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UNIVERSITY OF GLASGOW. APRIL, 1964.

Summary of Thesis:

Studies of Hormone Action on Cells in Vitro.

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(Manitoba)

for the Degree of

Doctor of Philosophy in the Faculty of Science.

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Attempts were made to maintain adult mammalian liver explants or cell aggregates in vitro by modifications of the Trowell organ culture technique. Tissue preparations survived for periods up to 6 days but results were not consistent enough to permit biochemical study of hormone action. Advantages of the use of the organ culture method in studies of hormone action are mentioned and a possible reason for the difficulty of maintaining liver in vitro is suggested.

Owing to the numerous advantages of cell strains for metabolic studies and investigations into the mechanisms of hormone action, these were employed for subsequent experiments. Addition of insulin to the culture medium induced an increase in the carbohydrate content of cell strains isolated from foetal human (HLM) and foetal calf liver. Possible mechanisms involved in this effect are discussed. Addition of prednisolone led to decreases in the cellular carbohydrate content and in cell multiplication. It is suggested that these effects may be secondary to other effects observed. This steroid also induced an increase in the content of DNA in HLM cells and in the lipid content of a strain of mouse fibroblastic cells.

Insulin addition led to a marked increase of glycolysis in cultures of HLM cells. It is suggested that the hormone may act by increasing the activity of an enzyme catalysing the phosphorylation of glucose and/or by increasing the rate of conversion of fructose-6-phosphate to fructose-1, 6-diphosphate.

Addition of prednisolone led to an inhibition of glucose utilization, but the keto acid content of the medium was increased in cultures containing this steroid. It is suggested that the latter effect may be primary to the former, since pyruvate inhibits glycolysis in isolated muscle.

Addition of insulin did not alter the levels of any enzyme consistently but increases in hexokinase activity were found on 2 out of 5 occasions. The induction of hexokinase or glucokinase could explain the insulin-induced increase in glycolysis. Addition of prednisolone caused a consistent increase in the esterase levels in HLM cells. The levels of other enzymes, such as glucose-6-phosphatase, glucose-6-phosphate dehydrogenase, lactic dehydrogenase and hexokinase were not consistently affected.

A technique for the study of glucose transport into HLM cells was developed. This involved the measurement of uptake of $[^{14}C]$- or $[^{3}H]$- glucose during a one minute exposure period. Experiments to determine the influx of labelled glucose and efflux of radioactivity over longer periods of time were also performed. The level of intracellular radioactivity approached a plateau after 20-30 minutes of exposure to labelled glucose. The efflux of radioactivity, following a 30 minute influx period, was more rapid than influx. Evidence is presented suggesting that 2 components can be distinguished during measurement of efflux of radioactivity. One of these was readily diffusible,

but the other remained within the cell even after a 30 minute efflux period.

Exposure of cells to insulin for periods as short as 10 minutes brought about a 2-fold increase in the rate of entry of glucose. An effect of insulin could be demonstrated under certain conditions at insulin concentrations as low as 10^{-4} units/ml.

The effect of physical factors on the rate of glucose transport and on the action of insulin was investigated. pH had no marked influence while increases in temperature from 20-30° induced increases in rate of transport of both control and insulin-treated cell cultures. The basal rate of glucose transport increased linearly with temperature and a Q_{10} (27-37°) of 1.7 was calculated. Below 30° the effect of insulin was absent but above this temperature insulin had an increasing stimulatory effect and a Q_{10} (27-37°) of 2.4 was calculated. The rate of glucose entry was increased in solutions of increased osmolarity in both control and insulin-treated cell cultures.

Omission of Ca^{2+} or Mg^{2+} did not affect the rate of glucose transport nor the effect of insulin. Omission of K^+, however, led to an increase in the rate of transport of glucose into control cell cultures to levels observed for insulin-treated ones. Reversing the molar ratio of Na^+ to K^+ led to a marked increase in the rate of glucose transport and the effect of insulin. No change was noted on omission of phosphate or in-

creasing the phosphate concentration by a factor of 10.

Glucose transport into insulin-treated and control cell cultures was found to obey Michaelis-Menten kinetics. Insulin apparently caused a decrease in the ratio Km/Vmax suggesting that it acts to increase the affinity of the glucose carrier for glucose or to increase the availability of the carriers themselves. The effect of insulin was apparent over a range of external glucose concentrations from 0.028 mM - 88.8 mM.

D-galactose or D-or L-arabinose could not be shown to exert any inhibitory effect on the rate of glucose transport into control or insulin stimulated cells. It is suggested that an inhibitory effect of these sugars may have been masked by a concomitant increase in transport rate induced by increased osmolarity.

Substances which inhibit glycolysis, such as fluoride, iodoacetate and pyruvate, induced an increase in the rate of glucose transport and masked or abolished the effect of insulin. It is suggested that this concurs with current concepts involving regulation of glucose transport in other insulin-sensitive cell types. Similarities and differences between this insulin-sensitive cell type and others are discussed. The effects of a number of other metabolic inhibitors were also investigated.

The rate of transport of glucose into cells grown in the presence of prednisolone was found to be decreased. It is suggested that this might explain the decrease in glucose utilisation, carbohydrate storage and cell multiplication observed

in prednisolone treated cultures. Insulin had an equally stimulatory effect on cells grown in the presence of prednisolone as on cells grown in its absence.

Studies of Hormone Action on Cells in Vitro

by

Robert James Hay

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ABBREVIATIONS

The following abbreviations will be used in this thesis:-

ACTH	Adrenocorticotrophic hormone.
AMP	Adenosine 5'-monophosphate.
ATP	Adenosine 5'-triphosphate.
CBH	Carbohydrate.
DNA	Deoxyribonucleic acid.
DNAP	Deoxyribonucleic acid phosphorus.
Glycogen synthetase	Uridine diphosphate glucose: α-1, 4-glucan α-4-glucosyl transferase.
NADP	Nicotinamide - adenine dinucleotide phosphate.
$NADH_2$	Reduced nicotinamide - adenine dinucleotide.
RNA	Ribonucleic acid.
RNAP	Ribonucleic acid phosphorus
tris	2-Amino-2-hydroxymethylpropane-1, 3-diol.
UDPG	Uridine 5'-diphosphate glucose.

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INTRODUCTION

In spite of persistent attempts to elucidate the means by which hormonal effects on cell systems are brought about, the primary mechanisms remain unknown. Hypotheses have been put forward implicating the cell membrane, mitochondria, ribosomes, the nucleus and other intracellular structures as the site of action of different hormones. It is generally agreed that hormones alter the rates of limiting reactions to initiate a sequence of events producing a recognizable change in cellular metabolism. The effect on rate limiting reactions may be direct or indirect. It may involve alterations in the availability of substrates, coenzymes or activating substances, alterations in amounts of apoenzymes, or changes in the structure of enzymes, thus altering their catalytic capacity. Thus hormonal effects on cells may involve changes in intracellular substrate availability by alterations in membrane permeability and/or changes in various enzyme activities. Both general effects could regulate cellular metabolism.

