of hyperosmolarity on glucose transport has since been demonstrated in several other preparations, including soleus muscle (Clausen, 1968; Clausen et al., 1970) and resting atria (Bihler and Sawh, 1977). In the perfused left atrium of the rat, hyperosmolarity increased sugar transport and also caused cell shrinkage, expansion of extracellular space and increase in intracellular Na^+ and ionic strength; opposite changes were induced by hypo-osmolarity (Bihler and Sawh, 1975).

Figure 11 shows the effect of hyperosmolar medium on 3OMG uptake in adrenal chromaffin cells. The addition of 100 mM mannitol to 145 mM Na^+ medium, producing hyperosmolar conditions, significantly ($P<0.001$) stimulated basal transport. The water content of the isolated adrenal chromaffin cells dropped from 84.1 $\pm$ 1.4% (11) under iso-osmolar conditions to 79.0 $\pm$ 2.2% (11) ($P<0.001$) under hyperosmolar conditions. It is unlikely that the hyperosmolarity-induced increase in transport rate was due only to cell shrinkage, as the percentage increase in transport rate of 37% was 6-fold greater than the percentage decrease of 6% in cell water.

In muscle, hyperosmolar conditions are associated with changes in cellular Ca^{2+} distribution which would presumably lead to increased cytosolic levels of free Ca^{2+}. The addition of nonpermeant solutes (100-400 mosmoles) increased the tension of rat soleus muscle in a

dose-dependent manner. This effect was reduced in the absence of external Na^+ or Ca^{2+}, and inhibited by the local anesthetic tetracaine. Tetracaine, which inhibits caffeine-induced contractures, originating from Ca^{2+} released by the sarcoplasmic reticulum, inhibited tension development in the rat soleus muscle. It was therefore suggested that hyperosmolarity-induced contractures (as with caffeine) originate from the release of Ca^{2+} from the sarcoplasmic reticulum. Hypertonicity (addition of 200 mM mannitol or sucrose to an isotonic medium) stimulated ^{22}Na and ^{45}Ca influx, and increased the efflux of ^{45}Ca from preloaded muscle in the presence and absence of external Ca^{2+} (Clausen et al., 1979). Addition of 200 mM mannitol markedly stimulated the fractional loss of ^{45}Ca followed by a similar rise in the washout of 3-[^{14}C] methylglucose in rat soleus and epididymal fat pads (Sorensen et al., 1980). Both in the presence and absence of extracellular Ca^{2+}, hyperosmolarity stimulated the efflux of ^{45}Ca from preloaded rat soleus muscles. In resting rat left atria, hyperosmolarity-induced stimulation of sugar transport was antagonized by omission of external Ca^{2+} and by the Ca^{2+} channel blocker D600. These observations suggest that hyperosmolar conditions lead to a rise in the intracellular concentration of free Ca^{2+}, partly due to mobilization of Ca^{2+} from intracellular pools, but to a considerable extent supplemented from extracellular sources.

Figure 11 demonstrates that the effect of hyperosmolarity on 3OMG uptake in chromaffin cells is also Ca^{2+}-dependent. The data show 3OMG uptake in the presence and absence of extracellular Ca^{2+} and the Ca^{2+} channel blocker methoxyverapamil (D600). Omission of external Ca^{2+}

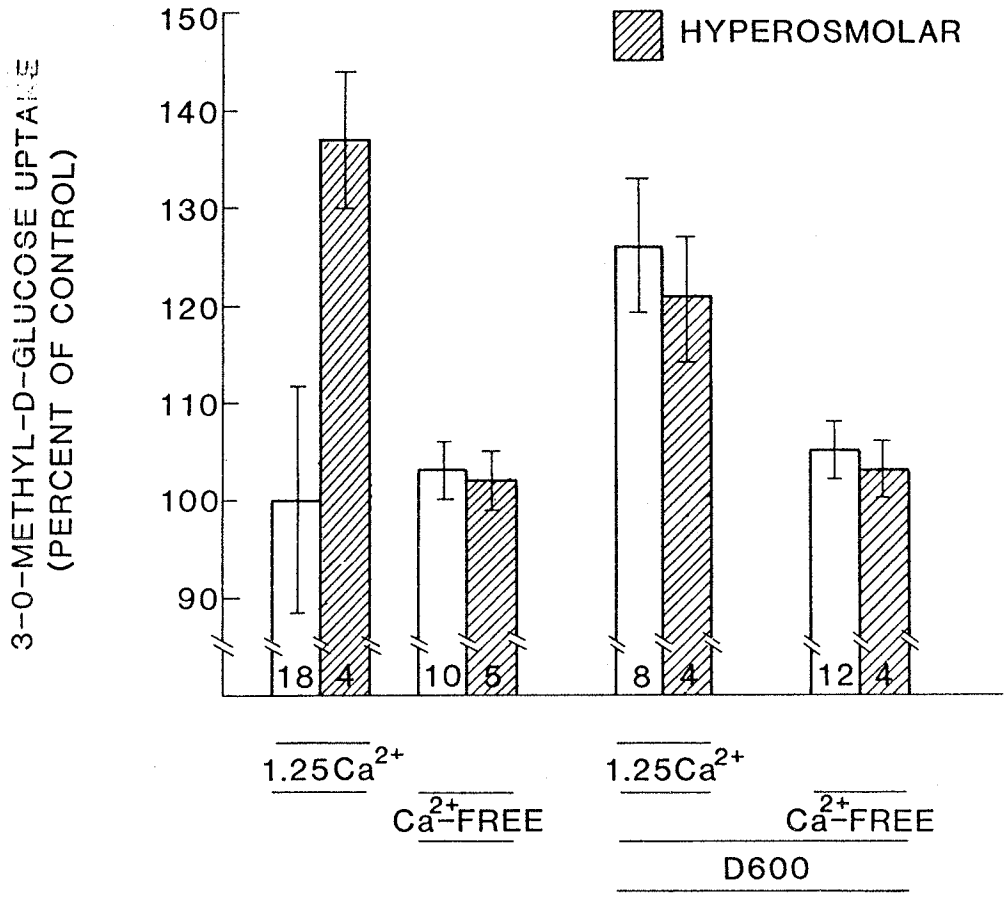


FIG. 11. Effect of hyperosmolarity on the 3OMG uptake in the presence and absence of extracellular Ca^{2+} and the Ca^{2+} channel blocker methoxyverapamil (D600). 100 mM mannitol was added to 145 mM Na^{+} medium to produce hyperosmolar conditions. The cells were incubated with 2 mM 3OMG as described in METHODS. The control uptake of 3OMG was 0.39 $\pm$ 0.05 (20) nmol/mg protein/min. Numbers in brackets denote the number of separate experiments, each done in triplicate.

did not alter basal transport but abolished the stimulatory effect of hyperosmolar medium. D600 (2 uM) significantly stimulated basal transport in the presence of external Ca^{2+}. As described previously (Figure 9), this stimulatory effect on basal transport may be related to the ability of D600 to displace Ca^{2+} from sites involved in maintaining membrane stability (Bihler and Sawh, 1980a). 3OMG uptake under hyperosmolar conditions was depressed by 2 uM D600 in the presence of external Ca^{2+} but was not altered in Ca^{2+}-free medium. This is consistent with the effects of D600 on hyperosmolarity-stimulated transport in rat atria (Bihler and Sawh, 1980).

Figure 12 shows the effect of hyperosmolarity on basal transport in the presence and absence of the Ca^{2+}-antagonistic ion La^{3+}. In medium buffered with triethanolamine, control uptake was significantly stimulated by hyperosmolar medium. The hyperosmolarity induced increase (14%) was less than in $NaHCO_3$-buffered medium (37%) as shown in Figure 11, perhaps because of the relatively high basal transport rate in triethanolamine-buffered medium. La^{3+} significantly decreased both basal and hyperosmolarity stimulated transport by 16% and 31%, respectively. Thus La^{3+} appears to inhibit hyperosmolarity-stimulated transport more than basal uptake. As described above, the ability of La^{3+} to inhibit basal transport may reflect inhibition by La^{3+} of unknown stimulatory effects under basal conditions. The observation that La^{3+} antagonized hyperosmolarity-stimulated transport is consistent with the effects of high concentrations of La^{3+} on stimulated transport in muscle (Bihler, 1972; Bihler et al., 1980a).

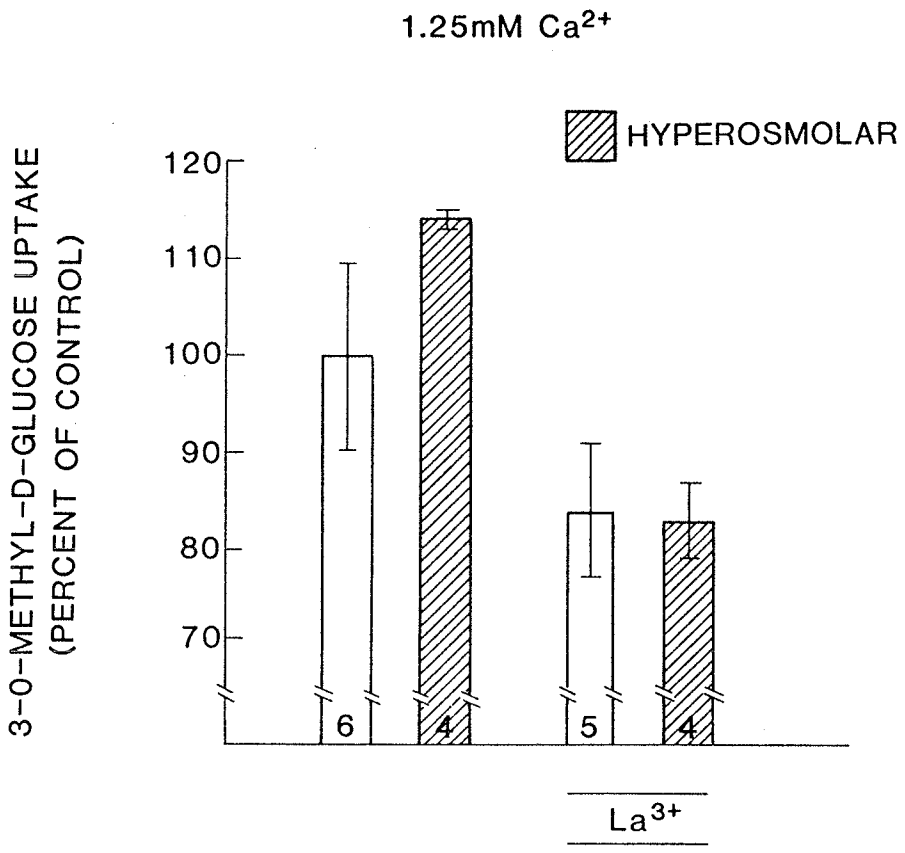


FIG. 12. Effect of 1.0 mM La^{3+} on basal and hyperosmolarity-stimulated 3OMG uptake in the presence of external Ca^{2+}. Experiments using La^{3+} were done in medium buffered with triethanolamine as described in Figure 10. The control uptake of 3OMG was 0.63 $\pm$ 0.06 (6) nmol/mg protein/min. Numbers in brackets denote the number of separate experiments, each done in quadruplicate.

In summary, 3OMG uptake in adrenal chromaffin cells was stimulated by hyperosmolarity, another factor shown to enhance glucose transport in muscle and adipose tissue. As with insulin, the activation of sugar transport under hyperosmolar conditions depends on the presence and perhaps influx of external Ca^{2+}.

(c) Secretory Stimuli

Douglas and Rubin (1961) first showed that the stimulation of catecholamine release from adrenal chromaffin cells by acetylcholine or K^+ depolarization was dependent on the presence of external Ca^{2+}. Several studies have since demonstrated that exocytosis is associated with a rise in the cytoplasmic free Ca^{2+}, and it is now well accepted that exocytosis is a Ca^{2+}-dependent process (Douglas, 1968; Putney, 1979). Although new techniques (fluorescent dyes, patch clamp, high voltage permeabilization) have further elucidated the nature of changes in cellular Ca^{2+} distribution associated with the secretory process (Baker and Knight, 1985), the underlying mechanism whereby the rise in cytosolic Ca^{2+} triggers catecholamine release has yet to be determined.

Ca^{2+} may be involved in dispersing cytoskeletal elements such as actin, which may act to stabilize or restrain the movement of chromaffin granules to the next stage of exocytosis. The fusion of liposomes in vitro was promoted by Ca^{2+} at concentrations of 1-2 mM, which are relatively high compared to those prevailing during exocytosis. However it is possible that even the small rise in cytoplasmic Ca^{2+} occuring during exocytosis may activate other factors which promote membrane fusion. These include the calcium binding proteins calmodulin and synexin. Trifluoperazine, a calmodulin inhibitor, and calmodulin antibodies were shown to block catecholamine secretion, suggesting that calmodulin may be involved in exocytosis. As described previously, synexin caused the aggregation of chromaffin granules, and the further addition of arachidonic acid resulted in the fusion of the aggregated granules. In addition, trifluoperazine and promethazine, which block synexin were shown to inhibit catecholamine

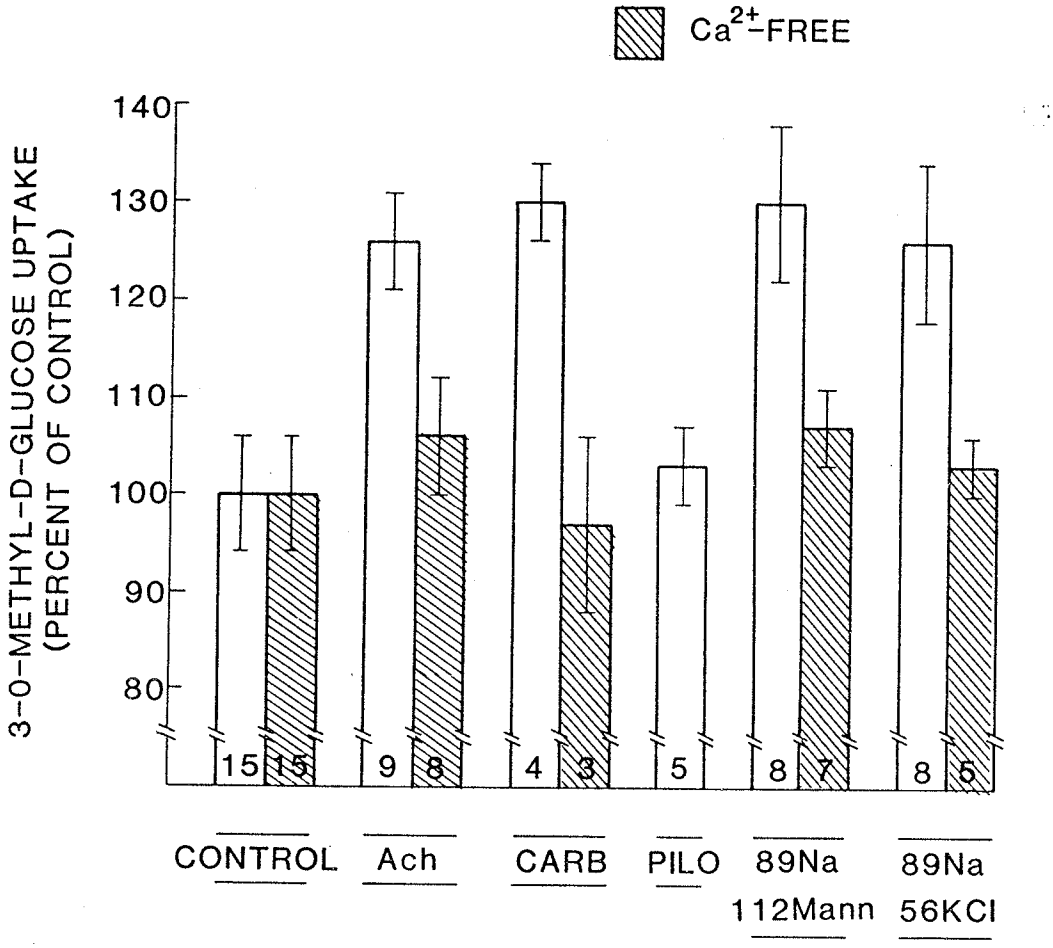


FIG. 13. Effect of secretory stimuli on 3OMG uptake in the presence and absence of extracellular Ca^{2+}. Cells were incubated with 3 mM 3OMG as described in METHODS. In low Na^{+} (89 mM) control, mannitol was used to replace Na^{+}. The control uptake was 0.50 $\pm$ 0.05 (14) nmol/mg protein/min in the presence of external Ca^{2+} and 0.70 $\pm$ 0.04 (15) nmol/mg protein/min in nominally Ca^{2+}-free medium. Numbers in brackets denote the number of separate experiments, each done in triplicate.

secretion. These observations are consistent with a role of the Ca^{2+} binding protein synexin in exocytosis (for review, see Pollard et al., 1985).

The rise in cytoplasmic Ca^{2+} associated with the secretory process may also result in the activation of the sugar transport system in secretory cells. Korc et al. (1979) showed that the cholecystokinin analogue caerulein stimulated in parallel amylase secretion, 2-DG uptake and ^{45}Ca efflux in isolated mouse pancreatic acini, another type of secretory cell preparation. In the same preparation, the Ca^{2+} ionophore A23187 also increased amylase release and 2DG uptake. Millaruelo et al. (1982a) reported that in adrenal chromaffin cells, acetylcholine stimulated glucose utilization and glucose oxidation, and this effect was most clearly seen within 30-60 min of exposure to acetylcholine. It was suggested that the increased glucose utilization was probably in response to a higher demand for energy, perhaps in order to replenish chromaffin granules.