This study is primarily concerned with such actions of insulin and the anti-inflammatory steroid, prednisolone, on cells in culture. Since insulin is also known to have a general anabolic action in vivo, attempts were made to determine if a similar response is promoted in this system.

CELL PERMEABILITY AND HORMONE ACTION

Insulin:

Effect on permeability to sugars

The passage of sugar molecules from extracellular fluid

to the cell interior has been known for some time to involve more than simple diffusion. Red blood cells and muscle have been used most extensively in defining the nature of the process and have provided information from which a partial model of the "transport mechanism" can be visualized. Monosaccharide transport in muscle and erythrocytes is not an active process; apparently no energy is expended during the transfer of the sugar from the extracellular to the intracellular space. It is an example of a type of transfer which is commonly referred to as facilitated diffusion. It is thought to involve the transitory, stoichiometric association of a sugar molecule with a specific site on the cell surface, these sites being limited in number. By virtue of this association the sugar is able to pass through the hydrophobic cell membrane barrier. It is generally believed that the sugar molecule combines with the cell receptor or carrier and that this complex diffuses across the membrane to release the free sugar to the cell interior. The entire process is reversible and formation of the sugar-carrier complex obeys Michaelis-Menten kinetics. Competition for the hypothetical carrier-sugar complex can be shown to exist among different sugars as well as between enantiomorphs. The evidence from which this concept has been derived is reviewed by Wilbrandt, (1961, 1963); Randle (1961); Randle & Morgan (1962); Le Fevre (1961); and Hokin & Hokin (1963).

Conclusive evidence that insulin exerts an effect on

the transport of sugars into muscle cells was obtained by Levine & Goldstein (1955). These workers showed that insulin increased the galactose space of eviscerated-nephrectomised dogs and suggested, therefore, that the primary action of insulin on muscle involved an acceleration of sugar transport.

Since these experiments were reported an overwhelming body of evidence has accumulated in support of their hypothesis. It is now accepted that insulin acts on muscle to promote increased membrane transport not only of galactose and other "non utilizable" sugars but also of glucose. (Levine, 1961; Park et al. 1959, 1961; Randle & Morgan, 1962).

The insulin effect on sugar transport in muscle was further defined by Randle and Smith (1958 a, b). They found that anoxia or inhibitors of oxidative phosphorylation increased the D xylose and D glucose space in rat diaphragm. Morgan, Randle & Regan (1959) reported similar findings of increased entry or exit rate of D glucose or L arabinose with the perfused rat heart under conditions where oxidative phosphorylation was impaired. Morgan, Henderson, Regen & Park (1961) studied the effects of insulin on the kinetics of glucose transport in the perfused heart and found that it caused increases in both the K_m and V_{max} in this preparation. The mechanism by which insulin increases glucose transport is still highly speculative and indeed the very nature of the glucose transport system is uncertain. Nevertheless an insight into the possible chemical definition of the

system was provided by Walaas, Borreback, Kristiansen & Walaas (1960). These workers separated from rat diaphragm a glucan-peptide into which labelled glucose is rapidly incorporated. Since insulin causes a marked increase in this incorporation, the authors suggested that the glucan-peptide may be part of the glucose transport system.

Effect on permeability to amino acids

Tomkins & Maxwell (1963) have suggested that an inter-relationship between sugar transport and amino acid transport may exist. Thus insulin-induced alterations of sugar transport could produce alterations in amino acid transport and vice versa.

Kipnis & Noal (1958) reported that insulin enhanced the rate of accumulation of α amino isobutyrate in rat diaphragm. Manchester & Young (1961) suggested that such an effect may be secondary to the insulin stimulation of protein synthesis. More recent work (Akedo & Christensen, 1962; Fritz & Knobil, 1963) however, supports the conclusion that insulin stimulates amino acid transport independently of its effect on protein synthesis.

Effect on permeability to cations

Since the transport of sugars (Bhattacharya, 1961) and amino acids (Christensen, 1960) is influenced by certain ions, it is conceivable that the effect of insulin on these processes and on ion transport might be related. Zierler (1959 a, b, 1960) reported that insulin increased the resting potential and the intracellular potassium of rat skeletal muscle in vitro.

Since this was demonstrable in the absence of added glucose it is probably not secondary to the effect on glucose transport. The uptake of potassium by the perfused liver is also increased by insulin (Kestens, Haxhe, Lambotte & Lambotte, 1963; Mortimore, 1961).

Effect on pinocytosis

Ball, Martin & Cooper (1959) and Barrnett & Ball (1959, 1960) observed that rat epididymal fat tissue cells in vitro showed a loss of granularity and development of pinocytic invaginations at the plasma membrane following the addition of insulin to the incubation medium. A system of cannalicular channels and vesicles developed deeper in the cytoplasm and changes in the mitochondria suggesting increased activity were also observed. Paul and Pearson (1960) reported that insulin promoted pinocytic vacuole formation in the human cerivical carcinoma cell, strain HeLa (Gey, Coffman & Kubicek, 1952).

At present there are serious objections to the hypothesis that increased pinocytosis could explain the stimulatory effect of insulin on cell permeability to sugars (Levine, 1961). The recognized stereospecificity in sugar transport, the apparent necessity for simultaneous movement of water in pinocytic vacuoles and the absence of any stimulation of pinocytosis in the insulin treated muscle cell firmly argue against such a mechanism. Moreover studies reported by Sheldon, Hollenberg & Winegrad, (1962) indicate that the presence of pinocytic vacuoles bears no consistent relation-

ship to the glucose metabolism of adipose tissue.

Alterations in cell permeability as secondary mechanisms of insulin action.

The cellular permeability changes occurring in response to insulin cannot readily explain all the effects of this hormone and it could be argued that they represent secondary events. For instance, the work of Larner, Villar-Palasi & Richman (1959, 1960), Bessman (1960), Norman, Menozzi, Reid, Lester & Hechter (1959) and others suggests that insulin favours glycogen synthesis in muscle out of proportion to other pathways of glucose metabolism.

Cahill, Ashmore, Earle & Zottu (1958) demonstrated that the hepatic cell is freely permeable to glucose. Nevertheless, there are many reports indicating effects of insulin on glycogen storage and glucose uptake or output by the liver (Krahl, 1961; de Duve, 1960; Miller, 1961; Leonards et al. 1961; de Bodo, Steele, Altszuler, Dunn & Bishop, 1963; and Mortimore, 1963).

Therefore, if a unitary concept of the mechanism of action of insulin were to be proposed, it would necessarily have to provide for the findings, cited above, which cannot be explained

solely on the basis of permeability alteration.

Steroid Hormones:

Effects on permeability to sugars

Manchester, Randle & Young (1959) presented evidence from glucose uptake studies suggesting that cortisol or growth hormone administration reduces the sensitivity of the isolated rat diaphragm to insulin.