Figure 13 shows the effects of secretory stimuli on 3OMG uptake in adrenal chromaffin cells in the presence and absence of external Ca^{2+}. Basal uptake was significantly stimulated ($P<0.001$) by the nicotinic agonists, acetylcholine (0.1 mM) and carbamylcholine (0.5 mM) but remained unchanged in the presence of the muscarinic agonist pilocarpine (0.5 mM). Reduction of extracellular Na^+ from 145 mM to 89 mM, with iso-osmolar replacement of Na^+ with mannitol, also significantly stimulated basal uptake. However, the addition of a depolarizing concentration of KCl (56 mM) with the simultaneous omission of mannitol from low Na^+ medium, did not cause any further

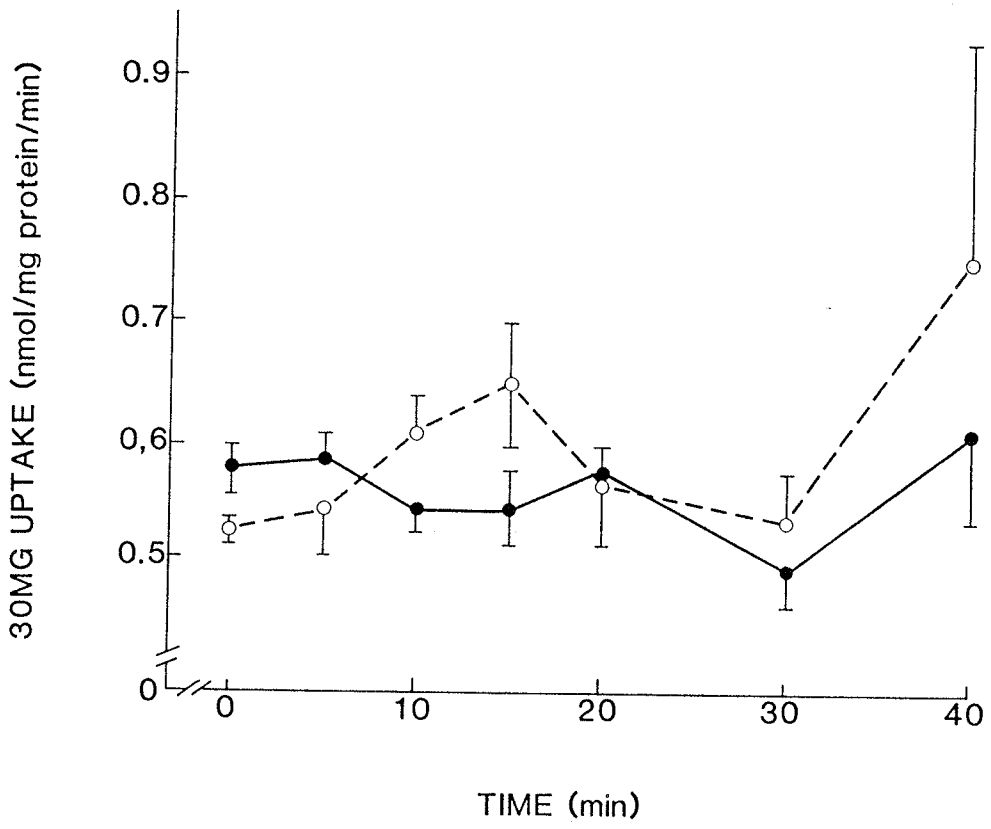


FIG. 14. Effect of preincubation time on the stimulation of 3OMG uptake by acetylcholine. The duration of preincubation in the presence (O---O) or absence (●——●) of 0.1 mM acetylcholine is shown on the abscissa. This preincubation period was followed by the standard incubation time of 2.5 min (Figure 1). Each point represents the mean ± S.E. of 2 separate experiments, each done in duplicate.

increases in 3OMG uptake over that produced by the low Na^+ (89 mM) control.

In nominally Ca^{2+}-free medium, basal transport was not significantly altered by 0.1 mM acetylcholine or 0.5 mM carbamylcholine. However reduction of external Na^+ from 145 mM to 89 mM caused a small but significant ($P < 0.005$) increase in 3OMG uptake even in the absence of extracellular Ca^{2+}. The addition of a depolarizing concentration of KCl (56 mM) to low Na (89 mM) medium did not cause any further increase in the value of 3OMG uptake.

If the activation of sugar transport by secretagogues depends on their effects on cellular Ca^{2+} distribution during the secretory process, the stimulation of glucose transport should coincide with the increase in catecholamine secretion, which is maximal within 5-10 min of exposure to 10^{-4}M acetylcholine (Hochman and Perlman, 1976). Alternatively or additionally, transport activation may be in response to a higher energy demand for replenishment of catecholamine stores (Millaruelo et al., 1982a), and hence would occur at a later stage.

Figure 14 shows the effect of preincubation time on acetylcholine stimulation of 3OMG uptake. Acetylcholine stimulation of sugar transport appeared to occur in two phases: the first after 10-15 min of exposure to acetylcholine and the second after 30 min preincubation with acetylcholine. Transport in the presence of acetylcholine was significantly increased to 117% and 120% of control after 10 and 15 min of exposure to acetylcholine, respectively. It dropped back to basal at 20 min, but was stimulated to 109% and 133% of control after 30 and 40 min exposure, respectively. This suggests that

both the above described mechanisms may be involved in the activation of sugar transport by acetylcholine.

In summary, basal 3OMG uptake was stimulated by factors or conditions (acetylcholine, carbamylcholine and low Na^+ medium) which have also been shown to stimulate the Ca^{2+}-dependent release of catecholamines from bovine adrenal chromaffin cells (Livett, 1984; Lastowecka and Trifaro, 1974). The muscarinic agonist pilocarpine, which does not elicit catecholamine secretion in bovine adrenal chromaffin cells (Livett, 1984) also did not alter sugar transport (Figure 13). Although a depolarizing concentration of KCl (56 mM) does stimulate catecholamine secretion, it did not stimulate sugar transport. It is possible that the sugar transport system is already maximally stimulated by the reduction of external Na^+, and the additional factor of depolarization will be unable to cause a further increase in activity. In addition, the effects of nicotinic agonists, acetylcholine and carbamylcholine to stimulate sugar transport appear to depend on the presence of external Ca^{2+}. The ability of low Na^+ (89 mM) medium to partially maintain its stimulatory effect on 3OMG uptake uptake in the absence of extracellular Ca^{2+} suggests that mechanisms other than Ca^{2+} influx may be involved. The observations also suggest that transport activation by secretagogues may be related to their effects on cellular Ca^{2+} distribution during the secretory process in that the stimulation of sugar transport coincided with the secretory process. The results also suggest that sugar transport may be stimulated after completion of the secretory process, perhaps in response to a higher energy demand for restoring the cell to the pre-stimulated state (e.g. replenishment of secretory granules).

(d) ^{45}Ca Uptake

As described earlier, some transport regulators cause parallel changes in sugar transport and Ca^{2+} influx. Ca^{2+} uptake was stimulated by insulin in developing muscle cells from chick embryo breast (Schudt et al., 1976) and in rat hemidiaphragm (Bigornia and Bihler, 1985) and by hyperosmolarity in rat soleus muscle (Clausen et al., 1979). The hyperosmolarity-induced increase in Ca^{2+} influx in muscle may be due to several mechanisms including the activation of Na^{+}/Ca^{2+} exchange by increased internal Na^{+} associated with cell shrinkage under hyperosmolar conditions. As discussed at length later, several other factors or conditions, which enhance Ca^{2+} influx via Na^{+}/Ca^{2+} exchange, have also been shown to stimulate glucose transport in muscle. These include ouabain, K^{+}-free medium, metabolic inhibitors (Bihler, 1968; Bihler and Sawh, 1975, 1977, 1980) and the Na^{+} ionophore monensin (Bihler et al., 1985a, 1985b).

Secretory stimuli may increase the intracellular concentration of free Ca^{2+} generally through their effects to enhance Ca^{2+} influx through Ca^{2+} channels. Three different types of Ca^{2+} channels are believed to exist in chromaffin cells: (1) Ca^{2+} channels associated with the nicotinic receptor complex that are insensitive to tetrodotoxin (TTX), Mn^{2+} and Co^{2+} and for which Na^{+} and Ca^{2+} appear to compete; (2) voltage-sensitive Ca^{2+} channels sensitive to Co^{2+} and (3) a separate D600-sensitive Ca^{2+} channel activated as a result of Na^{+} and Ca^{2+} entry through the voltage-sensitive channels (Livett, 1984). It has been demonstrated that an increase in Ca^{2+} uptake precedes the secretory response to depolarizing agents and nicotinic agonists. Depolarizing agents such as veratridine and 56 mM KCl stimulated Ca^{2+}

uptake through voltage-sensitive calcium channels, while nicotinic agonists stimulate Ca^{2+} entry through acetylcholine receptor ion channels, as well as through voltage-sensitive Ca^{2+} channels (Kilpatrick et al., 1982).

The effects of factors shown to stimulate sugar transport in adrenal chromaffin cells, including insulin, hyperosmolarity and secretory stimuli were examined on ^{45}Ca uptake.

The time course of ^{45}Ca uptake was measured in isolated bovine adrenal chromaffin cells, as shown in Figure 15, and found to be consistent with observations from other studies of ^{45}Ca uptake in isolated chromaffin cells (Garcia et al., 1984). On the basis of these results, a standard incubation period of 1.5 min was chosen, representing a point near the top of the linear portion of the curve, so that uptake values presented here reflect initial unidirectional influx rates.

La^{3+} is commonly used in calcium exchange experiments in muscle to block calcium fluxes after a test intervention. The 50 mM La^{3+} solution originally described by Godfraind (1976), which consists of 122 mM NaCl, 5.9 mM KCl, 1.25 mM $MgCl_2$, 50 mM $LaCl_3$, 11 mM glucose, and 15 mM Tris-maleate (pH 7.6) proved to be harmful to the integrity of the freshly isolated adrenal chromaffin cells, as indicated by the abnormally high extracellular space values. Adjustment of the Godfraind buffer to contain 1 mM La^{3+} did not improve the extracellular space values. Instead La^{3+} was dissolved in triethanolamine buffered standard medium used in sugar transport experiments described earlier (Figures 10 and 12). Table 4 shows that the addition of La^{3+} (0 to 10 mM) to the

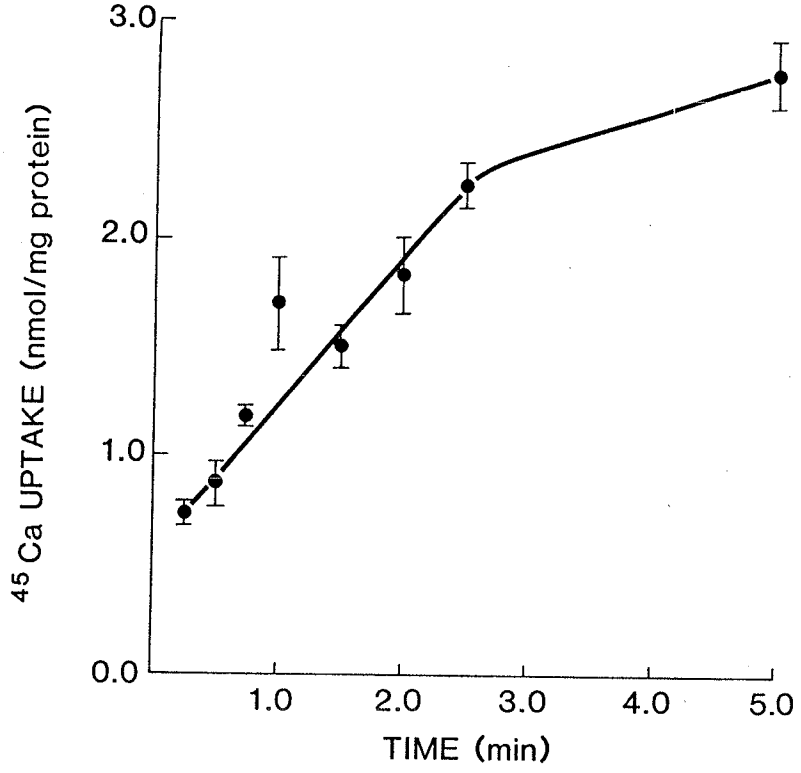


FIG. 15. Time course of ^{45}Ca uptake. Cells were incubated with 2 mM 3OMG as described in METHODS for the periods of time shown. Points represent the mean $\pm$ S.D. ^{45}Ca uptake rate (nmol/mg) of quadruplicate samples from one representative experiment.

TABLE 4

EFFECT OF La^{3+} IN THE STOPPING SOLUTION ON ^{45}Ca UPTAKE

^{45}Ca uptake was measured under basal conditions as described in METHODS and the reaction terminated in stopping solution containing various amounts of La^{3+}. Numbers in brackets indicate the number of experiments, each done in quadruplicate.

La^{3+} (mM)	^{45}Ca Uptake (nmol/mg protein/min)
0.0	0.39 ± 0.02 (2)
0.1	0.38 ± 0.02 (2)
1.0	0.40 (1)
10.0	0.39 (1)

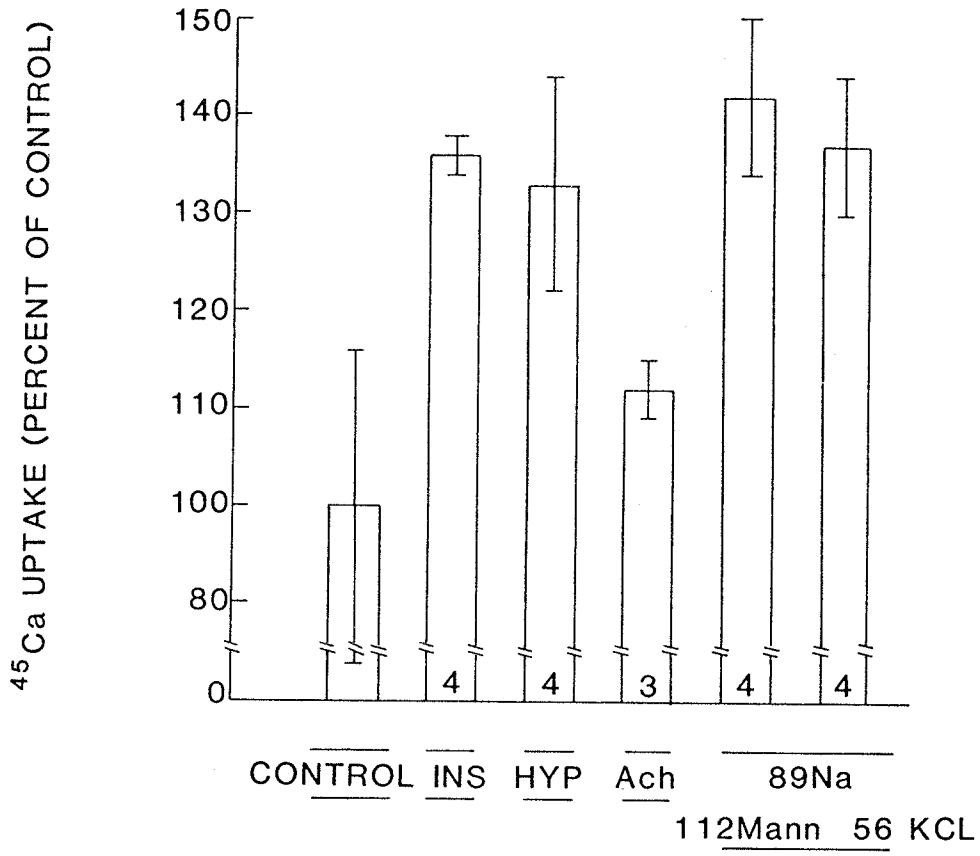


FIG. 16. Effect of 50 mU/ml insulin, hyperosmolarity, and secretory stimuli on ^{45}Ca uptake. Cells were incubated with 2 mM 3OMG as described in METHODS. The control uptake of ^{45}Ca was 0.90 $\pm$ 0.15 (10) nmol/mg protein/min. Numbers in brackets represent the number of experiments, each done in triplicate.

ice cold stopping solution did not alter basal ^{45}Ca uptake, and hence did not appear to help in rapidly terminating Ca^{2+} uptake. Hence it did not seem necessary to include La^{3+} in the stopping solution.

Figure 16 shows that insulin (50 mU/ml), acetylcholine, hyperosmolar and low Na^+ (89 mM) medium significantly stimulated ^{45}Ca uptake. The further addition of 56 mM KCl to low Na^+ medium did not cause any additional effects.

Thus in adrenal chromaffin cells, insulin, acetylcholine, hyperosmolar and low Na^+ medium caused parallel increases in sugar transport (Figures 7 - 14) and ^{45}Ca uptake (Figure 16). A temporal relation between the effects of regulators (insulin, secretory stimuli) on ^{45}Ca uptake and sugar transport was not examined. However, studies have shown that sugar transport activation by several factors was preceded or coincided with an increase in ^{45}Ca efflux from preloaded muscle and fat tissue (Sorenson et al., 1980; Clausen et al., 1981).