Morgan, Regen, Henderson, Sawyer & Park (1961), studied the effects of adrenalectomy and hypophysectomy on sugar transport and phosphorylation by the isolated, perfused rat heart. They concluded that no factor from either the hypophysis or the adrenal gland directly inhibits the transport of glucose in the diabetic heart. They did find, however, that growth hormone or hydrocortisone depressed the rate of glucose phosphorylation by the rat heart if these hormones were administered in vivo. It was also found that the administration of these hormones in vivo reduced the sensitivity of the glucose transport system of the isolated rat heart to insulin stimulation.

Maio & Rickenberg (1962) found that the artificial estrogen stilbestrol inhibits the transport of galactose into L cells cultivated in vitro. They suggested that this compound competes with galactose for the carbohydrate receptor sites at the cell surface.

Fain, Scow & Chernick (1963) reported that the 2α methyl derivative of cortisol and dexamethasone, a potent synthetic glucocorticoid inhibited glucose uptake and its conversion to

CO_2 by rat adipose tissue in vitro. Their results, however, do not indicate whether this is an effect on glucose transport, phosphorylation or some other step involved in glucose breakdown.

A General Hypothesis Explaining Hormone Action with Reference to Cell Permeability.

A general theory to explain hormonal alterations in permeability characteristics of a cell was proposed by Hechter & Lester (1960). They suggested that the changes in permeability may be secondary to alterations in internal architecture of the cell. They pointed out that hormone effects which apparently result in alterations of cell permeability, to sodium and potassium for instance, may be a reflection of a general change throughout the cell. It was postulated that "the high polymers of the cell are arranged to form a metastable system of ordered lattices representing a continuum throughout the cell". Furthermore, the existence of an interlayer aqueous region of bound and ordered water molecules was suggested. Hormones were supposed to alter the geometry of the system, thereby rendering additional volumes of the interlayer aqueous regions

accessible to sugars, amino acids or ions. Moreover it was postulated that proteins, including enzymes, form part of this ordered lattice network and that they are geometrically arranged to form multienzyme sequences. Thus, changes in the state of the lattice would not only influence substrate availability, via alterations of the state of the interlayer water, but enzyme activities as well. This suggestion is reminiscent of the hypotheses of Peters (1956) of hormone induced alterations in the cytoskeleton and of Krahl (1961) involving a "propagated disturbance" as a mechanism of insulin action. While these hypotheses are of value in that they provide a unitary concept of the mode of action of hormones, they suffer from the disadvantage that evidence to support or refute them is difficult to obtain.

ENZYME ACTIVITY AND HORMONE ACTION

Insulin:

Insulin is known to increase the activity of a glucose

phosphorylating enzyme in liver (Walker, 1962, 1963) and of an enzyme involved in glycogen synthesis in muscle (Friedman & Larner, 1963). The former effect may involve an activation of inactive enzyme precursor (Vester, 1963; Vester & Reino, 1963) or a stimulation of synthesis of enzyme protein (Salas, Vinuela & Sols, 1963). The latter involves an activation of the apparently less active glycogen synthetase (Friedman & Larner, 1963). These effects of insulin are discussed in more detail elsewhere.

Steroid Hormones:

A wide variety of enzymes in cell free extracts or in a purified state are influenced by steroid hormones (Tomkins & Maxwell, 1963). It is however, difficult to assess the physiological importance of these effects since it is not possible to demonstrate them in intact cells. Often, very high hormone concentrations are required to elicit the in vitro effect and for this reason also interpretation is made very difficult.

Steroid hormones also regulate the activity of numerous enzymes of intact cells both in vitro and in vivo (Tomkins & Maxwell, 1963). In many cases increases in enzyme activity have been shown to be a result of protein synthesis.

Weber, Banerjee & Bronstein (1961, 1962) have reported that cortisone administration to hypophysectomized or adrenalectomized rats causes increases in the activities of certain liver enzymes involved in gluconeogenesis whereas other enzymes of carbohydrate metabolism are not affected. The administration of the amino

acid analogue ethionine along with cortisone selectively inhibited the stimulation of the activities of these enzymes and this inhibitory effect was reversed by methionine. Ethionine is known to inhibit protein synthesis and its effect is reversed by methionine. The authors suggested, therefore, that the cortisone-induced increases in the activities of these enzymes are the result of synthesis of specific enzyme proteins.

Greengard, Smith & Acs (1963) reported effects on liver glycogen of the administration of cortisone or cortisone plus puromycin or actinomycin D to starved rats. Puromycin is known to inhibit protein synthesis whereas actinomycin D inhibits the deoxyribonucleic acid (DNA) - directed synthesis of ribonucleic acid (RNA) (Reich, Franklin, Shatkin & Tatum, 1961). The authors found that the cortisone-induced rise in liver glycogen in these animals, due presumably to gluconeogenesis, was inhibited by puromycin or actinomycin D. Weber, Singhal & Stamm (1963) similarly found that actinomycin inhibits cortisone induction of glycogen synthesis in rats. Furthermore, the induction of gluconeogenic enzymes by cortisone was also inhibited by this compound. The author stated that this evidence is in agreement with the suggestion that the stimulation of the synthesis of certain RNA species may be one of the primary actions of this hormone.

Other Hormones:

Glucagon and epinephrine

Glucagon promotes activation of liver phosphorylase

and epinephrine promotes activation of phosphorylases in liver and muscle, thus stimulating glycogen breakdown. The mechanism of this regulatory action has been partially elucidated and is now known to be mediated through production of cyclic adenylate (Stetten & Stetten, 1960: Sutherland & Rall, 1960: and Haynes, Sutherland & Rall, 1960). Friedman & Larner (1963) suggested that a similar mechanism may be involved in the insulin-stimulated conversion of the glucose-6-phosphate dependent form of uridine diphosphate glucose: α-1, 4-glucan α-4-glucosyl transferase (hereafter glycogen synthetase) to the independent form. This possibility is discussed in more detail elsewhere.

ACTH

Haynes (1958) reported that ACTH induces formation of cyclic adenylate by beef adrenal gland slices and that this in turn stimulates adrenal phosphorylase activity. Since ACTH also increases the permeability of the adrenal gland to sugars, Haynes et al. (1960) speculated that both of these effects may be mediated through production of cyclic adenylate. A similar suggestion might also be put forward to explain effects of insulin on enzyme activation and on cell permeability to sugars.

In this study some time was devoted to attempts at demonstrating specific enzyme induction by prednisolone or insulin in cultured cells. Such a system would seem an ideal tool for

the elucidation of the mechanism of enzyme induction and the influence of hormones on that process.

The hypothesis that hormones could act by regulating the rate of synthesis of specific "key" enzymes cannot be considered recent (Hechter, 1955). The elucidation of the steps involved in the synthesis of a protein, made in the last few years, provide for a more thorough insight into the possible loci for hormonal control.

An increase in the synthesis of a specific enzyme protein could be due to increased production or availability of the specific messenger RNA involved (Karlson, 1963). The mechanism by which a given hormone could act at the genetic level to induce an increased rate of formation of a specific messenger RNA species is still obscure.