(e) Na^+/Ca^{2+} Exchange

Factors which increase intracellular Na^+ stimulate sugar transport in muscle. In the presence of external Ca^{2+}, the Na^+ ionophore monensin increased Na^+ content and sugar transport in mouse diaphragm (Bihler et al., 1985a) and in avian erythrocytes (Bihler, 1985b). Monensin also increased ^{45}Ca influx in mouse diaphragm (Bihler et al., 1985a) presumably through activation of Na^+/Ca^{2+} exchange. These effects were not related to inhibition of the Na^+ pump, as monensin was shown to stimulate Na^+ pump activity in the mouse diaphragm. The stimulatory effect of monensin on sugar transport was maintained in Ca^{2+}-free medium in both mouse diaphragm and erythrocytes, suggesting that the release of intracellular stores of Ca^{2+} may also be involved in the activation of sugar transport by monensin. When rat hindlimb was perfused with Ca^{2+}-free medium, its mitochondrial Ca^{2+} content was decreased; monensin caused a further reduction perhaps by stimulating mitochondrial Na^+/Ca^{2+} exchange (Bihler et al., 1985a). These observations suggest that monensin may stimulate sugar transport by enhancing Ca^{2+} influx via sarcolemmal Na^+/Ca^{2+} exchange in muscle and/or by releasing intracellular Ca^{2+} stores in erythrocytes and muscle, presumably from the mitochondria.

When the Na^+ pump is inhibited, sugar transport was shown to be activated in the rat diaphragm (Bihler, 1968) and cardiac muscle (Bihler and Sawh, 1977; 1980). Conversely sugar transport is decreased when the Na^+ pump is stimulated by low concentrations of digitaloids (Bihler and Sawh, 1980) and adrenaline (Bihler and Sawh, 1976) or by anticonvulsant drugs such as diphenylhydantoin (Bihler and Sawh, 1978). The parallelism between the levels of intracellular Na^+ and rate of

sugar transport is also illustrated by the concentration-dependent effects of cardiac glycosides on sugar transport. In resting cardiac, skeletal and smooth muscle in vitro, ouabain and other cardiac glycosides have a dual effect on Na^+ and K^+ gradients. At very low concentrations (10^{-9}M) of ouabain, stimulation of the Na^+ pump and decreased intracellular levels of Na^+ were accompanied by inhibition of sugar transport. With high concentrations of ouabain (10^{-5}M) the classical inhibition of the Na^+ pump led to increases in intracellular Na^+ and in sugar transport (Bihler, 1968; Bihler and Sawh, 1975). Ouabain concentrations which inhibit the Na^+ pump, stimulated sugar transport in normoxia but did not cause a further increase in sugar transport in the diaphragm during anoxia. They did so in the detrusor of rat urinary bladder, a smooth muscle able to use anaerobic energy for Na^+ pumping. In contracting cardiac muscle, there is an additional stimulation of sugar transport which depends on the inotropic effect of ouabain and is unrelated to the Na^+ and K^+ distribution (Bihler, 1968; Bihler and Sawh, 1975).

As discussed in the Introduction, the effect of Na^+ pump inhibition to stimulate sugar transport is believed to depend on the presence of a Na^+/Ca^{2+} exchange system: Inhibition of the Na^+ pump results in accumulation of intracellular Na^+, which shifts Na^+/Ca^{2+} exchange towards net Ca^{2+} uptake across the plasma membrane (Reuter, 1974; Baker, 1970). This results in increased cytosolic levels of free Ca^{2+}, and presumably the stimulation of sugar transport (Elbrink and Bihler, 1975). If the Na^+/Ca^{2+} exchange system is present in adrenal chromaffin cells, reduction of the Na^+ gradient or increase in intracellular Na^+ levels should stimulate Ca^{2+} influx, inhibit Ca^{2+}

efflux and activate Ca^{2+}-dependent sugar transport or catecholamine secretion.

In the present study the effects on Na^+ content, Ca^{2+} fluxes and 3OMG uptake of factors which increase internal Na^+ (ouabain, K^+-free medium, monensin) or decrease the Na^+ gradient were investigated. As shown in Table 5, in chromaffin cells, cellular Na^+ content was significantly ($P < 0.001$) increased by 0.1 mM ouabain, K^+-free medium, and the Na^+ ionophore monensin. Cellular K^+ content was significantly decreased ($P < 0.001$) by ouabain and K^+-free medium. Monensin also decreased K^+ but to a lesser extent. These observations are consistent with data on other tissues and chromaffin cells (Livett, 1984).

In addition, Figure 17 shows that ouabain (0.1 mM) and K^+-free medium produced large increases in ^{45}Ca influx, whereas monensin caused a lesser increase. Complete replacement of external Na^+ with LiCl or mannitol increased ^{45}Ca uptake to 110% and 165% of control, respectively. The observations that K^+-free medium, ouabain and monensin increase intracellular Na^+ content and Ca^{2+} influx are consistent with the operation of a Na^+/Ca^{2+} exchange mechanism.

The presence of a plasma membrane Na^+/Ca^{2+} exchange system was further assessed by examining the effects of partial or complete omission of external Na^+ or addition of monensin on ^{45}Ca efflux from preloaded adrenal chromaffin cells. ^{45}Ca efflux into Ca^{2+}-containing medium was not significantly altered by 7.5×10^{-7}M monensin or complete replacement of external Na^+ with LiCl (Figure 18).

TABLE 5

EFFECT OF OUABAIN, K^+-FREE MEDIUM, AND MONENSIN ON CELLULAR Na^+ AND K^+ CONTENTS

In K^+-free medium, NaCl was increased by 5.4 mM to compensate for the loss of KCl. Data represent the millimolar concentrations of Na^+ and K^+ in the intracellular water. Numbers in brackets indicate the number of separate experiments, each done in quadruplicate.

	Na^+	K^+
CONTROL	46.0 ± 3.0 (14)	90.6 ± 8.5 (13)
0.1 mM OUABAIN	114.0 ± 5.0 (5)[c]	36.0 ± 3.0 (6)[c]
K^+-FREE	134.0 ± 11.0 (4)[c]	65.0 ± 4.0 (4)[c]
MONENSIN		
1×10^{-9} M	108.0 (1)	77.0 (1)
1×10^{-8} M	113.0 (1)	65.0 (1)
1×10^{-7} M	105.0 (1)	72.0 (1)
1×10^{-6} M	166.0 (1)	66.0 (1)

[c] $P < 0.001$

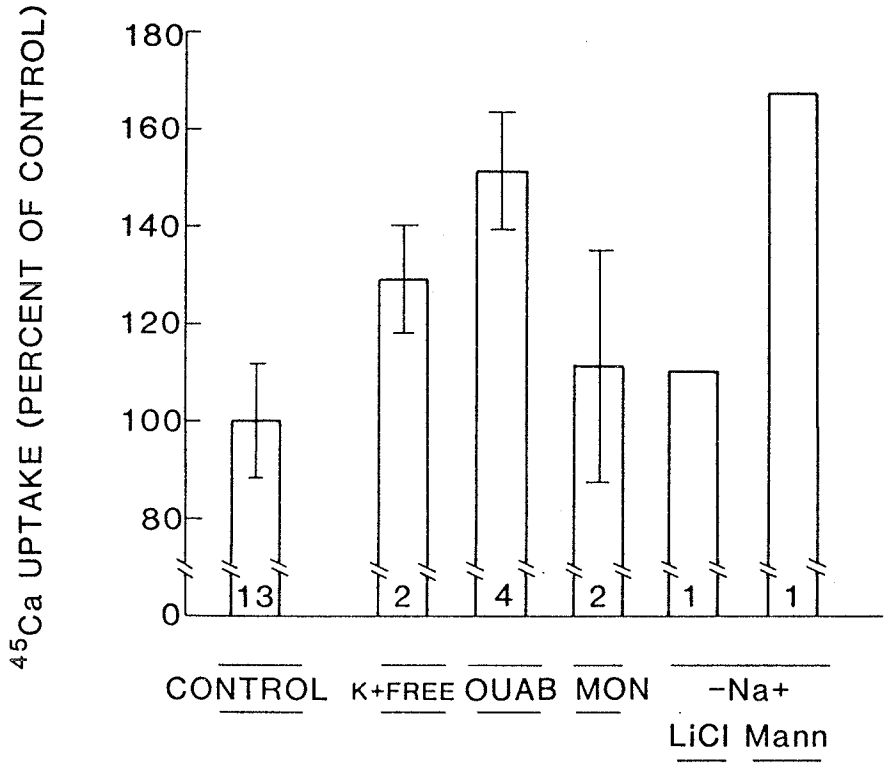


FIG. 17. Effect of K^+-free medium, 0.1 mM ouabain, monensin (7.5×10^{-7}M) and Na^+-free medium on ^{45}Ca uptake. In Na^+-free medium, 143.4 mM NaCl was replaced with an equimolar amount of LiCl or with 0.3M mannitol and the medium buffered with 25 mM triethanolamine. The cells were incubated with 2 mM 3OMG as described in METHODS. The control uptake for ^{45}Ca was 0.42 ± 0.05 (6) nmol/mg protein/min. Numbers in brackets denote the number of experiments, each done in triplicate.

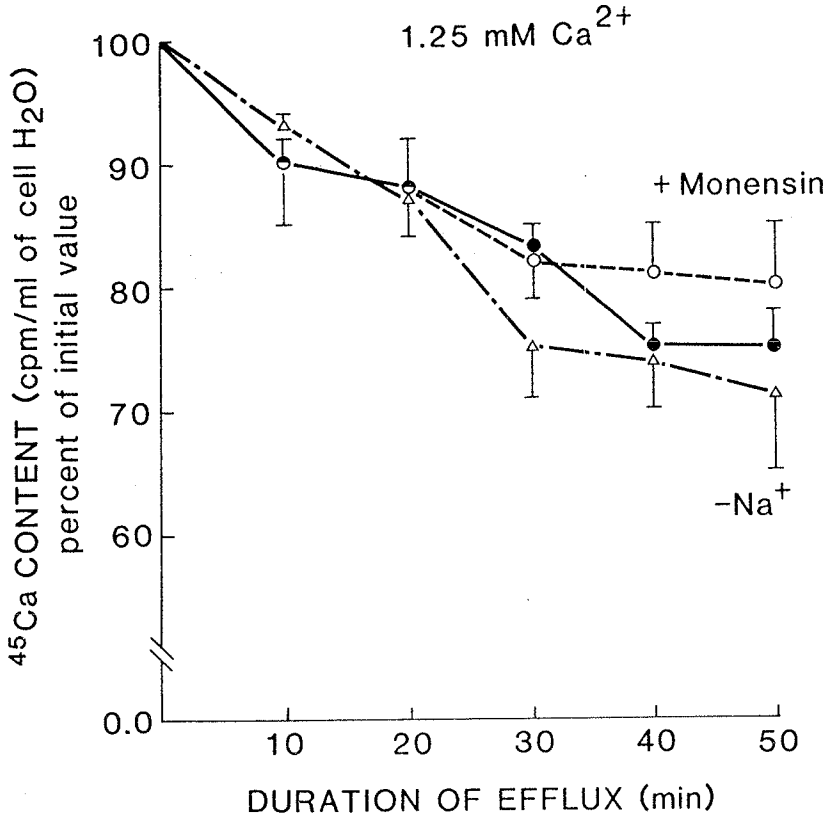


FIG. 18. ^{45}Ca efflux in the presence of 1.25 mM Ca^{2+} under control conditions (●——●); with the addition of 7.5 X 10^{-7}M monensin (0---0); and Na^+-free medium (Δ-·-Δ). Experiments were carried out in HEPES (25 mM) -buffered standard medium as described in METHODS. In Na^+-free medium, 143.4 mM NaCl was replaced with and equimolar amount of LiCl. Data are presented as the percent of cell ^{45}Ca content (cpm/ml cell water) at the start of incubation. Points represent the mean ± S.E. from 4 separate experiments, each done in duplicate.

Figure 19 shows that omission of extracellular Ca^{2+} significantly decreased ($P < 0.001$) the amount of Ca^{2+} remaining in the cells at all times tested after loading, i.e. it increased ^{45}Ca efflux. Reduction of extracellular Na^+ from 145 mM to 89 mM and iso-osmolar mannitol replacement decreased this loss in Ca^{2+}-free medium. These observations are consistent with the operation of a Na^+/Ca^{2+} exchange mechanism. In the absence of external Ca^{2+}, Na^+ more successfully competes for the external site on the Na^+/Ca^{2+} exchange protein. Consequently Na^+ influx and hence Ca^{2+} efflux should increase. However, when external Na^+ is reduced in Ca^{2+}-free medium, Na^+ competes less effectively. Thus Na^+ influx and Ca^{2+} efflux should be reduced.

Complete replacement of external Na^+ with mannitol in the absence of external Ca^{2+} also reduced ^{45}Ca efflux but 7.5×10^{-7}M monensin had no effect (Figure 20). These observations show that omission of external Ca^{2+} increases ^{45}Ca efflux and partial or complete replacement of external Na^+ with mannitol appears to reduce this effect. However complete replacement of external Na^+ with LiCl (Figure 18) or the addition of the Na^+ ionophore monensin (Figure 19 and 20) did not appear to affect ^{45}Ca efflux.

The effects of the factors or conditions described above on 3OMG uptake were investigated. Table 6 shows that Na^+ pump inhibition with K^+-free medium and 0.1 mM ouabain, metabolic inhibition with 2 mM KCN, and monensin (10^{-9}M and 10^{-8}M) and A23187 (1, 5, 10, 20 uM) did not significantly affect 3OMG uptake under basal conditions. In fact sugar transport was significantly ($P<0.01$) decreased to 83% of control by higher concentrations (10^{-7}M to 10^{-6}M) of monensin.

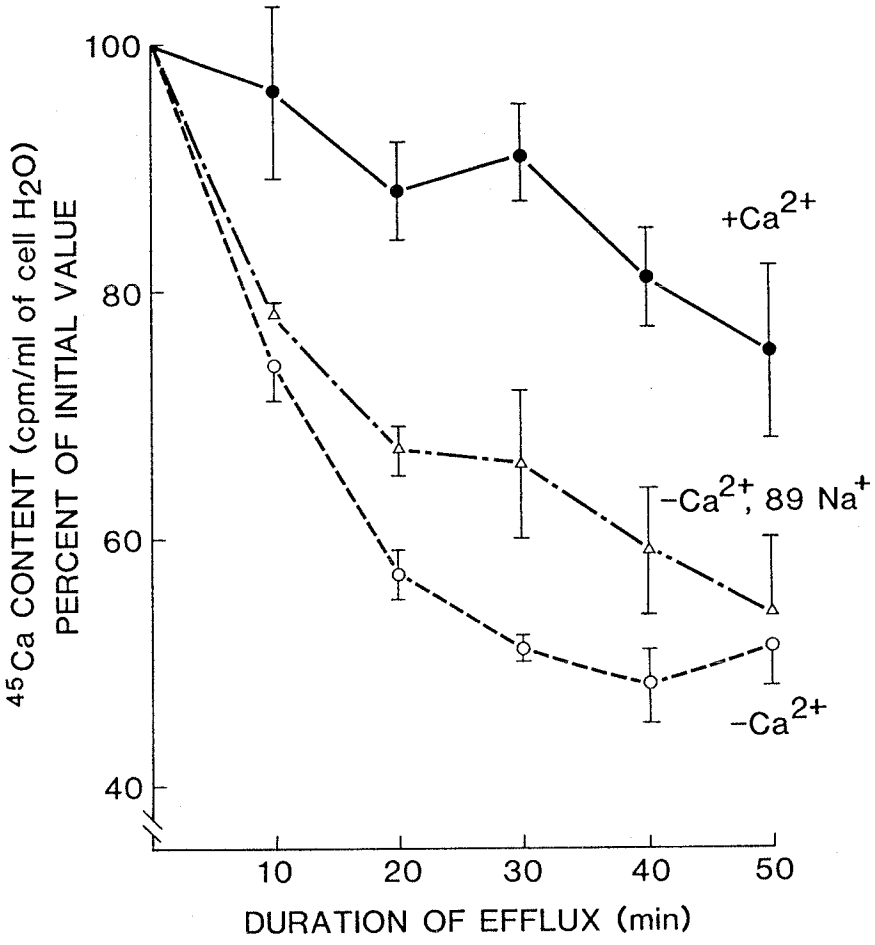


FIG. 19. ^{45}Ca efflux in the presence (●——●) and absence (0---0) of extracellular Ca^{2+} (1.25 mM) and in Ca^{2+}-free, low Na^+ (89 mM) Na^+ medium (Δ-·-Δ). Experiments were carried out in $NaHCO_3$-buffered medium, as described in METHODS. In low Na^+ medium osmolarity was maintained with 112 mM mannitol. Data are presented as described in Figure 17. Points represent the mean ± S.E. from four separate experiments, each done in duplicate.

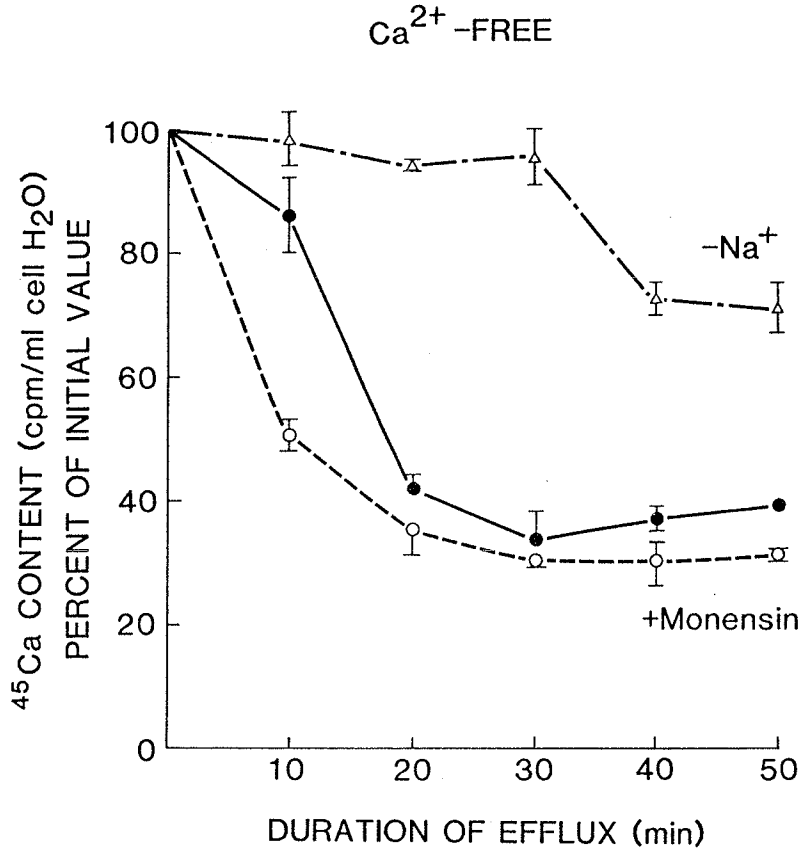


FIG. 20. ^{45}Ca efflux in the absence of extracellular Ca^{2+} under control conditions (●—●); with the addition of 7.5×10^{-7}M monensin (0---0); and in Ca^{2+}-free, Na^{+}-free medium (Δ-·-Δ). Experiments were done in triethanolamine-buffered medium as described in METHODS. In Na^{+}-free medium, Na^{+} was replaced with an iso-osmolar amount of mannitol. Data are presented as described in Figure 17. Points represent the mean ± S.D. of duplicate samples from one representative experiment.

TABLE 6

EFFECTS ON 3OMG UPTAKE OF SEVERAL FACTORS WHICH AFFECT Na^+ and Ca^+ FLUXES

Cells were incubated with 2 mM 3OMG as described in METHODS. In K^+-free medium, NaCl was increased by 5.4 mM to compensate for the loss of KCl. The control uptake was 0.34 ± 0.03 (21) nmol/mg protein/min. Numbers in brackets denote the number of separate experiments, each done in quadruplicate.

	3OMG UPTAKE % control
K^+-FREE	93 ± 3 (6)[c]
0.1 mM Ouabain	99 ± 7 (5)
2 mM KCN	97 ± 6 (6)
MONENSIN	
1.0×10^{-7} M	83 ± 3 (3)[c]
5.0×10^{-7} M	85 ± 13 (2)
7.5×10^{-7} M	79 ± 1 (2)[c]
1.0×10^{-6} M	88 ± 5 (3)[b]
A23187	
1 uM	91 ± 5 (3)[a]
5 uM	88 ± 2 (3)[c]
10 uM	93 ± 4 (3)[a]
20 uM	89 ± 6 (3)[a]

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$

In summary, ouabain and K^+-free medium increased intracellular Na^+ levels (Table 5) and Ca^{2+} uptake (Figure 17), and partial or complete replacement of external Na^+ with mannitol reduced Ca^{2+} efflux in a Ca^{2+}-free medium (Figures 19 and 20). Although these observations would be consistent with the presence of a Na^+/Ca^{2+} exchange system, other observations do not support the existence of such a mechanism: Monensin increased intracellular Na^+ but did not significantly increase ^{45}Ca uptake (Figure 17). In addition, monensin had no effect on ^{45}Ca efflux in the presence or absence of external Ca^{2+}. While complete replacement of Na^+ with mannitol affected Ca^{2+} fluxes, replacement with LiCl did not (Figures 18 and 20), confirming that the effects of Na^+ withdrawal depend to a large extent on the substance with which Na^+ is replaced (Rink, 1977). This lack of correlation between Ca^{2+} fluxes thought to be mediated by Na^+/Ca^{2+} exchange and activity of the Ca^{2+}-dependent process has also been observed in studies of catecholamine release from adrenal chromaffin cells.

Ca^{2+} Dependent Catecholamine Release in Adrenal Chromaffin Cells

The presence of a Na^+/Ca^{2+} exchange system in adrenal chromaffin cells is supported by observations that Ca^{2+}-dependent catecholamine release was stimulated by factors or conditions which may increase intracellular Na^+ or decrease the Na^+ gradient.

K^+-free medium stimulated ^{45}Ca uptake and catecholamine release in a Ca^{2+}-dependent manner in the intact perfused adrenal gland and isolated chromaffin cells (Douglas and Rubin, 1961, 1963; Banks et al., 1969; Sorimachi et al., 1981; Sorimachi and Nishimura, 1983). Ouabain also stimulated Ca^{2+} uptake in isolated chromaffin cells (Sorimachi et

al., 1981; Sorimachi and Nishimura, 1983) and catecholamine release in the intact adrenal gland (Banks, 1967) and isolated chromaffin cells (Sorimachi et al., 1981; Sorimachi and Nishimura, 1983). Banks (1967) reported that the stimulatory effect of ouabain was dependent on extracellular Ca^{2+}.

Reduction of external Na^+ stimulated basal catecholamine release in the intact adrenal gland (Douglas and Rubin, 1961; Lastowecka and Trifaro, 1974; Aguirre et al., 1977). Complete replacement of external Na^+ with sucrose or choline stimulated basal secretion in the intact adrenal gland (Douglas and Rubin, 1961), isolated chromaffin cells (Sorimachi et al., 1981) and chromaffin cell cultures (Trifaro and Lee, 1980). The effect of Na^+ deprivation was not dependent on the presence of external Ca^{2+} in the intact adrenal gland (Douglas and Rubin, 1961; Lastowecka and Trifaro, 1974). In adrenomedullary chromaffin cell slices, replacement of external Na^+ with Li^+ or choline caused a Ca^{2+}-dependent increase in catecholamine output and parallel increase in Ca^{2+} uptake. However sucrose and Tris solutions produced Ca^{2+}-independent secretory responses with quite different time courses (Rink, 1977).

Na^+-free medium decreased Ca^{2+} efflux in Ca^{2+}-free medium. It was suggested that removal of Na^+ reduced Na^+/Ca^{2+} exchange and hence Ca^{2+} efflux. In Ca^{2+}-containing medium, Na^+ deprivation increased Ca^{2+} efflux. It was suggested that although Na^+ deprivation reduces Na^+/Ca^{2+} exchange, the presence of external Ca^{2+} permits Ca^{2+}/Ca^{2+} exchange (Aguirre et al., 1977). In Ca^{2+}-containing medium, replacement of external Na^+ with Li^+, K^+ or choline increased ^{45}Ca efflux and uptake in bovine adrenomedullary slices (Rink, 1977). Nishimura and Sorimachi

(1984) showed that replacement of Na^+ with sucrose, choline Cl or Tris Cl reduced the rate of ^{45}Ca efflux in a Ca^{2+}-deficient medium, consistent with the presence of Na^+-dependent ^{45}Ca efflux mechanism. In addition, they reported that Li^+ partially replaced Na^+ in maintaining ^{45}Ca efflux.

Other observations do not support the presence of a Na^+/Ca^{2+} exchange system in chromaffin cells. Rink (1977) reported that ouabain had no effect on Na^+-dependent Ca^{2+} efflux, Ca^{2+} uptake or catecholamine release in bovine adrenomedullary slices. Pocock (1983b) found that omission of external Na^+ did not affect catecholamine secretion, ^{45}Ca uptake or ^{45}Ca efflux. Anoxic conditions increased the intracellular level of Na^+ but did not alter catecholamine secretion or inhibit ^{45}Ca efflux. Ouabain stimulated catecholamine secretion and inhibited Ca^{2+} efflux, but did not stimulate Ca^{2+} influx. In addition, the stimulatory effect of ouabain did not appear to depend on the presence of external Ca^{2+}. Pocock therefore proposed that the effect of ouabain was not related to inhibition of the Na^+ pump and net Ca^{2+} influx via Na^+/Ca^{2+} exchange but to inhibition of active Ca^{2+} extrusion via the plasma membrane Ca^{2+}-ATPase. Suchard et al. (1982) showed that monensin induced a slow prolonged release of catecholamines, and this effect was dependent on external Na^+ but not external Ca^{2+}. They proposed that monensin increased intracellular Na^+, which decreased Ca^{2+} binding to intracellular sites, thereby increasing the amount of cytosolic Ca^{2+} available for exocytosis.

In summary, the results of the present study and observations from studies of catecholamine secretion have not clearly established a link between Ca^{2+} influx via Na^{+}/Ca^{2+} exchange and the regulation of sugar transport and catecholamine secretion in the adrenal chromaffin cell. The presence of a Na^{+}/Ca^{2+} exchange system remains in doubt and it is not unexpected that factors which increase internal Na^{+} do not cause an increase in 3OMG transport. These observations in chromaffin cells are in contrast to those in muscle, where the link between increased Ca^{2+} influx via Na^{+}/Ca^{2+} exchange and increased sugar transport is well documented (Bihler, 1980).

Ca^{2+} Influx and the Activation of Sugar Transport

Data from present study have clearly demonstrated a relationship between Ca^{2+} influx and the stimulation of sugar transport by insulin, acetylcholine, hyperosmolar and low Na^{+} medium in adrenal chromaffin cells. Under certain conditions however, this relation between net Ca^{2+} influx and sugar transport activation is not so straightforward.

A23187

In the perfused left atria and intact hemidiaphragm of the rat, the Ca^{2+} ionophore A23187 affected sugar transport and Ca^{2+} uptake in parallel, with low concentrations inhibiting (0.0625 uM, 0.25 uM) and higher concentrations (1, 6, 10 uM) stimulating influx under basal conditions. A23187 antagonized the stimulatory effects of insulin, K^{+}-free medium and high concentrations of ouabain and adrenaline (Bihler et al., 1980b). It was suggested that, in addition to enhancing

Ca^{2+} influx, A23187 may also cause the release of Ca^{2+} from intracellular stores and thereby increase the cytosolic level of Ca^{2+}. However, A23187 may also cause an indiscriminate "washout" of Ca^{2+} gradients within the cell, thereby depleting various cell compartments and/or binding sites, including the hypothetical sites involved in the regulation of sugar transport. This may explain the neutralization by A23187 of both stimulatory and inhibitory effects of various sugar transport modulators.

In adrenal chromaffin cells, A23187 did not appear to affect basal sugar transport (Table 6). The control rate of 3OMG uptake in the series of experiments with A23187 was relatively high (group C, see Table 1) and may actually reflect the stimulation due to unknown factors. As discussed earlier, if transport is already maximally stimulated, other regulators of sugar transport may have no effect. The failure of A23187 to stimulate basal transport under these conditions may then be due in part to inhibition of the stimulatory effects of these unknown factors.

Na^{+}/Ca^{2+} Exchange

In contrast to muscle, sugar transport in adrenal chromaffin cells was not affected by factors or conditions which may enhance Ca^{2+} influx via Na^{+}/Ca^{2+} exchange. This difference in transport regulation between muscle and adrenal chromaffin cells may be related to the differences in the characteristics of their cellular Ca^{2+} pools and transport systems.

The observation that sugar transport activation in muscle can persist much longer than the perturbation in free cytoplasmic Ca^{2+}

(Bihler, 1980) suggests that sugar transport regulation may not be directly related to the average free cytosolic Ca^{2+} concentration, but may depend on Ca^{2+} binding to a membrane-associated regulatory site which is in contact with the cytosolic Ca^{2+} pool. Ca^{2+} binding would therefore reflect alterations in Ca^{2+} level of the cytoplasmic Ca^{2+} pool (or in its time-averaged concentration) but would also reflect the characteristics of the binding sites themselves, expressed as a lag in binding and release of Ca^{2+}. The cytoplasmic pool may differ between cell types and may account for the varying nature and degree of Ca^{2+} dependence of sugar transport regulation.

It has been demonstrated that sugar transport activation in cardiac and skeletal muscle varies in its dependence on external Ca^{2+} (Bihler, 1980). Dependence on external Ca^{2+} was greater in atria than in diaphragm, in parallel to the greater sensitivity to external Ca^{2+} exhibited by contractile activity and the greater effect of Ca^{2+} antagonists (Bihler and Sawh, 1980). In a smooth muscle, the detrusor of the urinary bladder, the same pattern of effects but a still greater sensitivity to La^{3+} than in atria was observed (Bihler et al., 1977) again paralleling the greater sensitivity of contractile activity of this muscle to changes in external Ca^{2+}. Differences between various types of muscle may be related to known differences in the nature of Ca^{2+} fluxes and in Ca^{2+}-dependent processes. In nucleated avian red blood cells, there is no Na^{+}/Ca^{2+} exchange and Ca^{2+} permeability is very low. Sugar transport was independent of external Ca^{2+} under normal conditions but was stimulated by factors which release intracellular Ca^{2+} stores (anoxia, catecholamines) or which cause a large increase in Ca^{2+} influx (the Ca^{2+} ionophore A23187) (Bihler et al., 1982, 1982a).

Similarly in adrenal chromaffin cells, Na^+/Ca^{2+} exchange, if present, may be of little significance in determining the cellular Ca^{2+} distribution in the vicinity of the postulated regulatory binding site. As discussed above, a role for Na^+/Ca^{2+} exchange has not been clearly demonstrated in stimulus-secretion coupling which is a major physiological function of these cells (Livett, 1984). Other Ca^{2+} transport mechanisms (Pocock, 1983b) may play a greater role in controlling cellular Ca^{2+} distribution.

The observation that low Na^+ stimulates Ca^{2+} and sugar transport in parallel (Figures 13 and 16) could be taken as favouring a role for Ca^{2+} influx mediated by Na^+/Ca^{2+} exchange in the activation of sugar transport. However other or additional mechanisms may be involved in the stimulatory effect of low Na^+ medium, as the effect is partially maintained in the absence of external Ca^{2+} (Figure 13). For example, hyperpolarization may be involved. Douglas et al. (1967) found that the reduction of external Na^+ increases the membrane potential and reduces the depolarizing effect of acetylcholine in isolated adrenal chromaffin cells. Some studies in muscle and adipose tissue have suggested that hyperpolarization of the cell membrane may be involved in the mechanism of sugar transport activation by some factors. Pershadsingh and McDonald (1984) proposed that the combined effects of insulin to reduce Ca^{2+} bound to the sarcolemma and to hyperpolarize the cell membrane might cause altered permeability properties. Zierler and Rogus (1980) showed that a hyperpolarization of only 1.5 mV specifically enhanced D-glucose transport by 40% in the caudofemoralis muscle of the rat.

B. BOVINE ADRENAL CHROMAFFIN CELL CULTURES

Within 9 hr in culture, bovine adrenal chromaffin cells adhere to the plating surface and begin to flatten or spread out. Their round shape becomes bipolar, and from each end, growth cones begin to form. By day 1, the growth cones develop into neurites or processes which extend to other cells, making contact with their cell bodies and processes. After 5 days in culture most of the cells have developed neurites or processes, which have increased both in number and branching. In comparison with chromaffin cells in vivo, the cultured chromaffin cell has a predominance of noradrenaline-storing cells, a loss of catecholamines as indicated by histochemistry, a reduction in the number and size of intracellular chromaffin granules and an increase in free ribosomes, in addition to exhibiting the formation of processes (Unsicker and Chamley, 1977).

The morphological changes of adrenal chromaffin cells in culture have been well documented (Trifaro and Lee, 1980; Livett, 1984), but it remains uncertain as to what triggers the formation of processes. It was suggested that process outgrowth in bovine adrenal chromaffin cells is induced by a factor or factors produced by nonchromaffin cells in culture. Despite the use of specific measures (FDU, cytosar) to inhibit the growth of nonchromaffin cells, there always remains a small percentage of fibroblasts in the culture (Livett, 1984).

Nerve growth factor (NGF) was shown to be required for neurite outgrowth from embryonic and neonatal rat and bovine adrenal chromaffin cells, but not from adult rat and bovine adrenal chromaffin cells. Factors, other than NGF, that induce process outgrowth from cultured chromaffin cells have only been recently characterized. These

substances have been extracted from a diverse group of sources including: (a) conditioned media from a cultured C6 glioma cell line; (b) media conditioned over adrenal fibroblast-like cells; (c) bovine seminal vesicles; and (d) conditioned media from SV3T3 cells. The non-NGF growth factors were not inhibited by antibodies to NGF or by dexamethasone. The predominant cell type, which produced these rat non-NGF growth factors, was described as fibroblast-like in its manner of growth. Nonchromaffin cells from bovine adrenal glands also were shown to have a factor that elicits fiber outgrowth from cultured bovine adrenal chromaffin cells. These non-NGF factors appear to be both organ- and species-specific (Livett, 1984).

Because adrenal chromaffin cells undergo changes in their morphology in culture, developing into neuron-like structures, chromaffin cell cultures may serve as a useful model for the study of some aspects of neuronal cell development. Kenigsberg and Trifaro (1980) investigated the catecholamine uptake system in adrenal chromaffin cells after 2 days in culture when the cells were closer to their morphological state in vivo, and also after 7 days in culture when the cells had developed processes of considerable length. They demonstrated in both 2 day and 7 day old cultured chromaffin cells the presence of a high affinity catecholamine uptake system, which exhibited saturation (Michaelis-Menten) kinetics, Na^+ dependence and sensitivity to inhibition by low concentrations of desipramine. The kinetic parameters of the high affinity uptake system did not appear to change in parallel with the outgrowth of neurites.

As described in the Introduction, the use of secondary neural cell lines for the study of transport and metabolism has been questioned. These cell lines are usually derived from tumors. In addition, it is uncertain whether some of their characteristics may be due to culturing. The use of adrenal chromaffin cell cultures, a primary cell culture, excludes uncertainties due to tumour origin. The present study examined the effects of culturing by comparing characteristics of sugar transport in cultured cells to those in freshly isolated cells. In addition, transport characteristics were examined in relation to morphological changes of the chromaffin cells in culture. Specifically, the kinetics parameters, insulin and Ca^{2+} sensitivity of 3OMG uptake were determined in bovine adrenal chromaffin cells after 1 day in culture, when growth was rapid and morphologic changes were extensive, and after 5 days in culture, when the cells appeared to be at a stationary phase in growth and morphologic change.