Jacob & Monod (1961) have provided evidence indicating that enzyme synthesis in microorganisms is controlled by "regulator" genes. These regulator genes are thought to influence the so-called structural genes which are supposed to act as templates for messenger RNA synthesis. The regulator genes are thought to act by means of specific cytoplasmic compounds termed "repressors" which inhibit the formation or availability of messenger RNA.

This concept has arisen from studies with microorganisms and was proposed to explain induction of enzyme synthesis. Nevertheless it is possible that hormones exert their influence on similar genetic systems in mammalian cells. If such control

mechanisms are present, it would be possible to visualize an interaction of a given hormone or a product formed through its influence, with the repressor substance. Such an interaction could then prevent the repressor substance from inhibiting the formation of messenger RNA and thereby stimulate the synthesis of a given protein.

A suggestion to explain the simultaneous induction of several enzymes was presented by Jacob & Monod (1961). Several structural genes grouped together constitute an operon and are supposed to be controlled by another special gene, the "operator gene". In this case the repressor substance, produced by the regulator gene, would act on the operator gene to alter production of messenger RNA by all of the genes under its control. Thus for example genes responsible for the production of gluconeogenic enzymes might constitute an operon and be controlled by an operator gene. The action of cortisone on the liver could be supposed to interact in some manner with repressors produced by the regulator gene for this operon. Inhibition of the synthesis of specific messenger RNA, involved in the production of these enzymes, could thereby be released. Hence gluconeogenic enzyme synthesis would proceed at a greater rate.

Szilard (1960) presented a somewhat different hypothesis. He proposed that the repressor substance is synthesized in the cytoplasm under the influence of a regulator gene. The repressor exerts its effect by causing the enzyme to adhere to the ribosome

thereby rendering the template messenger RNA unavailable for further enzyme synthesis. The repressor is thought to combine with a specific controlling site on the enzyme molecule remote from the active catalytic center. Induction of enzyme synthesis is envisaged as a result of competition of the inducer with the repressor for this controlling site. The hypothesis that hormones may act in this manner is reflected by the explanation suggested by Feigelson, Feigelson & Greengard (1962) to explain tryptophan pyrrolase induction by cortisone. This might be accomplished by the direct action of the inducer on the enzyme attachment site (Halvorson, 1960) or by an action on enzymes which catalyse the removal of the polypeptide chain from the ribosome (Chantremme, 1961).

GENERAL ANABOLIC ACTION OF HORMONES ON NUCLEIC ACID AND PROTEIN SYNTHESIS

The more general anabolic action of such hormones as the estrogens, androgens, growth hormone, insulin, thryroid hormones, and the insect hormone ecdysone may also prove to be due, at least in part, to a general stimulatory effect of these on messenger RNA and protein synthesis (Tepperman & Tepperman, 1960; Karlson, 1963; and Tomkins & Maxwell, 1963).

Many anabolic hormones induce increases in RNA synthesis in target organs but this does not necessarily reflect synthesis of messenger RNA specifically.

Wool (1963) studied the effect of insulin on the incorporation

of label from 8- ^{14}C adenine and ^{14}C glucose into total nucleic acid and RNA of isolated rat diaphragm. Although insulin did not affect the incorporation of these compounds into DNA it did stimulate the incorporation into the RNA fraction. It also caused an increase in the actual amount of RNA which could be extracted from the tissue after a two hour incubation period. This worker suggested that the stimulation of RNA synthesis may be the primary point at which insulin acts to increase protein synthesis. Insulin has been shown to exert this effect on RNA metabolism even in the absence of added substrate (Wool, 1960). It was suggested therefore that it cannot be a reflection of an hormonal effect on the sugar transport mechanism. Wool & Munro (1963) reported experiments which indicate that insulin can stimulate the formation of a rapidly labelled nucleic acid having the characteristics of messenger RNA. In other instances insulin was found to effect the persistence or appearance of messenger RNA from the isolated rat diaphragm. These findings were, however, reported to be somewhat variable, although a consistent increase in the total ribonucleo - protein RNA synthesis as a result of insulin stimulation was observed. The authors postulated that RNA synthesis is the molecular site of action of insulin in the promotion of protein synthesis. They also suggested that this action may explain other biochemical and physiological effects of the hormone.

Eboue-Bonis, Chambaut, Volfin & Clauser (1963), however, present evidence arguing against this hypothesis. These workers studied the effects of puromycin and actinomycin D on the insulin

stimulation of incorporation of 8- ^{14}C adenine into RNA, incorporation of labelled amino acids into protein, and of glucose uptake and ^{32}P incorporation into high energy phosphates of rat diaphragm in vitro. They reported that although actinomycin D inhibited insulin-stimulation of RNA synthesis it had no such inhibitory effect on the insulin-stimulation of amino acid incorporation. Nor did it effect the insulin-stimulation of incorporation of ^{32}P into high energy phosphate compounds. Similarly puromycin blocked the incorporation of amino acids into protein. It did not affect insulin-stimulated glucose transport or the enhanced incorporation of ^{32}P into high energy phosphate. The authors concluded that it is therefore improbable that the actions of insulin on these processes can be explained on the basis of an effect on messenger RNA synthesis.

It is however conceivable that the longer term anabolic effects of insulin could still be explained on the basis that the hormone influences nucleic acid stability, synthesis or turnover.

Insulin is known to cause increased amino acid incorporation into muscle, fat and liver protein as well as into protein of subcellular structures under certain conditions. It seems very probable that this reflects a direct action of the hormone on the protein synthesizing systems of the cell (Manchester & Young, 1961; Krahl, 1961).

APPRAISAL OF TECHNIQUES USED IN ELUCIDATION OF HORMONE ACTION

As indicated in the foregoing outline, the present

understanding of the loci and mechanisms of hormone action evolved through a variety of general experimental avenues of investigation. One category of experimental approach has involved the destruction or extirpation of the endocrine gland or cells producing a given hormone. Subsequently, studies of a physiological, morphological or biochemical nature were performed either on the intact animal or on organs, organ segments or subcellular fractions prepared from it. The findings were then compared with those obtained from studies performed with similar material from control animals which had received replacement hormone injections.

The main difficulty in this type of experiment is interpretation. No single organ is exposed to the administered hormone alone and the presence of numerous tissue types may be involved in its action on the whole animal. It is therefore very difficult, if not impossible, to interpret any single effect as being induced primerily or directly by the hormone involved.

A second category of experiments involved the removal of an organ or tissue, believed to be influenced by a given hormone, from a normal donor animal. Direct effects of the hormone were then determined by adding it either to a perfusing medium or to medium maintaining fragments or slices of the tissue being examined.

The advantages of being able to observe the effects of a biological regulator in a system removed from the complexities of the whole animal are obvious.