1. Day 1 Cultures

(a) Time Dependence

The time course of 3OMG equilibration was determined in day 1 cultures of bovine adrenal chromaffin cells incubated with 5 mM 3OMG for up to 1 min. Figure 21 shows that the time curve consisted of an initial linear portion followed by a progressively decreasing rate, characteristic for an equilibrating process. On the basis of these data, a standard incubation time of 0.2 min was chosen, representing a point on the upper linear portion of the time curve. The data presented here thus represent unidirectional influx rates.

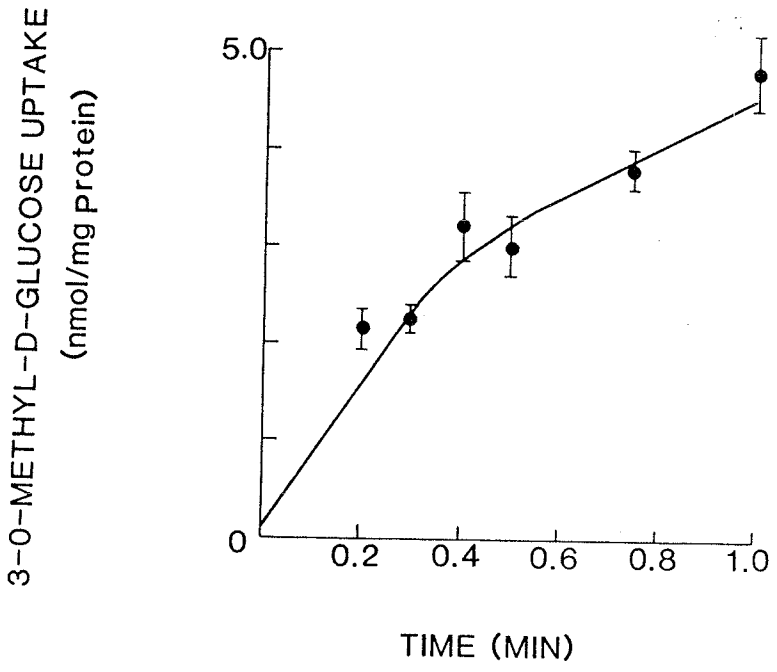


FIG. 21. Time-course of 3OMG uptake in day 1 cultures of adrenal chromaffin cells. Cell cultures were incubated with 5 mM 3OMG as described in METHODS for various periods of time. Points represent the mean $\pm$ S.E. of 5 separate experiments, each done in quadruplicate.

In comparison, the transport rate in freshly isolated adrenal chromaffin cells was much lower and the standard incubation time was substantially longer at 2.5 min (Figure 1). The rate of sugar transport in cultured chromaffin cells may be faster because of the change in morphology of the cells in culture. In culture, when adrenal chromaffin cells adhere to and spread out onto the plating surface, their cell surface area may become large relative to their cytoplasmic volume. This morphologic attribute, also characteristic for adipocytes, usually leads to more rapid (within secs) equilibration of the sugar (Czech, 1980). Short incubation times (0.25 to 1.0 min) have also been used with other neural cells cultures including cultured human glioma cells (Edstrom et al., 1975; Walum and Edstrom, 1976a) and cultured mouse neuroblastoma cells (Walum and Edstrom, 1976b).

(b) Concentration Dependence

Figure 22 shows the concentration dependence of 3OMG uptake in day 1 cultures of adrenal chromaffin cells. The plot of 3OMG uptake as a function of 3OMG concentration yields a hyperbolic curve, suggesting that sugar transport in day 1 cultures of adrenal chromaffin cells is mediated by a facilitated diffusion mechanism, similar to freshly isolated chromaffin cells (Figure 2) and most other animal cell types. Reciprocals of the substrate concentrations and of mean uptake rates were fitted to a linear regression equation to obtain the values for the kinetic parameters. The straight line double-reciprocal plot (inset, Figure 22) is consistent with Michaelis-Menten kinetics. The kinetic constants were Vmax = 139 nmol/mg protein/min and Km = 15.4 mM.

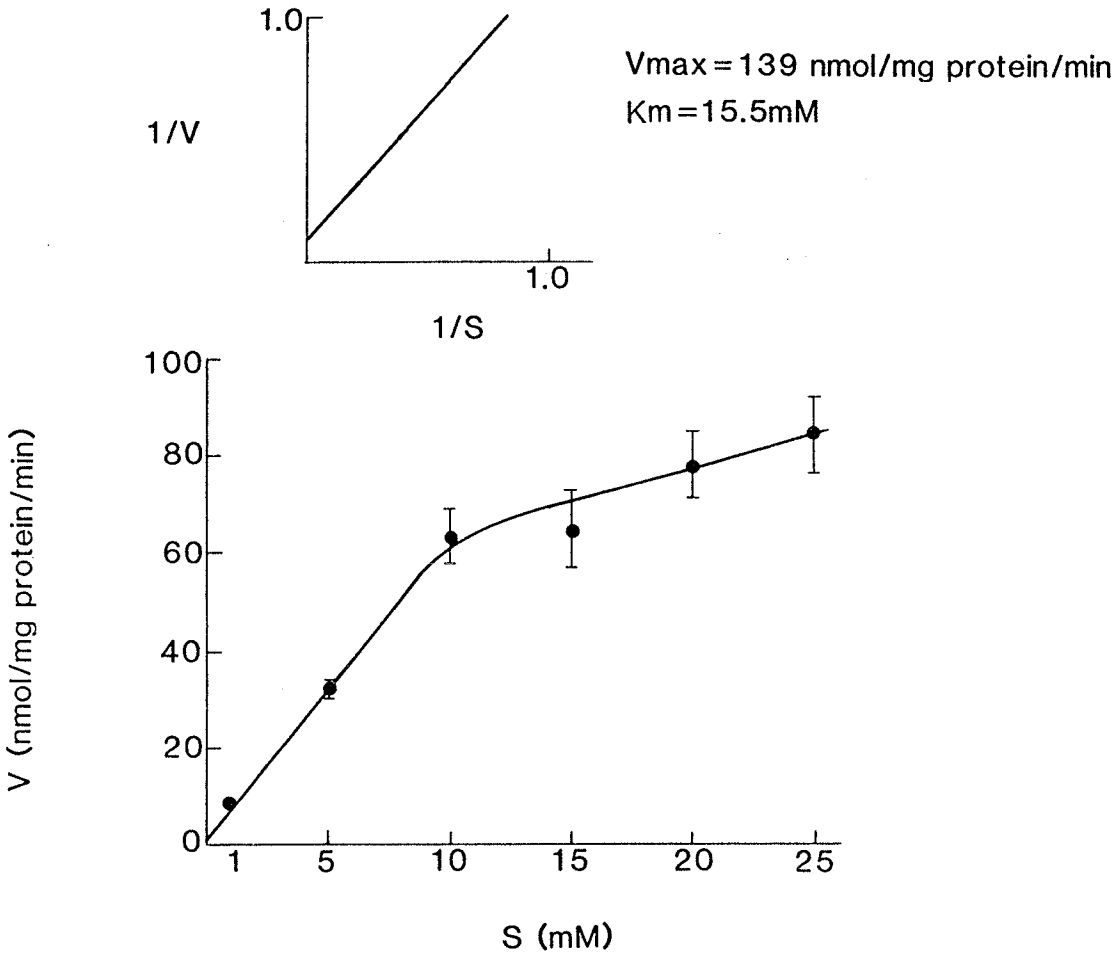


FIG. 22. Concentration curve of 3OMG uptake in day 1 cultures of adrenal chromaffin cells. Cell cultures were incubated as described in METHODS with various concentrations of 3OMG. Points represent the mean ± S.E. of 4 separate experiments, each done in quadruplicate. The inset is a double-reciprocal plot of the same data calculated by linear regression.

In comparison with freshly isolated cells (Vmax = 0.69 nmol/mg protein/min, Km = 8.2 mM), the maximal capacity of sugar transport was increased 200-fold in day 1 chromaffin cell cultures. It is possible that rapid growth and extensive morphological change, involving protein synthesis, intracellular transport of structural proteins, etc., is associated with a higher demand for energy-yielding substrates, and the cell may undergo changes to adapt to the increased energy metabolism. As described earlier, Vmax is a function of the number of carriers present and their mobility i.e. rate of reorientation or translocation through the cell membrane. Hence, an increase in the maximal capacity of transport would indicate an increase in the total or functional number of glucose transporters. This may be accomplished by increased synthesis of new glucose transporters. Neural cell differentiation is often associated with a quantitative increase in protein synthesis (Patrick et al., 1978). Alternatively, an increased maximal capacity may be due to increased translocation of glucose transporters from an intracellular storage site to th plasma membrane. As discussed earlier in the Introduction, insulin stimulated glucose transport in fat cells by facilitating the translocation of glucose transporters to the plasma membrane from an intracellular storage site (Cushman and Wardzala, 1980; Suzuki and Kono, 1980).

Other studies have shown an enhancement of sugar transport with increased cell growth and morphologic differentiation. Edstrom et al. (1975) examined the uptake of 3OMG in different morphological states of cultured glioma cells (138MG). After treatment with dibutyryl cyclic AMP (dbcAMP) or prostaglandin E_1, these cells develop the morphologic characteristics of differentiated glial cells. They reported that

dbcAMP-induced morphological differentiation was associated with a 44% increase in the specific uptake of 3OMG. It was speculated that the dbcAMP-induced change in morphology was an energy-requiring process and the increase in energy metabolism could be reflected in glucose uptake. This effect was further investigated by Walum and Edstrom (1976a), who examined the kinetics of 2DG uptake in dbcAMP-induced differentiated 138MG cell cultures at different cell densities. Although morphological changes were induced at all cell densities, 2DG uptake was affected only under certain conditions. Rapidly growing sparse cultures, exposed to dbcAMP, increased their Vmax for 2DG transport without any change in Km. In contrast, the morphologicial changes induced by dbcAMP in dense, growth-inhibited cultures of 138MG cells were not accompanied by any change in 2DG uptake. As described earlier, sugar transport was reported to be faster in virus-transformed cells than in their normal counterparts (Kalckar, 1976).

(c) Effect of Insulin

The effect of insulin on 3OMG uptake was investigated in day 1 chromaffin cell cultures. Insulin increased 3OMG uptake (nmol/mg protein/min) from 32.25 $\pm$ 2.25 (3) to 41.15 $\pm$ 4.9 (3) ($P<0.05$), 65.25 $\pm$ 8.05 (3) to 82.5 $\pm$ 8.9 (3) ($P<0.05$), 77.7 $\pm$ 7.0 (3) to 97.15 $\pm$ 5.25 (3) ($P<0.01$), and 83.5 $\pm$ 7.6 (2) to 100.0 $\pm$ 7.05 (3) at 3OMG concentrations of 5, 15, 20 and 25 mM, respectively. The reciprocals of substrate concentrations and means of uptake rates were fitted to a linear regression equation to obtain double-reciprocal plots (Figure 23) and and values of kinetic constants (Table 7). The data show that insulin increased Vmax 1.2-fold to 169 nmol/mg protein/min, but did not alter Km of 3OMG uptake in day 1 chromaffin cell cultures.

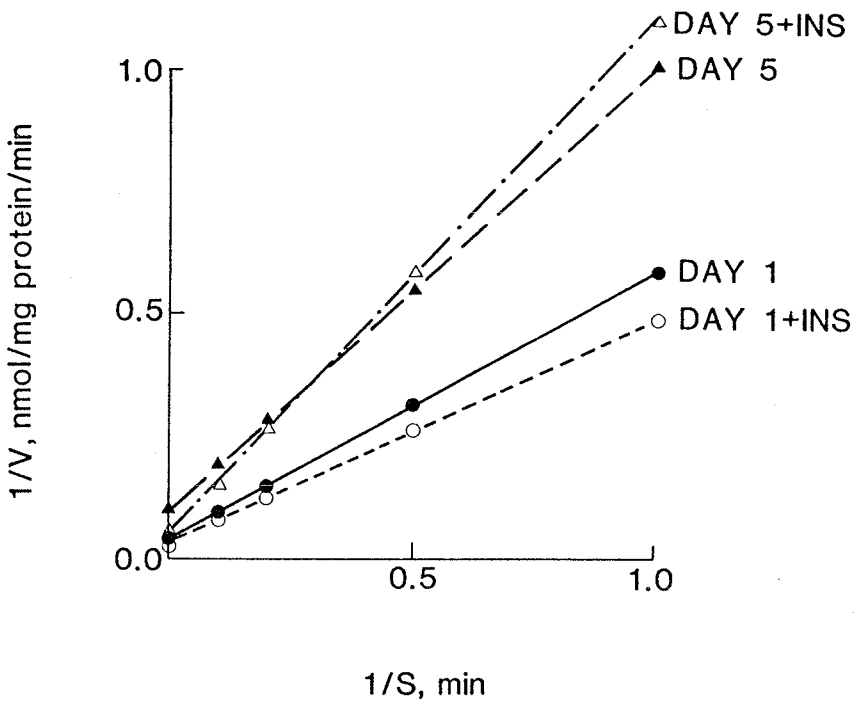


FIG. 23. Double-reciprocal plots of 3OMG uptake in Day 1 adrenal chromaffin cell cultures in the presence of 1.25 mM Ca^{2+} (●——●) and with Ca^{2+} and 50 mU/ml insulin (0---0); and in Day 5 adrenal chromaffin cell cultures in the presence of Ca^{2+} (▲---▲) and with Ca^{2+} and 50 mU/ml insulin (△-·-△). Cell cultures were incubated as described in Table 7. The mean ± S.E. rates of 3OMG uptake were determined from 3 separate experiments, each done in quadruplicate. Reciprocals of substrate concentrations and mean uptake rates were fitted to a linear regression equation to obtain the double-reciprocal plots.

TABLE 7

EFFECT OF INSULIN ON KINETIC PARAMETERS OF 30MG UPTAKE IN DAY 1 AND DAY 5 CULTURES OF BOVINE ADRENAL CHROMAFFIN CELLS

Day 1 and day 5 cultures of adrenal chromaffin cells were incubated in Ca^{2+}-containing medium with various concentrations of 30MG in the presence and absence of 50 mU/ml insulin. The mean rates of 30MG uptake was determined from 3 separate experiments, each done in quadruplicate. Reciprocals of the substrate concentrations and of mean transport rates were fitted to a linear regression equations to obtain the values for the kinetic parameters. Vmax is given in nmol/mg protein/min and Km in mmol/L.

	- Insulin	+ Insulin
DAY 1 CULTURES		
Vmax	139.0	165.0
Km	15.4	15.4
DAY 5 CULTURES		
Vmax	50.3	98.2
Km	9.2	20.8

During this stage of rapid morphologic change, growth factors may be present which stimulate the glucose transport system. As described in METHODS, fetal calf serum (F.C. = 10%) was routinely added to the cell culture medium. Serum contains many growth-promoting factors, including multiplication-stimulating activity (MSA), somatomedins and insulin-like growth factors (IGF). IGF receptors were found to be present in all cells. IGF can also bind to the insulin receptor and exert insulin-like effects at all target cells, but these factors are generally less potent than insulin (Froesch et al., 1985). IGF may also down regulate the insulin receptor (Rechler and Nissley, 1985). It is therefore conceivable that these factors may exert long-lasting effects (half-life of IGF is 4 hr), which may interfere with insulin action. Thus if the sugar transport system is already maximally activated by such factors, insulin will have no effect under these conditions. In contrast, freshly isolated cells were never exposed to serum, and hence were not subject to the effects of IGF.

In summary, within the first days of culturing, adrenal chromaffin cells undergo extensive changes in their morphology. This may be associated with a higher demand for energy-yielding substrates, which may in part be satisfied by increasing the maximal capacity of glucose transport. The maximal capacity, which is a function of the number of active glucose transporters in the plasma membrane, may be increased by the synthesis of transport proteins or by the translocation of transporters to the plasma membrane from an intracellular site. Kinetic studies, however, do not distinguish between these two processes. In addition, the changes in morphology of

cultured chromaffin cells may contribute to a more rapid transport rate. For example, the cell surface area would be increased due to the formation of processes. A large cell surface area to cytoplasmic volume ratio with a corresponding increase in the number of functional glucose transporters in the plasma membrane would contribute to more rapid equilibration of the sugar. In addition, the morphological changes may be associated with changes in the physico-chemical properties of the membrane. It was shown that transformed fibroblasts (Barnett et al., 1974a) and phytohemagglutinin-stimulated lymphocytes (Barnett et al., 1974b) have a more fluid membrane structure than growth-inhibited fibroblasts and unstimulated lymphocytes. Insulin did not stimulate sugar transport in day 1 cultures. During this initial period of rapid growth, the glucose transport system may be maximally activated by insulin-like growth factors in the serum, and hence, significant stimulation by insulin will not be evident.

2. Day 5 Cultures

(a) Concentration Dependence and Insulin Effect

After 5 days in culture, adrenal chromaffin cells have flattened and spread out onto the plating surface and show extensive development of neurites. At this stage of growth, the cells closely resemble peripheral adrenergic neurons and do not appear to change further from this form. This negligible rate of morphologic change may also be due to the increase in cell density as the cells appear larger and more densely packed. Studies have reported that an increase in plating density inhibits the formation of processes from adrenal chromaffin cells in culture (Livett, 1984). 3OMG uptake in day 5 cultures of adrenal chromaffin cells was saturable and characterized by a straight line double reciprocal plot (Figure 23), consistent with Michaelis-Menten kinetics. Vmax was 50.2 nmol/mg protein/min and Km was 9.2 mM.

In comparison with the kinetic parameters of sugar transport in day 1 cultures (Vmax = 139 nmol/mg protein/min, Km = 15.4 mM), the maximal capacity was reduced by 64% and the affinity increased by 40% in day 5 chromaffin cell cultures. The reduction in maximal capacity in day 5 chromaffin cells cultures coincides with the slower rate of morphologic change and may be related to the lower energy demand during at this stage of growth.

Walum and Edstrom (1976) examined 2DG uptake in rapidly growing and stationary phase culture of mouse neuroblastoma cells. In rapidly growing cultures, Km and Vmax for the transport of 2DG were 0.8 mM and 18.3 nmol/mg protein/min, respectively. As the cultures entered the stationary phase, the number of morphologically differentiated cells

increased and this was associated with changes in the kinetic parameters. Km and Vmax for 2DG transport were 4.9 mM and 9.1 nmol/mg protein/min, respectively in the stationary, differentiated phase. It was suggested that variations in the Michealis-Menten constants between rapidly growing and stationary phase cultures indicate that differentiation was accompanied by both quantitative and qualitative alterations in the glucose transport systems.

It is possible that sugar transport may be accelerated in response to higher energy demands associated with recovery of the cells from possible damage due to mechanical or enzymatic disaggregation during cell isolation. This may explain why the maximal rates of 3OMG uptake were considerably enhanced in chromaffin cell cultures in comparison with freshly isolated cells. With increasing time in culture, chromaffin cells may have recovered considerably such that their energy demands are lowered. Hence, Vmax of 3OMG uptake was lower in day 5 chromaffin cell cultures than in day 1 cultures.

However, in comparison with freshly isolated cells (Vmax = 0.69 nmol/mg protein/min, Km = 8.2 mM), the maximal capacity of 3OMG uptake in day 5 cultures was still increased about 70-fold. Hence, recovery from cell damage may not fully account for the differences in maximal rates of 3OMG uptake between freshly isolated and cultured chromaffin cells. One may speculate that in culture, the adrenal chromaffin cell may assume new or additional functions, which may have different nutrient requirements than that of freshly isolated chromaffin cells. For example, freshly isolated chromaffin cells may primarily function in neurosecretion, whereas cultured chromaffin cells with their dendritic-like processse, may be geared to neurotransmission. These two

different functions vary greatly in energy requirements, and the cell may undergo changes, such as an increase in the maximal capacity of glucose transport, to adapt to the varying energy requirements. Thus the differences in certain characteristics of sugar transport between freshly isolated and cultured chromaffin cells may be related to differences in the energy requirements and cell functions of freshly isolated and cultured adrenal chromaffin cells.

In Day 5 chromaffin cell cultures, insulin stimulated basal transport to 180%, 134% and 123% of control at 3OMG concentrations of 5, 10 and 15 mM 3OMG. As shown in Figure 23, Vmax increased from 50.2 nmol/mg protein/min in the absence of insulin to 98.2 nmol/mg protein/min with the addition of 50 mU/ml insulin. The magnitude of insulin-induced increase in Vmax in day 5 chromaffin cell cultures (1.95-fold) was similar to that obtained with freshly isolated cells (1.8-fold). The data show that when the glucose transport system is working at a lower capacity, as in day 5 cultures, the effect of insulin to stimulate sugar transport becomes evident.

In summary, after 5 days in culture, the adrenal chromaffin cells appear to have reached a stationary phase in morphological change, and may have a lower demand for energy-yielding substrates than in the rapid initial stages of differentiation (i.e. Day 1 cultures). Hence, the reduction in energy metabolism may be reflected in the reduction in maximal capacity of the sugar transport system in comparison to day 1 chromaffin cell cultures. In addition, when operating at less than maximal rates, the sugar transport system appears to become again sensitive to the stimulatory effects of

insulin. Thus, the data show that the stimulatory effect of insulin on 3OMG uptake is maintained in slow growing Day 5 chromaffin cell cultures.

(b) Effect of Dexamethasone

Pharmacological doses of glucocorticoids retard or interrupt growth in man, reflected for example in an adverse effect on the growth of epiphyseal cartilage. Inhibition of growth is a widespread effect of glucocorticoids. For example, they inhibit cell division or the synthesis of DNA in thymocytes, fibroblasts, normally developing and regenerating liver, gastric mucosa, developing brain, developing lung and epidermis. The effect is somewhat selective and glucocorticoids do not characteristically produce the bone marrow depression or the enteritis that follows exposure to nonspecific antimitotic agents. The mechanism of this effect of steroids is not known (Loeb, 1976)

Dexamethasone is a synthetic analogue of the naturally occuring glucocorticoid hydrocortisone. A few studies have shown that 10^{-5}M dexamethasone inhibits the formation of processes by adrenal chromaffin cells in culture (Unsicker and Chamley, 1977; Unsicker et al., 1978; Soliman et al., 1985). Unsicker et al. (1978) speculated that the glucocorticoids may inhibit fiber outgrowth by medullary chromaffin cells in vivo. Hence, adrenal chromaffin cells may develop processes in culture because they are released from glucocorticoid "restraint". It was therefore of interest to examine whether dexamethasone affected glucose transport in relation to its effect on the outgrowth of processes from chromaffin cells in culture.

The effects of dexamethasone on the morphological changes of bovine adrenal chromaffin cell cultures were qualitatively examined; however, quantitative measurements of number and size of processes and rate of process outgrowth were not done. The present study showed that 10^{-5}M and 10^{-4}M dexamethasone appeared to have no effect on the formation of processes in day 5 chromaffin cell cultures, whereas 10^{-3}M dexamethasone appeared to reduce process outgrowth and to cause the cells to assume a spindle shape (Figures 24 and 25). In addition, the number of cells in cultures treated with 10^{-3}M dexamethasone appeared to be lower than in untreated controls, suggesting cytotoxicity at this concentration of the glucocorticoid. The observation that 10^{-5}M dexamethasone had no effect on the outgrowth of processes in chromaffin cell cultures is consistent with observations from several other laboratories, which showed that fibre outgrowth from adult adrenal chromaffin cells in cultures was not affected by glucocorticoids (Trifaro, personal communication).

Sugar transport was measured in Day 5 cultures of adrenal chromaffin cells, which had been cultured in the presence and absence of 10^{-5}M to 10^{-3}M dexamethasone. Adrenal chromaffin cell which were cultured in medium containing 10^{-3}M dexamethasone, showed significant permeability to L-glucose, which normally is restricted to the extracellular space. These observations suggest considerable damage to the structural integrity of the cell membrane and are consistent with the cytotoxicity of this concentration of dexamethasone. The effect of lower concentrations (10^{-4}M and 10^{-5}M) of dexamethasone on 3OMG uptake was also examined. 10^{-5}M dexamethasone decreased 3OMG uptake to 28%, 20%, 45% and 45% of control at 1, 10, 20 and 25 mM 3OMG, and 10^{-4}M

FIG. 24. Day 5 culture of adrenal chromaffin cells cultured in DMEM medium containing (a) no drug, phase contrast (X37.5); and (b) 10^{-5}_{M} dexamethasone, phase contrast (X75).

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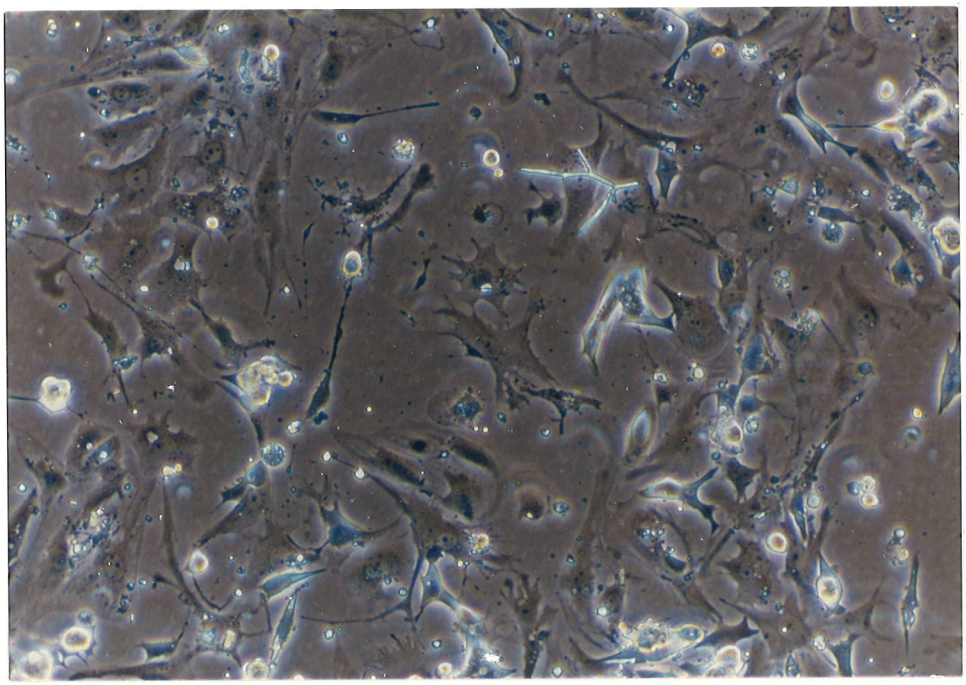

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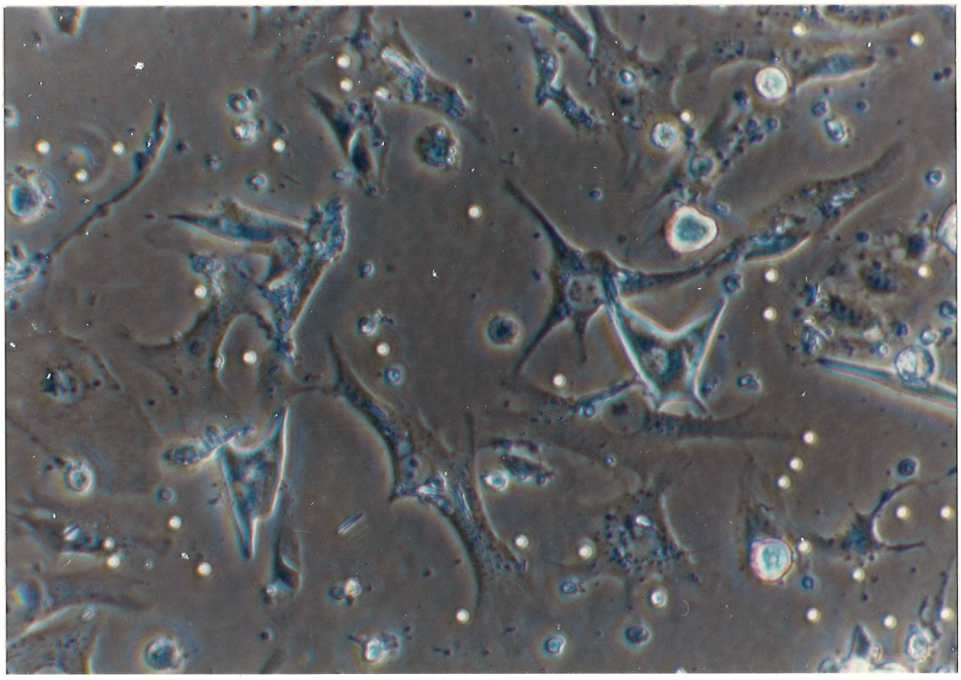

FIG. 25. Day 5 culture of adrenal chromaffin cells cultured in DMEM medium containing (a) 10^{-4}M and (b) 10^{-3}M dexamethasone. Both phase contrast (X75).

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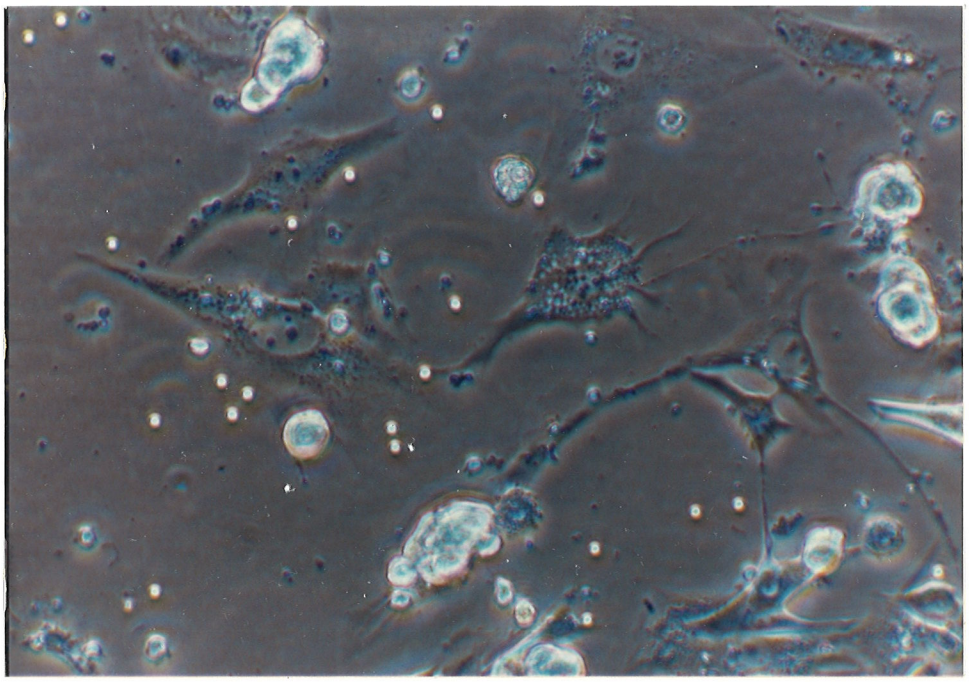

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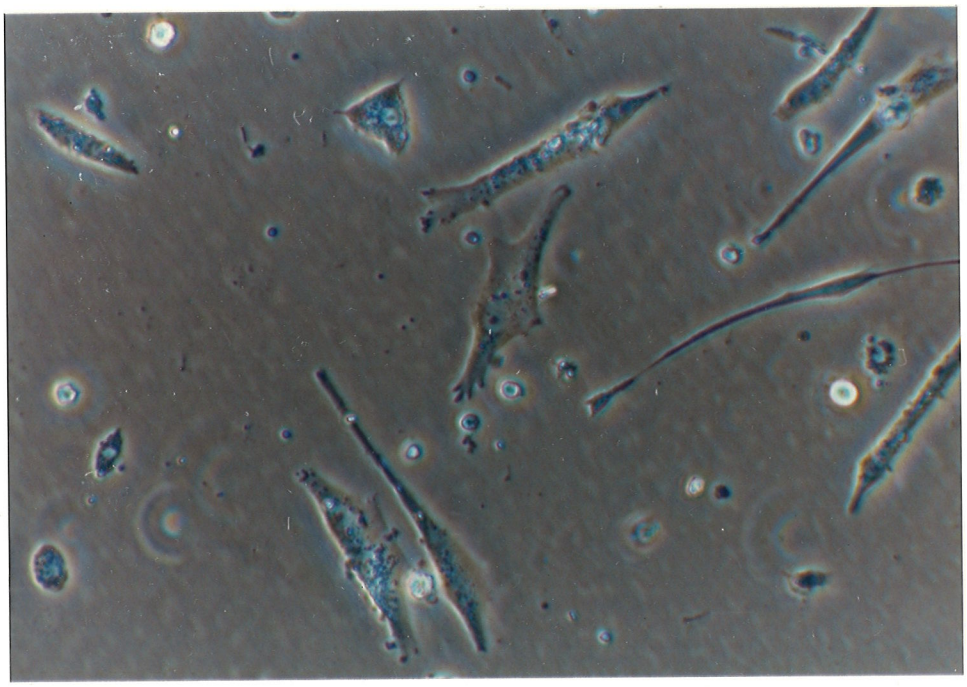

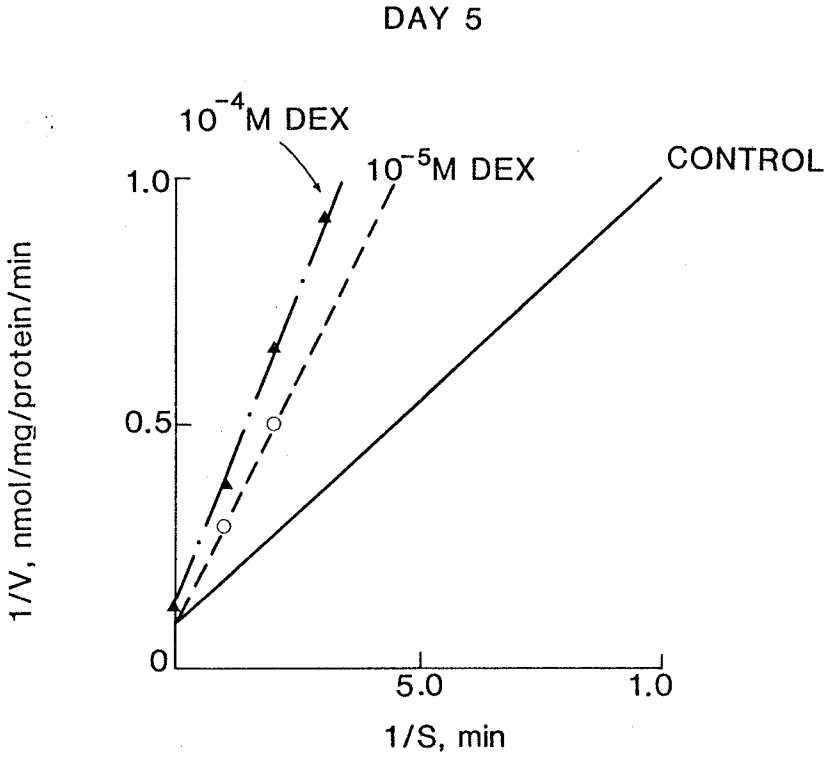

	Vmax	Km
CONTROL	50.3	9.2
10^{-5}M DEX	53.0	21.6
10^{-4}M DEX	41.4	22.0

FIG. 26. Double reciprocal plots of 3OMG uptake in the presence of 1.25 mM Ca^{2+} in Day 5 cultures of adrenal chromaffin cells cultured in DMEM medium containing no drug (●——●); 10^{-5}M dexamethasone (0---0); and 10^{-4}M dexamethasone (▲-•-▲). Data were calculated as described in Table 7.

dexamethasone decreased 3OMG uptake to 21%, 15%, 38% and 30% of control at 1, 10, 15 and 25 mM 3OMG. As shown in Figure 26, in day 5 cultures treated with 10^{-4}M and 10^{-5}M dexamethasone, Km was increased 2.4-fold in comparison with untreated day 5 cultures, but Vmax was generally not affected. Thus dexamethasone inhibits sugar transport primarily through an effect on the affinity. These observations suggest that dexamethasone competitively inhibits 3OMG uptake in adrenal chromaffin cell cultures, and are consistent with observations of Taylor and Gagneja (1975).