Although there are considerable advantages to this type of in vitro study, certain disadvantages should also be recognized. Perfused organs such as the heart and liver are excellent tools for short term experimentation and have certainly yielded much valuable information. The effects of some hormones, however, require a considerable time to become apparent. These preparations and indeed the tissue slice and diaphragm preparations also, do not survive for extended periods of time. For this reason they cannot be used for the elucidation of the mechanism of longer term hormonal effects. Some hormones do not appear to exert any influence on these systems when applied in vitro but do exert an effect when administered in vivo over longer periods of time. While these effects could be manifestations of an indirect action, it would also be possible that they occur only after longer periods of exposure of the tissue to the hormones involved.

The use of organ or cell culture techniques has long been recognized as a valuable tool in the study of the effects of metabolically active compounds on whole cell preparations. The main advantage of these techniques is the precision with which it is now possible to define the conditions under which the cells are studied. With other in vitro study methods the tissue preparations have a very limited survival time. Results are often extremely variable owing perhaps to tissue trauma on being excised from the donor animal or to differences in these

animals themselves. The preparations often require complex nutrient solutions containing largely blood plasma or other material of unknown composition.

With a recently developed organ culture technique, however, it is possible to maintain a given target organ or organ segment in a completely synthetic medium for extended periods (Trowell, 1959). In this and older organ culture methods the tissue is in a healthy state throughout the study. Furthermore it is possible to accustom it to culture prior to exposure to a given hormone. This ensures that hormonal effects persisting at the time of removal of the tissue from the animal due to endogenous hormone, are minimized. This of course is not always possible with short term in vitro preparations.

Difficulties are also encountered with this technique however. The possibility that hormone and nutrients do not penetrate the tissue and effect all cells present to the same extent must be recognized. Furthermore, while many tissues can be maintained by these methods certain tissues do not survive.

Several main advantages of cell as opposed to organ culture techniques for biochemical studies should be emphasized. Cell strains can be cultivated permanently in vitro as monolayers or in suspension under well defined conditions. Their growth stages can be readily recognized and their metabolism is readily studied. Cells in a given culture can be equally exposed to hormones and nutrients alike. Conversely, with the organ culture

technique the problem of inward diffusion of hormones or nutrients and outward diffusion of end products of metabolism may complicate interpretation.

Certain difficulties must also be acknowledged in this case. Cellular characteristics may be lost or altered during adaptation to growth in vitro. One metabolic change commonly observed during this transition is an increase in aerobic glycolysis as compared to complete oxidation of glucose. Furthermore the identity of the cell type eventually isolated from a particular target tissue may be uncertain. Nevertheless certain cell strains must possess the necessary "reactive sites" rendering them responsive to hormonal stimulation.

HORMONAL EFFECTS ON CELL SYSTEMS IN CULTURE

Lasnitzki (1958) reviewed work concerning the effects of hormones, carcinogens and vitamins on organ cultures. The emphasis was placed on morphological changes and influence on growth and development. Most of the studies referred to were performed using media of non defined nature containing high concentrations of serum, plasma or tissue extracts. More recently developed organ and cell culture techniques however, allow use of media which are either completely synthetic or require only slight fortification with serum. These techniques can undoubtedly be of value in studies of metabolic alterations following hormone addition.

Insulin:

Effects on growth and cell multiplication

Growth stimulatory effects of insulin on chick embryonic heart explants cultured in vitro (Leslie & Davidson, 1951; Leslie & Paul, 1954) and on organ cultures of embryonic bone (Chen, 1954; Hay, 1958) have been reported. Similar studies with established cell strains however, have given negative or inconsistent results (Leslie, Fulton & Sinclair, 1957; Paul & Pearson, 1960). Crockett & Leslie (1963) reported a consistent growth promoting effect of insulin on a strain of cells, designated HLM, derived from human foetal liver (Leslie, Fulton & Sinclair, 1956). They suggested that the previous variability of results may reflect differences in the response to the hormone due to differences in media employed. The growth rate of established cell types is markedly effected by pH (Paul, 1959 ; Broda, Suschny, Rucker & Kellner, 1959). Since it is extremely difficult to maintain a constant pH between insulin-stimulated and control cultures this may also complicate interpretation.

Lieberman & Ove (1959) reported that insulin addition to a cell culture medium containing no serum stimulated the growth of HeLa cells and of cells (strain A1) isolated from normal human appendix (Chang, 1954). The authors claimed that this was not a non-specific effect since the addition of inactivated insulin did not enhance growth.

Franks (1961) reported that organ cultures of mouse ventral prostate grown in either a chemically defined medium

or medium containing serum showed marked epithelial hyperplasia after 5 to 7 days exposure to insulin.

The addition of insulin to medium maintaining adult mouse ear fragments caused a doubling of the mitotic rate if glucose was used as the substrate but was ineffective if fructose or lactate was used. It was suggested that the effect of insulin was due to an increase in entry and/or phosphorylation of glucose (Bullough, 1954).

Effects on metabolism

Perhaps the most consistent observation made with cultured cells and organs maintained in the presence of insulin has been an increase in glucose utilization often with a concomitant increase in lactate production. Paul & Pearson (1960) reported that insulin consistently stimulates glycolysis by cultured strain L mouse fibroblasts (Earle, 1943) and by primary cultures of cells from the chick embryonic carcass. An increase in the utilization of pyruvate from the incubation medium was also noted. The authors were unable to detect any effect of insulin on the accumulation of protein, lipid, RNA or DNA. Earlier work with other cell strains indicated that increments in RNA phosphorus and in glycolytic rate occurred after insulin addition. No increment in DNA phosphorus was found (Leslie, Fulton & Sinclair, 1957).

Crockett & Leslie (1963) confirmed that insulin stimulates glycolysis in cultured HLM cells. They also showed that insulin does not alter the distribution of labelled carbon atoms from

glucose in various components of the cell. Furthermore insulin was not found to alter pentose shunt activity significantly.

Vann, Nerenberg & Lewin (1963) determined the effect of temperature, osmotic pressure and various incubation media on glucose uptake and insulin action on HeLa cells. These variables affected basal glucose uptake and insulin-stimulated uptake to different extents. Insulin stimulation was negligible in the absence of bicarbonate.

The authors interpreted these results as indicating that two pathways for glucose utilization exist in HeLa cells, one dependent and the other independent of insulin. Their suggestion is similar to that of Shaw & Stadie (1959) who proposed that two independent pathways of glucose utilization exist in rat diaphragm. According to these workers, an insulin dependent pathway operates only in the presence of bicarbonate while the insulin independent pathway occurred in either bicarbonate or phosphate-buffered media.

Insulin has also been found to influence the metabolism of adipose tissue cultured in vitro (Sidman, 1956b). Typical adipose cells were shown to differentiate from the mesenchyme of the interscapular region of late foetal rats within a few days after culture in vitro (Sidman, 1956a). The addition of insulin was found to stimulate the synthesis of glycogen, hasten the deposition of fat and increase the survival time of this preparation.

Insulin is one of the hormones required for optimum maintenance and secretory activity of mouse mammary explants in organ culture (Rivera & Bern, 1961). This tissue in culture was recognized as being very suitable for the study of effects and mechanisms of action of insulin, cortisol, mammotrophin and somatotrophin.