As discussed earlier, Taylor and Gagneja (1975) developed a molecular model, describing the spatial distribution of four oxygen atoms, thought to be involved in hydrogen bonding of glucose to the transport protein. They also found that the molecular structures of the naturally occuring glucocorticoid hydrocortisone and the synthetic glucocorticoid prednisolone overlapped with that of glucose and cytochalasin B with respect to the four oxygen atoms involved in binding. In addition, kinetic data indicated that hydrocortisone and prednisolone competitively inhibited glucose transport in human erythrocytes. It may also be possible that, through effects on the genetic apparatus, transcription or protein synthesis, glucocorticoids may in some way affect the structure of the carrier protein in such a way as to decrease its affinity for the substrate.

It may be argued that the dexamethasone induced decrease in Km may reflect nonspecific damage to the cell membrane, a well known phenomenon with high concentrations of any steroid hormone. However, as shown below in Figure 27, although day 5 chromaffin cell cultures treated with 10^{-4}M and 10^{-5}M dexamethasone exhibited depressed basal

3OMG uptake rates, they were still sensitive to the stimulatory effects of insulin.

Glucocorticoids have manyfold effects on carbohydrate metabolism, including the storage of glucose as glycogen especially in the liver, inhibition of glucose utilization by peripheral tissues, and the stimulation of glucose formation. Prolonged exposure to large doses of glucocorticoids leads to the exaggeration of these effect on glucose metabolism, so that a diabetic-like state is produced: elevated plasma glucose levels in the fasting subject, increased insulin resistance, decreased glucose tolerance and glycosuria (Haynes and Murad, 1980). It was suggested that glucocorticoid inhibition of glucose transport, as demonstrated in adipose tissue, skin, fibroblasts and thymocytes, may contribute to the mechanism whereby glucocorticoids inhibit the utilization of glucose by peripheral tissues (Loeb, 1976).

(c) Ca^{2+}-Dependence and Insulin Response in Dexamethasone-treated Chromaffin Cell Cultures

The stimulatory effects of insulin, hyperosmolarity and secretory stimuli on 3OMG uptake in freshly isolated chromaffin cells were shown to be dependent on the presence of external Ca^{2+}. Thus, it was of interest to determine if the Ca^{2+}-dependence of insulin action was maintained in day 5 chromaffin cell cultures. The effect of insulin (50 mU/ml) in the presence and absence of external Ca^{2+} was examined on the uptake of 5 mM 3OMG in day 5 cultures of adrenal chromaffin cells, which were cultured in the presence and absence of dexamethasone (10^{-4}M and 10^{-5}M) (Figure 27). Results were expressed as a percentage of the control (presence of Ca^{2+}, absence of dexamethasone or insulin).

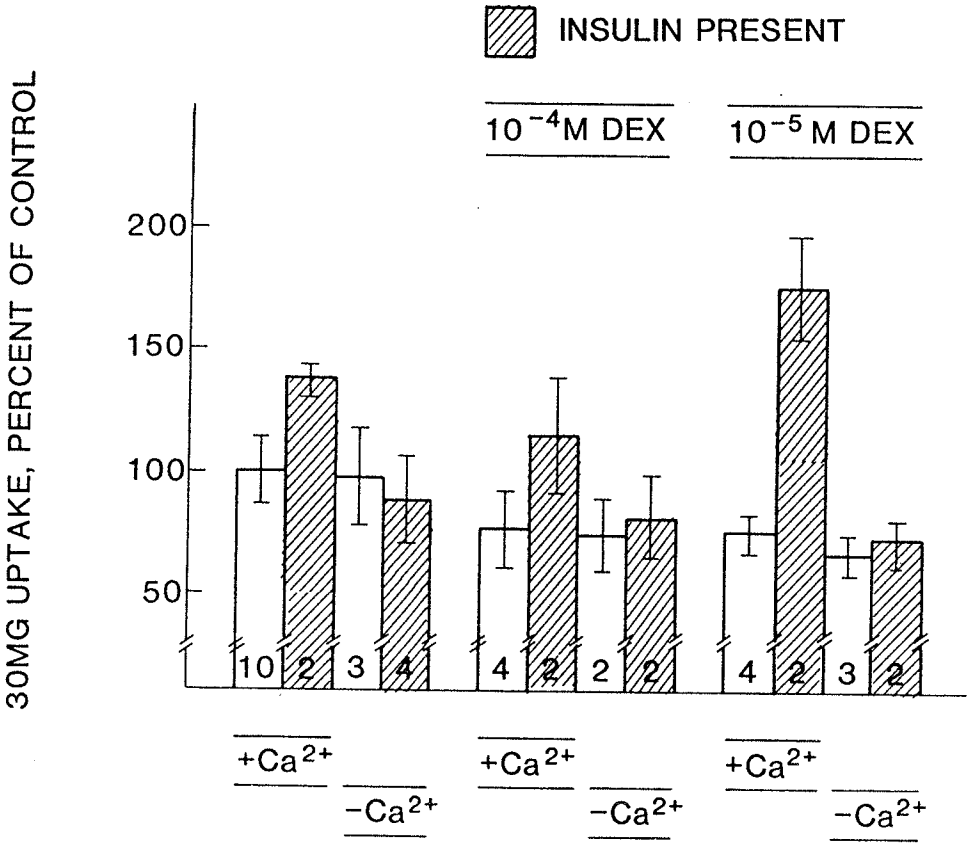

FIG. 27. Effect of dexamethasone (10^{-5}M and 10^{-4}M) and insulin (50 mU/ml) on 3OMG uptake in Day 5 cultures of adrenal chromaffin cells in the presence and absence of extracellular Ca^{2+} (1.25 mM). Adrenal chromaffin cells were cultured in the presence and absence of dexamethasone. After five days in culture, control and dexamethasone-treated cells were incubated with 5 mM 3OMG in the presence and absence of extracellular Ca^{2+} (1.25 mM) and insulin (50 mU/ml). Data were presented as percentage of the control (Ca^{2+} present, no drug). Control uptake for 3OMG was 11.85 $\pm$ 1.62 (10) nmol/mg protein/min. Experiments were done in triplicate.

Figure 27 shows that in the presence of external Ca^{2+}, insulin significantly stimulated ($P<0.01$) sugar transport to 137% of control. Omission of external Ca^{2+} had no effect on basal transport, but abolished the stimulatory effect of insulin. These observations confirm the stimulatory effect of insulin on sugar transport in adrenal chromaffin cell cultures. In addition, they also show that the Ca^{2+}-dependence of the insulin effect, observed in freshly isolated chromaffin cells, was maintained in culture.

Adrenal chromaffin cells cultured in the presence of 10^{-4}M and 10^{-5}M dexamethasone exhibited 3OMG uptake rates that were significantly decreased to 76% ($P<0.025$) and 75% ($P<0.01$) of control values, respectively. Insulin significantly stimulated 3OMG uptake from 76% to 114% ($P<0.05$) and from 75% to 175% ($P<0.001$) in day 5 chromaffin cell cultures treated with 10^{-4}M and 10^{-5}M dexamethasone, respectively. The omission of external Ca^{2+} did not alter the depressed 3OMG uptake rates in dexamethasone-treated chromaffin cell cultures, and further addition of insulin to this nominally Ca^{2+}-free medium also had no effect (Figure 27).

The stimulatory effect of insulin was much greater (100% increase) in day 5 cultures treated with 10^{-5}M dexamethasone than that observed in freshly isolated chromaffin cells (37%), untreated (i.e. with dexamethasone) cell cultures (37%) and cultures treated with 10^{-4}M dexamethasone (38%). This suggests that 10^{-5}M dexamethasone may potentiate the stimulatory effect of insulin on sugar transport. The increased insulin sensitivity with the presence of 10^{-5}M dexamethasone is consistent with the findings of other studies examining the interaction of glucocorticoids and insulin. For example, it was shown

that primary cultures of hepatocytes incubated with dexamethasone and insulin were hyper-responsive to the ability of insulin to stimulate lipogenesis chronically (Amatruda et al., 1983).

In summary, the results show that: (a) with external Ca^{2+} present, insulin stimulated 3OMG uptake in untreated (with dexamethasone) cell cultures and dexamethasone-treated cell cultures; (b) the effect of insulin was consistently abolished in the absence of external Ca^{2+}; and (c) sugar transport was depressed in dexamethasone-treated cultures in the presence and absence of external Ca^{2+}. These observations confirm the stimulatory effects of insulin and inhibitory effects of dexamethasone on sugar transport in day 5 cultures of adrenal chromaffin cells. They also indicate that the Ca^{2+}-dependence of sugar transport activation by insulin was maintained in culture.

Adrenal chromaffin cells, which had been maintained in culture for longer than 5 days, did not appear to undergo further changes in their morphology. Sugar transport was not measured in cultures maintained longer than 5 days. However, the results described above suggest that changes in characteristics of sugar transport may be related to the rate of growth and morphologic change. For example, a rapid rate of growth and morphologic change in day 1 cultures coincided with an increase in the maximal capacity of sugar transport in comparison to freshly isolated cells and slow growing stationary day 5 cultures. Hence, if the rate of growth and morphologic change is negligible after 5 days in culture, the characteristics of sugar transport also would not be expected to change after 5 days.

SECTION IV

GENERAL DISCUSSION

AND

CONCLUSIONS

Adrenal Chromaffin Cells: Model of a Neuronal Cell Population

Glucose is the main energy substrate of the brain and nerve and its uptake into neural cells is mediated by facilitated diffusion. Whether the transport step is regulated by insulin and other factors has not been clearly established. This is due to several technical difficulties with currently available neural preparations. These complications include cell heterogeneity, the presence of a blood brain barrier in the central nervous system and a perineurial membrane barrier in the peripheral nervous system, and an uncertain value for the extracellular space. The suitability of secondary neural cell cultures has been questioned because these cell lines generally originate from tumors; in addition, some of their characteristics may be related to the effects of culturing. Chromaffin cells isolated from the adrenal medulla resemble peripheral adrenergic neurons in their ectodermal origin and some aspects of their function (catecholamine secretion) and morphology (neurite outgrowth). These cells can be isolated in large quantities and with relatively good purity. The transport of glucose into neural cells was investigated using bovine adrenal chromaffin cells as a model of a homogeneous neuronal cell population. Sugar transport was measured by following the cell/medium distribution of the nonmetabolizable glucose analogue 3-O-methyl-D-glucose (3OMG).

Characteristics of Sugar Transport in Adrenal Chromaffin Cells

The uptake of 3OMG in adrenal chromaffin cells was characterized by saturability, competitive inhibition, countertransport and inhibition by phloretin and cytochalasin B. Similar characteristics

of glucose transport were reported in glioma and neuroblastoma cells, synaptosomes, brain slices, whole perfused brain and peripheral nerve (Lund-Andersen, 1979; Baker and Carruthers, 1984). Thus, similar to other neural tissue preparations and most animal cell types, sugar transport in adrenal chromaffin cells was mediated by a facilitated diffusion mechanism.

In comparison with other cells of the brain, the sugar transport system in isolated bovine adrenal chromaffin cells resembles glucose transport in glial cells, which is also of low affinity (Bachelard, 1983). In addition, Passonneau (1976) showed that glucose uptake by astrocytoma (a type of glial cell) was stimulated by insulin. In C6 glioma cells, glucose transport was found to be the rate-limiting step for glucose utilization (Keller et al, 1981). Although the present study presents no direct evidence that sugar transport is rate-limiting in the adrenal chromaffin cell, the nature of the countertransport response (Figure 4) suggests that these cells have low levels of intracellular glucose. However unlike cultured glioma cells, isolated bovine adrenal chromaffin cells do not show a high Vmax characteristic of cells of tumour origin. For example, 3OMG uptake in human glioma cells (strain 138 MG) had a Km of 20 mM and Vmax of 500 nmol/mg protein/min (Edstrom et al., 1975). Adrenal chromaffin cells may therefore more closely reflect sugar transport in nonmalignant neural cells. In addition to similarities in some aspects of their morphology, function and ectodermal origin, adrenal chromaffin cells appear to resemble peripheral neurons in some characteristics of sugar transport, including its low affinity (Baker and Carruthers, 1984) and sensitivity to insulin (Field and Adams, 1964). Thus adrenal chromaffin cells may

be useful for the study of sugar transport in at least some but not all types of neural cells.

Regulation of Sugar Transport in Adrenal Chromaffin Cells

Adrenal chromaffin cells share many characteristics of tissues in which sugar transport is subject to regulation. The present study shows that 3OMG uptake in adrenal chromaffin cells is stimulated by insulin and hyperosmolar medium, factors which have been shown to stimulate glucose transport in muscle and adipose tissue. Sensitivity of sugar transport to secretory stimuli was also demonstrated, and may be a regulatory feature characteristic of this cell type. It appears that glucose utilization in adrenal chromaffin cells is variable, increasing in response to secretory stimuli (Millaruelo et al., 1982a). In addition, chromaffin cells also contain energy reserves of glycogen (Millaruelo et al., 1982b) and have the capacity for both aerobic and anaerobic metabolism (Millaruelo et al., 1982a). As described above, these cells appear to have low intracellular glucose levels indicating that the rate of glucose utilization is rapid and determined by the rate at which glucose gains access to the intracellular enzymes. Thus, the membrane transport of glucose may be rate-limiting for glucose utilization and may serve as a point of regulation by various metabolic and hormonal factors.

Ca^{2+}-Dependence

Although the factors or conditions (insulin, secretory stimuli, hyperosmolarity) which may increase glucose utilization vary between different cell types, they all have the ability to stimulate sugar

transport, and may share some common aspect of the molecular mechanism underlying the regulation of sugar transport. The data suggest that Ca^{2+} may serve as a commom mediator for transport regulation. The stimulatory effects of insulin and hyperosmolarity were abolished in the absence of external Ca^{2+} and depressed in the presence of the Ca^{2+}-antagonistic ion La^{3+} and the Ca^{2+} channel blocker methoxyverapamil. Basal transport was stimulated by factors, including acetylcholine, carbamylcholine, and low Na^{+} medium, which have been shown to stimulate Ca^{2+}-dependent catecholamine secretion from adrenal chromaffin cells, and their effects were abolished in Ca^{2+}-free medium. In addition, insulin, acetylcholine, hyperosmolar and low Na^{+} medium stimulated ^{45}Ca uptake. Thus glucose transport in adrenal chromaffin cells resembles that in muscle and adipose tissue with respect to the Ca^{2+}-dependence of transport activation by insulin and hyperosmolarity, and also exhibits Ca^{2+}-dependence of transport activation by secretory stimuli.

Variation in Ca^{2+}-Dependence

It may be speculated that sugar transport regulation involves Ca^{2+} binding to a membrane associated regulatory site which is in contact with the cytoplasmic Ca^{2+} pool or a particular region of the cytoplasm. The binding of Ca^{2+} to this site would depend on the characteristics of the binding site and the availability of Ca^{2+} in the cytoplasmic pool. Differences in the cytoplasmic Ca^{2+} pool between different cell types may account for the varying nature and degree of Ca^{2+} dependence of transport regulation. In different muscle cell types, the degree of Ca^{2+} dependence of transport activation varied in

parallel to the sensitivity of contractile activity to changes in external Ca^{2+} and to effects of Ca^{2+} antagonists. Ca^{2+} dependence of sugar transport activation was greatest in smooth muscle and least in skeletal muscle (Bihler et al., 1977; Bihler, 1980; Bihler and Sawh, 1980). Avian red blood cells differ from muscle cells in the nature of their Ca^{2+} dependence of transport activation. Sugar transport in avian red blood cells was largely dependent on the release on intracellular stores of Ca^{2+} rather than on Ca^{2+} influx across the plasma membrane (Bihler et al., 1982, 1982a).

Differences in the cytoplasmic Ca^{2+} pool may also explain some differences in transport regulation between muscle and chromaffin cells. As described earlier, in muscle, factors which increase intracellular Na^+ also enhance ^{45}Ca uptake via sarcolemmal Na^+/Ca^{2+} exchange. The subsequent rise in cytosolic Ca^{2+} presumably leads to stimulation of sugar transport. Hence monensin (Bihler et al., 1985a,b) and inhibition of the Na^+ pump (Bihler, 1968; Bihler and Sawh, 1977; 1980) stimulated sugar transport in muscle. In adrenal chromaffin cells, only some of the factors which increase intracellular Na^+, also increased ^{45}Ca influx. In fact, the presence of Na^+/Ca^{2+} exchange in adrenal chromaffin cells has not been definitely established. In contrast to muscle, factors which increased both intracellular Na^+ and ^{45}Ca uptake (ouabain, K^+-free medium) did not stimulate sugar transport in adrenal chromaffin cells. These observations suggested that Ca^{2+} influx via Na^+/Ca^{2+} does not play a significant role in the stimulation of sugar transport in adrenal chromaffin cells. Na^+/Ca^{2+} exchange in these cells may be less significant than other transport mechanisms in contributing to the availability of Ca^{2+} in the vicinity of the

postulated regulatory binding site. Similarly, a role for Na^+/Ca^{2+} exchange has not been clearly demonstrated in catecholamine secretion, a major physiological function of these cells.

Effects of Culturing on Sugar Transport

As described above, the suitability of secondary neural cell cultures has been questioned because of their malignant origin and the possibility that some of their characteristics may be due to culturing (Kalckar, 1976). The problem of malignant origin may be excluded by the use of adrenal chromaffin cell cultures, which are a primary cell culture. The effects of culturing on the characteristics of sugar transport were examined by comparing the kinetic parameters, insulin sensitivity, and Ca^{2+}-dependence of transport activation by insulin of 3OMG uptake in cultured adrenal chromaffin cells to that in freshly isolated cells. It was also of interest to examine whether the synthetic glucocorticoid dexamethasone altered sugar transport in relation to its effects on the morphologic changes of adrenal chromaffin cells in culture.

Sugar transport in day 1 cultures of adrenal chromaffin cells was mediated by a facilitated diffusion mechanism, as in freshly isolated chromaffin cells and most other animal cell types. In day 1 cultures, the maximal capacity of the sugar transport system was increased in comparison with that of freshly isolated cells. Within the first few days of culturing, adrenal chromaffin cells undergo extensive morphologic changes, and this process may be associated with a higher demand for energy-yielding substrates. The increase in maximal capacity

of the sugar transport system may be an an adaptation to this increased energy metabolism. An increase in the rate of sugar transport with morphologic change was also observed in dbcAMP-induced differentiated human glioma (138 MG) cell cultures (Edstrom et al., 1975; Walum and Edstrom, 1976a).

The regulation of sugar transport in several animal tissues is correlated with the metabolic requirements of each tissue. In muscle, regulation of glucose transport by activity or demand is exemplified by transport activation by feedback from an energy-consuming activity, such as muscular exercise, or in response to greater metabolic requirements stemming from less efficient energy generation under anoxic conditions. Regulation of glucose transport by substrate supply or storage is exemplified by insulin, which when secreted in response to the increased availability of substrates (i.e. glucose) stimulates glucose transport. Another example of supply regulation is the inhibition of glucose transport by alternative oxidative substrates, such as free fatty acids, which may act via feedback from altered cellular metabolism (Elbrink and Bihler, 1975).

Insulin had no effect on 3OMG uptake in day 1 cultures of adrenal chromaffin cells. In addition to its already accelerated rate, sugar transport may have been activated by insulin-like growth factors present in the fetal calf serum (Froesch et al., 1985), which was routinely added to the cell culture medium. Hence, if the sugar transport system is already maximally stimulated, insulin should have no effect under these conditions.

After 5 days in culture, adrenal chromaffin cells appear to have reached a stationary phase in morphologic change, and the maximal capacity of sugar transport was decreased. At this stationary phase of growth, chromaffin cells may have a lower energy demand than in the rapid initial stages of morphologic change (i.e. day 1 cultures). Hence, the decrease in energy metabolism may lead to a reduction in maximal capacity of glucose transport in comparison to day 1 chromaffin cell cultures. This difference in kinetic parameters of sugar transport between cells undergoing rapid growth and cells at a stationary phase of growth was also observed in mouse neuroblastoma cells (Walum and Edstrom, 1976b).

In comparison with freshly isolated cells, day 5 cultures still had a greater maximal capacity of 3OMG uptake. It is possible that the cultured chromaffin cell assumes new or additional cell functions with different energy requirements than freshly isolated cells, and the cell may undergo changes to adapt to the increased energy requirements. Similarly, in muscle, sugar transport is stimulated by feedback from energy-consuming muscular exercise. Thus, the differences in characteristics of sugar transport between freshly isolated and cultured adrenal chromaffin cells may be related to differences in their energy requirements and cell functions.

Insulin stimulation of 3OMG uptake was observed again in day 5 cultures of adrenal chromaffin cells. It appears that when operating at a lower maximal rate, the sugar transport system can again be stimulated by insulin. This is somewhat analogous to what is observed in freshly isolated cells, where the stimulatory effect of insulin is reduced under conditions where "basal" transport may have been

increased by unknown stimulatory effects. Thus the stimulatory effect of insulin is maintained in culture, but is only evident in chromaffin cell cultures at a stationary phase of growth and morphologic change (i.e. day 5 cultures).

Dexamethasone (10^{-5}M and 10^{-4}M) had no effect on the formation of processes in adrenal chromaffin cell cultures, but 3OMG uptake in dexamethasone-treated day 5 cultures was depressed primarily through an effect on the affinity of sugar transport. A competitive inhibition of glucose transport by dexamethasone has also been observed in other cell types (Loeb, 1976; Taylor and Gagneja, 1975).

The stimulatory effect of insulin in day 5 chromaffin cell cultures was abolished in the absence of external Ca^{2+}, consistent with observations in freshly isolated cells, muscle and adipose tissue. In contrast, the inhibitory effect of dexamethasone was not dependent on the presence of external Ca^{2+}. Insulin also stimulated 3OMG uptake in dexamethasone-treated cell cultures, and transport activation by insulin was potentiated in cultures treated with 10^{-5}M dexamethasone. Such a potentiation of insulin action by glucocorticoids has also been observed in other cell types (Amatruda et al., 1983).

In summary, characteristics of sugar transport, including saturation kinetics, sensitivity to insulin, and Ca^{2+}-dependence of transport activation by insulin, are maintained in culture. However, if the energy requirements of the cell change as a result of rapid growth and extensive morphologic change and/or the acquisition of new cell functions, the extent of transport activity is altered. In addition, if the sugar transport system is maximally activated as in the initial

stage of rapid growth and morphologic change (day 1), the stimulatory effect of insulin on sugar transport will not be evident. It remains to be determined whether culturing-associated factors other than rapid growth and morphologic change may affect the characteristics of sugar transport. Since dexamethasone did not inhibit the formation of processes in adrenal chromaffin cells cultures, the dexamethasone-treated cell is not representative of the cultured cell, which has not been morphologically altered. In addition, it was shown that dexamethasone had a direct and competitive effect on glucose transport in the cultured adrenal chromaffin cells, as also observed in other cell types.

In conclusion, it was shown that adrenal chromaffin cells transport glucose by facilitated diffusion as do other neural cells. More importantly, in this neuronal cell preparation, sugar transport was shown to be regulated by insulin, Ca^{2+}, secretory function, growth and morphologic change, indicating a link between the activity of the glucose transport system and cellular energy demand and hormonal influence. Thus, adrenal chromaffin cells have provided a useful model for the qualitative and quantitative investigation of sugar transport regulation.

Future Considerations

Sugar Transport Regulation in Adrenal Chromaffin Cells

The present study did not directly examine whether the membrane transport of glucose was rate-limiting. As described earlier, this question may be approached by determining the intracellular glucose content or by comparing the rates of glucose transport and phosphorylation. If glucose transport in adrenal chromaffin cells was found to be rate-limiting, this would confirm the present observations that sugar transport is an important regulatory step in glucose utilization by adrenal chromaffin cells.

In addition, the regulatory effects of insulin and secretory stimuli may be further examined by: (a) comparing the time courses of 3OMG uptake in response to insulin and secretory stimuli, (b) measuring the combined effects of insulin and secretory stimuli on 3OMG uptake and (c) comparing the time courses of 3OMG uptake with that of catecholamine secretion and ^{45}Ca uptake. This information would contribute to a better understanding of the mechanism underlying the regulation of sugar transport in the adrenal chromaffin cell.

Model For Sympathetic Neuropathy

The adrenal chromaffin cell may serve as an uncomplicated model for the study of other aspects of carbohydrate utilization in nervous tissue under normal conditions, in relation to neuronal cell development and under pathophysiological conditions such as diabetic neuropathy.

The widespread involvement of the autonomic nervous systems in diabetic neuropathy is indicated by symptoms of disordered autonomic

function. Pathologic changes affecting the sympathetic nervous system have been described (Low et al., 1975), but it is not clear whether the sympathetic lesion is postganglionic (Leveston et al., 1979) or preganglionic (Hilsted, 1982). The mechanisms underlying the development of diabetic neuropathy have not been fully defined, although several hypotheses have been proposed, as described in the Introduction. The cultured adrenal chromaffin cell, which resembles peripheral adrenergic neurons in its ectodermal origin and some aspects of its morphology and function, may provide a useful model for testing the biochemical mechanisms postulated in the development of diabetic neuropathy, such as the sorbitol hypothesis.

The sorbitol pathway consists of the reactions catalysed by aldose reductase, which converts glucose to sorbitol, and sorbitol dehydrogenase, which converts sorbitol to fructose. Normally, the activity of the sorbitol pathway in nerve is low. In persistent hyperglycemia, as in diabetes, cellular glucose levels would increase and enhance the activity of the sorbitol pathway leading to accumulation of sorbitol. Studies in the lens have shown that increased activity of the sorbitol pathway may enhance nerve myo-inositol efflux, and thereby reduce peripheral nerve myo-inositol content. Myo-inositol is a substrate for the synthesis of membrane phosphatidyl inositol, which has been postulated as a regulator of the nerve Na^+/K^+ ATPase activity. It is possible that a decrease in nerve myo-inositol content results in altered phosphoinositide metabolism, reduced Na^+/K^+-ATPase activity and depressed nerve conduction velocity (Brown and Greene, 1984). In diabetic nerve, there was a concommittant development of reduced motor nerve conduction velocity and accumulation of sorbitol

and fructose (Gabbay, 1975). In addition, it was shown that recently developed inhibitors of aldose reductase prevented the accumulation of sorbitol, the inhibition of myo-inositol uptake and the development of the nerve conduction velocity deficit (Tomlinson et al., 1982; Yue et al., 1982; Gillon and Hawthorne, 1983; Kikkawa et al., 1983).

As described above, enhanced sorbitol pathway activity leads to the accumulation of sugar alcohols, such as sorbitol. Because of its poor diffusibility across cell membranes, sorbitol may serve as an important osmotic factor contributing to the pathogenesis of the "sugar cataract". Kinoshita et al. (1962) showed that in the lens in diabetes, massive accumulation of sorbitol and fructose generates a considerable increase in lenticular water which disaggregates the internal fibers and causes cataract formation. Cataract formation has also been observed in galactosemia. This hereditary disease is characterized by genetic defects in the enzymes which convert D-galactose to D-glucose in the liver. In galactosemia, D-galactose is instead converted by aldose reductase to galactitol, a sugar alcohol, which accumulates and causes the formation of cataracts.

Sympathetic neuropathy appears to develop later than parasympathetic neuropathy (Hilsted, 1982; Clarke et al., 1979). It may be hypothesized that the greater resistance of sympathetic nerves may be related to low intracellular levels of glucose. In other words, if sympathetic nerves were able to maintain low intracellular levels of glucose, the abnormal increase in activity of the sorbitol pathway may be prevented or at least delayed. Adrenal chromaffin cells may serve as a useful model for diabetic changes in sympathetic nerve. Determinations of intracellular glucose levels in cells exposed to

normal and high external glucose, and investigation of the sorbitol pathway and of myo-inositol uptake should be of great help for understanding metabolic changes associated with diabetic neuropathy.

Integration of Cellular Function and Metabolism

A role for Ca^{2+} has been demonstrated or implicated in several cellular functions, including membrane events, contractile mechanisms, secretory mechanisms, activation or inhibition of specific enzyme systems and other cell events. In addition to glucose transport, other membrane events which have been shown or postulated to be mediated by Ca^{2+}, include cell adhesion, platelet aggregation, cell communication via gap junctions (inhibition), membrane fluidity (phase transition), membrane fusion, K^{+} conductance and stimulus-response coupling (receptor occupancy, action potentials, excitation-concentration coupling, stimulus-secretion coupling) (for review, see Case, 1980).

In a wider sense, Ca^{2+} may provide the link between cellular function and cellular metabolism. This does not exclude the possibility that other well known second messengers or mediatory mechanisms may participate in the integration of cellular processes. For example, it is well known that some cellular events such as the hormonal control of glucose-glycogen metabolism and postsynaptic effects of certain neurotransmitters, are mediated by the interaction of Ca^{2+}, the cyclic nucleotides and protein phosphorylation.

Much attention is currently centered on the phosphatidyl-inositol (PI) system. Certain factors, most notably Ca^{2+} mobilising neurotransmitters and hormones, activate the hydrolysis of the membrane phosphoinositide 4,5-biphosphate to form two important

products: (1) diacyglycerol, which activates the membrane protein kinase C and hence the phosphorylation of membrane proteins, the release of arachidonic acid, and the activation of guanylate cyclase; and (2) inositol triphosphate, which has been implicated in the release of intracellular stores of Ca^{2+}. Thus these two metabolites, which are second messengers themselves, may release other second messengers, including Ca^{2+}. The PI system has been implicated as a molecular mechanism underlying the regulation of several cellular processes, including cell growth, exocytosis, and most recently, sugar transport (for reviews, see Berridge, 1984; Farese, 1984).

Observations in isolated adipocytes suggest that the phosphatidyl-inositol (PI) second messenger system may participate in the regulation of basal and insulin-stimulated transport activity. Components of the PI system, including phospholipase C, 1,2-diolein (diacylglycerol) and inositol triphosphate stimulated basal glucose transport (Kirsch et al., 1985a; McDonald and Christensen, 1985). The phorbol esters, 12-O-tetradecanoyl-beta-phorbol-13-acetate (TPA) and PdBu (4-beta-phorbol-12,13, dibutyrate), which mimic the action of diacylglycerol, also stimulated basal transport, but inhibited the insulin-stimulated glucose transport and insulin binding in isolated rat adipocytes (Kirsch et al., 1985b). In mouse embryo fibroblast Swiss 3T3 cells, TPA increased the translocation of glucose transporters from the microsomal membranes to the plasma membranes (Kitagawa et al., 1985). Koepfer-Hobelsberger and Wieland (1984) showed that insulin activated the phospholipase C.

Based on these observations, Kirsch et al. (1985b) speculated that when insulin stimulates phospholipase C and thereby activates the

PI system, diacylglycerol is produced and it activates protein kinase C. The phosphorylation by protein kinase of both inhibiting and activating sites in the insulin receptor may then result in the simultaneous stimulation of basal glucose transport and inhibition of insulin-stimulated glucose transport. Thus, activation of protein kinase C consequent to insulin binding may provide a feedback mechanism to inhibit insulin signal transmission.

Another cell function which may be regulated by the PI system is the muscarinic receptor-mediated rise in cytosolic Ca^{2+}. Kao and Schneider (1985) showed that a component of the acetylcholine-evoked rise in cytosolic free Ca^{2+} in bovine chromaffin cells was independent of external Ca^{2+} and was mediated by muscarinic receptors. The muscarinic receptor-mediated rise in cytosolic Ca^{2+} was small (50-100 nM) in comparison to the rise in Ca^{2+} (uM range) caused by nicotinic agonists, and may explain why muscarinic agonists do not stimulate catcholamine secretion in bovine adrenal chromaffin cells. They suggested that this component may serve as an additional mechanism whereby acetylcholine increased cytosolic Ca^{2+}, and was distinct from the well known nicotinic pathway, involving membrane depolarization and net Ca^{2+} influx through voltage-gated Ca^{2+} channels. Although it was not known how muscarinic receptor activation stimulated the rise in cytosolic Ca^{2+}, several observations were consistent with a mediatory role of the PI system: (1) Muscarinic receptors in other tissues altered PI metabolism. (2) The extent and kinetics of the rise in cytosolic Ca^{2+} mediated by inositol triphosphate in other cells was roughly similar to that observed for muscarinic stimulated chromaffin

cells, i.e. a rapid rise in seconds to levels of a few hundred nanomolar. (3) Muscarinic agonists stimulated ^{32}P labelling of phosphoinositides and phosphatidic acid in bovine chromaffin cells, and this was independent of external Ca^{2+} (Kao and Schneider, 1985; for review, see Baker and Knight, 1984).

Other observations support a mediatory or modulatory role of the PI system in Ca^{2+}-dependent exocytosis. Phorbol esters, which activate protein kinase C, shifted the Ca^{2+} activation curve for catecholamine secretion to the left in leaky chromaffin cells, indicating that they increase the sensitivity of the secretory response to cytosolic Ca^{2+} (Baker and Knight, 1984). Holz (1985) showed that treatment of digitonin-permeabilized bovine adrenal chromaffin cells with phorbol esters enhanced phosphorylation and subsequent Ca^{2+}-dependent secretion, suggesting that protein phosphorylation can modulate Ca^{2+}-dependent secretion in adrenal chromaffin cells.

Thus, adrenal chromaffin cells may also provide a suitable model for examining the role of Ca^{2+} and its interaction with other mediatory mechanisms and potential second messengers in the integration of cellular metabolism (e.g. glucose transport) and cellular function (e.g. exocytosis).

SECTION V

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