It should be emphasised that in the majority of these studies concentrations of insulin have been employed which are considerably in excess of that to be expected in vivo. This factor must be borne in mind when the results are assessed.

Steroid Hormones:

Effects on organ cultures

Fell & Thomas (1961) studied the effects of hydrocortisone on organ cultures of the cartilagenous long bone rudiments from chick embryos and on well ossified limb bones from foetal mice. The chick bone rudiments were found to grow well in culture. Hydrocortisone inhibited this and also decreased the formation of intercellular material and the size of the hypertrophic cells of the bone shaft. The mouse radii grew very slowly in culture but hydrocortisone was found to arrest the resorption of cartilage, a process which was very evident in the control cultures.

It was also noted that hydrocortisone partially inhibited the effect of vitamin A to cause disintegration of chick or mouse cartilage and of mouse bone in culture.

The effects of cortisone or hydrocortisone on skeletal

tissue in culture is qualitatively similar to effects on the growing skeleton of the intact animal. However the interaction of vitamin A and hydrocortisone in vitro is opposite to the interaction in vivo. It was emphasized that this apparent discrepancy can probably be explained on the basis that other systemic factors are involved in the in vivo response.

A suggestion as to the role of the hydrocortisone in diminishing the effects of vitamin A arose from other experiments. Dingle (1961) reported that vitamin A promotes release of at least one proteolytic enzyme from rat liver lysosomes. Lucy, Dingle & Fell (1961) extracted proteolytic material from normal chick cartilagenous rudiments which is capable of producing effects on cartilage matrix similar to those produced following vitamin A treatment. Fell & Thomas (1961) therefore suggested that hydrocortisone may inhibit the release, synthesis or activity of the lysosomal proteases. In this respect Weissman & Dingle (1962) and Weissman (1962) presented evidence suggesting that hydrocortisone retards the release of potentially harmful enzymes from lysosomes of rat or rabbit liver.

Rivera & Bern (1961) reported that cortisol is necessary to promote optimum maintenance and secretory activity of mouse mammary tissue in organ culture. As mentioned earlier, other hormones were also required and appear to act synergistically in vitro as they do in vivo.

Effects on cultured cells

(a) Growth Inhibition

Corticoid hormones and related compounds inhibit multiplication of numerous cell types (Holden & Adams, 1957; Grossfeld, 1959; Wellington & Moon, 1962; Cox & MacLeod, 1962). Gabourel & Aronow (1962) using cultured mouse lymphoma cells (ML-388) (Herzenberg & Roosa, 1960) found that the growth inhibitory potency of various steroids generally paralleled their anti-inflammatory activity. Cell multiplication was inhibited by 50% in the presence of 10^{-7} Molar hydrocortisone. Steroid concentrations used by other workers with other cell strains were usually much higher.

Jaffe, Fisher & Welch (1963) determined the growth inhibitory potency of a variety of glucocorticoid derivatives on cultured mouse leukemic cells (strain L5178Y) (Fischer, 1958). They reported that methylation at C16, halogenation at C6 or C9 and introduction of a 16α, 17α-O- propylidene derivative to the basic cortisol molecule enhanced the growth inhibition properties.

(b) Morphological Changes

Holden & Adams (1957) reported that L cells grown in the presence of hydrocortisone appear swollen and more transparent than controls. Treated cells were also found to have enlarged, irregular shaped nuclei and numerous cytoplasmic vacuoles. Cox & Macleod (1962) also noted increases in the volume of steroid-treated epithelial cell strains but no increase was found in

the volume of L cells. This discrepancy may be due to the large differences in steroid concentrations used in these two studies.

Wellington & Moon (1961) found that the nuclei of a strain of epithelial cells isolated from human liver (Chang, 1954) were larger and more irregular after growth in the presence of hydrocortisone. The cytoplasm was more abundant but less dense.

(c) Changes in Cell Composition and Metabolism

Low concentrations of adrenal and related steroids decreased the cellular protein, DNA and amino acid content of mouse lymphoma cells (ML-388). Protein synthesis, as judged by incorporation of ^{14}C leucine was depressed (Gabourel & Aronow, 1962). Conversely, increases in cell protein, RNA and DNA were found in strains of human epithelial cells cultivated in the presence of prednisolone (Cox & MacLeod, 1962).

Greenberg & Stewart (1961) found that hydrocortisone decreased glucose uptake and glucose storage by HeLa cells. Grossfeld (1959) on the other hand, reported that massive doses of hydrocortisone had an immediate inhibitory effect on respiration and a stimulatory effect on glycolysis of strain L fibroblasts. However, cells cultivated in the presence of smaller doses of the hormone for 24 or 96 hours respired more actively than controls.

These qualitative differences emphasize the probability that steroid action varies with concentration and cell type used.

(d) Alkaline Phosphatase Induction

Cox & MacLeod (1962) determined the alkaline phosphatase activity of several types of primary cell cultures and certain established cell strains. The primary cell cultures, several human fibroblastic cell strains and the mouse fibroblastic L line did not contain alkaline phosphatase. Human epithelial strains either contained these enzymes constitutively or could be induced by prednisolone to produce them.

Various other factors such as medium composition (Cox & MacLeod, 1962; 1963), presence of substrate (Cox & Pontecorvo, 1961), and osmolarity (Nitowsky, Herz & Geller, 1963) influence the level of alkaline phosphatase in certain cell strains. Maio & De Carli (1963) separated variants, having diverse alkaline phosphatase levels, from a human, embryonic, epithelial cell strain EUE (Maio & De Carli, 1962). Variants with lower levels of this enzyme activity responded to prednisolone, hydrocortisone and organic monophosphate with formation of increased amounts of these enzymes. The same compounds however, repressed formation of alkaline phosphatase in variants with a high enzyme activity.

Other Hormones:

Thyroid and parathyroid hormones

(a) Effects on Organ Cultures

Gaillard (1963) studied the effects of parathyroid and thyroid hormones on mouse embryo radius rudiments in organ culture. Effects of a parathyroid extract on such parameters

as bone resorption, decrease in osteoblasts and bone formation, increase in osteoclasts, together with effects on cartilage and bone shaft connective tissue were observed. The effects of thyroxine were very slight and inconsistent. However, the production of hypertrophic cells in the proximal and distal zones of the rudiments was consistently and significantly depressed by thyroxine addition. In spite of the variability in the results, the author concludes that thryroxine is able to induce a concentration-dependent, mild and limited parathyroid like effect.

Mecca, Martin & Goldhaber (1963) studied the effects of parathyroid extract on the metabolism of whole mouse calvaria in organ culture. Marked bone resorption was observed by the fourth day of culture in the presence of parathyroid extract. Conversely, control cultures were virtually unchanged as judged by total calcium and phosphorus analysis. Accompanying the resorption of bone was an increased production of citrate and an increase in glucose utilization and lactate and hydroxyproline production. The authors interpret the results as supporting the hypothesis that parathyroid hormone acts to cause increased bone resorption by inducing increased citric acid formation. The increased hydroxyproline appearance in the medium supporting parathyroid extract treated calvaria, was thought to suggest that organic matrix was being destroyed at a greater rate.

(b) Effects on Cultured Cells

Halevy & Avivi (1960) observed effects of thyroxine and triiodothyronine on primary cultures of chick embryo cells.

They reported that these hormones at very low concentrations induced an increase in the glycolytic rate. Epinephrine, estradiol and insulin had no effect.

Eaton, Adler, Bond & Scala (1956) reported that the addition of small amounts of thyroxine to cells cultured from the chorioallantoic membrane increased their oxygen consumption after a lag period of twelve hours. This effect was observed in the absence of added substrate and in the presence of glucose or pyruvate.

Leslie and Sinclair (1959) examined the effects of thyroxine and triiodothyronine on cells from foetal lung and kidney and on several established cell strains. No effects were found if physiological concentrations of these hormones were used but at higher concentrations glycolysis was stimulated. In addition, the growth of foetal lung cells was inhibited but no such effect was observed with the other cell types.

Since high concentrations of thyroid hormones have been shown to uncouple oxidative phosphorylation in isolated mitochondria, the possibility of a similar action on cultured cells was discussed. The inhibition of foetal lung cell growth might be explained on that basis. The authors suggest that insensitivity to this action of thyroid hormone, shown by the other cell types, may be due to alterations in their oxidative metabolism occurring during transition to growth in vitro.

Growth hormone

(a) Effects on Organ Cultures

Bullough (1954) studied the influence of growth hormone on mitotic rate of mouse ear epidermis in organ culture. This hormone depressed the number of mitoses if glucose was used as substrate but had no effect if fructose or lactate was used. The author suggested therefore that growth hormone acts at some initial stage of the entry of glucose into the cell metabolic pathways.

Bullough also noted an antagonism between growth hormone and insulin. The inhibitory effect of growth hormone on mitosis could be partially offset by large doses of insulin and the stimulatory effect of insulin on mitosis could be inhibited by large amounts of growth hormone.

It is interesting that Henderson et al. (1961a) and Morgan, Regen, Henderson, Sawyer & Park (1961) found a similar antagonism of the insulin stimulatory effect on glucose uptake by the perfused rat heart. In this case, however, the effect could only be demonstrated after administration of growth hormone to hypophysectomized rats some time before removing the heart. Their results indicate that growth hormone inhibits phosphorylation of glucose rather than glucose transport. Conversely, insulin primarily affects glucose transport. The authors suggested that the inhibitory effect of growth hormone (and corticosteroids) on glucose phosphorylation may represent an indirect action.

The results of Bullough (1954) however, strongly argue against this as a general supposition. Although no direct measurements of glucose uptake were made in that study, initial stages in the metabolism of glucose are implicated in the action of both hormones on this in vitro system.

Hay (1958) studied the effects of growth hormone on bone rudiments in organ culture. A slight stimulation in growth of chick embryo tibia and femur was noted.

(b) Effects on Cultured Cells

Moon, Jentoft & Li, (1962) reported that growth hormone caused an increase in nuclear proliferation in a strain of cells isolated from human liver (Chang, 1954). This effect increased with increasing doses of the hormone and with increasing exposure times. Although the cell numbers were increased as was the total protein per culture, the protein per cell was decreased.

ACTH

Schaberg, De Groot & Gelpke (1959) and Schaberg (1961) reported that adrenal cortical explants and pituitary explants could be maintained in a combined organ culture. Morphological changes in the adrenal explants were induced by ACTH or by combination with anterior hypophysis explants. By direct measurement and by inference, the authors concluded that these organ cultures produce corticosteroids and ACTH. Steroid synthesis by the adrenal explants was stimulated by culture with the pituitary explants.

The ability to reproduce in vitro, effects of hormones which are similar in essential details to those which occur in vivo, would be of infinite value in the elucidation of primary action mechanisms. The foregoing review emphasizes the fact that numerous hormone actions have been demonstrated on cell and organ culture systems. Many of these are very like effects which occur on isolated tissues and in the intact animal. These techniques were therefore put to use in this study, in an attempt to define further the effects of insulin and steroids at the cellular level.

MATERIALS AND METHODS

CELL CULTURE TECHNIQUES

Cell Strains

The cell strain used most extensively in these studies was isolated from human foetal liver and designated HLM (Leslie, Fulton & Sinclair, 1956). The cells of this strain are epithelial-like in appearance. Other epithelial cell strains used were the strain KaLe isolated from foetal calf liver (Pieck & Kuyper, 1961), the human cervical carcinoma cell, strain HeLa (Gey, Coffman & Kubicek, 1952) and a substrain of this, designated HeLa (A.P.). This latter strain can be induced by hydrocortisone or prednisolone to produce increased amounts of alkaline phosphatase.

A variant of the mouse subcutaneous fibroblast cell, strain L, clone 929 (Sanford, Earle & Likely, 1948) which grows in suspension was isolated in this laboratory and was used in one experiment of this study. It was designated strain LS.

Maintenance of Cell Cultures

Cell cultures were maintained as described by Paul (1960). All strains with the exception of the L cell variant were grown in Waymouth's medium (see Appendix) supplemented with 5% (V/V) calf serum and 2% (V/V) human serum. Unless otherwise specified this medium supplemented with serum, will be referred to as Waymouth's medium. Calf serum was prepared from blood obtained at a local abattoir and was sterilized by millipore filtration. Human serum was obtained from the blood bank. Sera

were tested for toxicity and cloning efficiency before use.

The variant of the L cell strain was grown in Eagle's medium (see Appendix) supplemented with 5% (V/V) calf serum and 0.5% (W/V) Bacto-peptone (DiFco, obtained through W. B. Nicolson, Ltd., Glasgow). Stock cells were grown as monolayers, with the exception of strain LS, in Roux flasks containing 50 ml. Waymouth's medium. When the monolayer had become confluent, the overlying medium was removed and 10 ml. of 0.5% (W/V) trypsin solution (see Appendix) was added. The vessel was then rocked gently to distribute the solution over the cell monolayer. After 1-3 minutes the trypsin solution was removed and the culture vessels were incubated for a further 5-10 minutes. After this treatment the cell monolayer had disintegrated from the vessel wall. Cells were then suspended in Hank's balanced salt solution (BSS) or tris-citrate buffered BSS (see Appendix) depending on which of these had been used in the growth medium. This cell suspension was usually checked under low power objective to ensure that it consisted of single cells. An aliquot was taken for a cell count which was performed using a Coulter Electronic particle counter, model D.

Fresh Roux flasks were seeded with 5×10^6 cells in 50 ml. of Waymouth's medium and incubated for 3-5 days at 37°. After this period the medium was replaced and cultures were incubated for an additional 3-5 days. At this time the cell monolayer had usually become confluent and the culture cycle

was repeated as described above.

With the LS strain no trypsinisation was necessary. Instead of removing "spent" medium after 3-4 days as was done with monolayer cultures, an additional 50 ml. of growth medium was added for the second incubation period. Cell inocula were prepared by centrifuging the LS cells and resuspending the pellet in Hank's BSS.

Insulin and Prednisolone.

Glucagon free insulin was obtained from Boots Pure Drug Co.Ltd. as a sterile stock solution containing 200 units/ml.

Prednisolone-disodium phosphate was obtained from Glaxo Laboratories, Ltd. A stock solution in Hank's BSS was made to contain the equivalent of 250 µg of prednisolone per ml. and was sterilized by millipore filtration.

Both stock solutions were stored at 4° C.

ANALYTICAL METHODS

All reagents, unless otherwise stated, were prepared using "Analar" (if available) or "laboratory grade" chemicals obtained from British Drug Houses Ltd.

Colorimetric determinations were made using a Beckman DB spectrophotometer or a Unicam S.P. 600 with a light path length of 1 cm. In some cases, which will be specifically mentioned, a Beckman/Spinco spectro-colorimeter 151 was used. This instrument is equipped with a capillary cuvette and can measure the intensity of as little as 0.1 ml. of coloured solution. Reagent blanks containing water or other appropriate solvent instead of the tissue extract or deproteinized solution were included with each series of estimations.

Methods of Analysis for Cell Components

Harvesting procedure

Cell monolayers were harvested by the trypsinization procedure described for stock culture maintenance. Cells in tube cultures were harvested by the addition of 2 ml. trypsin solution followed by an incubation period of 5 minutes. The cells, suspended in the trypsin solution, were then removed by centrifugation before further treatment. The volumes of reagents used in these cases were reduced as compared with those employed for larger cell numbers.

After trypsinization, the cells were suspended in 10 ml. Hank's BSS at 4° and an aliquot was taken for estimation

of cell number. The cells were then removed by centrifugation at 4° and treated by the fractionation procedure described by Paul (1958).

Fractionation procedure

Two ml. of an ethanol-ether mixture (3 : 1 V/V) was added to the centrifuge tube containing the pellet of washed cells. The resulting mixture was thoroughly stirred with a platinum wire and set aside at room temperature for 15-20 minutes. It was then centrifuged and the supernatant was collected. This procedure was repeated, first with 2 ml. of an ether-carbon tetrachloride mixture (3 : 1 V/V) and then with a further 2 ml. of the ethanol-ether mixture. The supernatant liquids were combined and saved for lipid estimation.

Two ml. of ice-cold N H_2SO_4 were then added to the residue in the tube. The contents were mixed and left in an ice bath for 10 minutes with occasional stirring. The residue was separated by spinning in a refrigerated centrifuge. This procedure was repeated twice and the supernatant liquids were combined.

Two ml. of N perchloric acid was added to the material remaining in the tube and the contents were stirred as before. The tube was then placed in a water bath at 70° for 20 minutes during which time the contents were stirred occasionally.

The residue was again separated by centrifugation and the supernatant liquid was collected. This procedure was repeated with a further 2 ml. of N perchloric acid and the supernatant was combined with the first. This perchloric acid extract was used for estimation of DNA and RNA. An aliquot of this fraction was combined with an aliquot of the H_2SO_4 extract and used for estimation of carbohydrate (CBH).

Two ml. of N trichloracetic acid was added to the residue and the mixture was stirred as before. The acid wash was separated by centrifugation and was discarded.

Protein was estimated in the remaining material.

Lipid estimation

Principle

The lipid extract was analysed by a modification of the method of Bloor (1947). This involved the oxidation of lipids by Chromic acid and the determination of the intensity of the green colour produced by reduction of the chromate ion.

Reagents

$NaHCO_3$ - 10 g./litre.

Chromic acid. Prepared by dissolving 12.5 g. of silver dichromate or 8.5 g. of sodium dichromate in 500 ml. of 25 N H_2SO_4. (If concentrated H_2SO_4 was used a precipitate was formed).

Ether.

Procedure

The ethanol-ether-carbon tetrachloride layer was shaken with 2 ml. of the $NaHCO_3$ solution and 2 ml. of ether was added. The organic-solvent layer was removed and the $NaHCO_3$ layer was washed twice with 2 ml. of ether each time. The washings were added to the organic-solvent fraction. The volume of this was adjusted if necessary and aliquots were taken for total lipid estimation.

The aliquots were placed in tubes and evaporated to dryness in a hot air bath. Chromic acid solution (0.6 ml.) was added to each tube and they were placed in a boiling water

bath for 15 minutes. The tube was removed, cooled in a crushed ice bath and 0.6 ml. of water was added. The green colour was measured at 620 mu. Standards were prepared by dissolving pure lard in carbon tetrachloride. The range of the method was 50-500 ug. of fat. Organic solvent fractions from individual cell specimens were divided into 3 aliquots. Each of these was analysed as described above and the lipid content was calculated from the mean of the three estimations.

Carbohydrate estimation

Carbohydrate was estimated by the anthrone method as described by Trevelyan and Harrison (1952).

Reagents

Anthrone reagent. Freshly prepared by dissolving 0.2 g. of anthrone in 100 ml. of 25 N H_2SO_4.

Procedure

Five ml. of anthrone reagent was added to each of a series of tubes and these were placed in a crushed ice bath. After 10-15 minutes 1 ml. of the acid extract to be tested was added to each tube so as to form a layer on the top of the reagent. Each tube was then sealed with a glass stopper, shaken thoroughly and returned to the ice bath. The tubes were then transferred to a vigorously boiling water bath. After 10 minutes they were removed and plunged immediately into an ice bath. When the solutions had cooled, the intensity of the green colour produced was measured at 620 mu.

Standard solutions were prepared from a solution (1 g./litre) of glucose in saturated benzoic acid. Standards, containing 50 or 100 ug. of glucose, were included with each series of estimations. Results, expressed in terms of glucose, are means of duplicate determinations. The range of the method is 10-150 ug. of glucose.

In experiments where the number of cells available was small, the volumes of anthrone reagent, acid extract, standards and blanks were reduced to one-fifth of those described above.

DNA estimation

DNA was estimated by the colour produced on heating of the cell extract in the presence of indole and HCl (Ceriotti, 1952).

Reagents

Indole solution (0.4 g./litre). The indole was dissolved in 1 ml. of ethanol and this was added to distilled water.

Indole-HCl mixture. Prepared just prior to use. Equal volumes of the indole solution and concentrated HCl were mixed.

Chloroform. Anaesthetic grade (obtained from McCulloch Bros. & Wilson, Glasgow).

Procedure

Two ml. of the test solution were placed in a tube and 2 ml. of the indole-HCl mixture were added. The tube was

placed in a boiling water bath for 10 minutes and then removed and cooled. The reactions mixture was extracted 3 times with 4 ml. chloroform and the chloroform extracts were discarded. The tube was then centrifuged and the intensity of the coloured solution was measured at 490 mμ. Standards containing 1.58 or 3.16 μg. DNA phosphorus were included with each series of estimations. The range of the method is 1-6 μg. of DNA phosphorus.

In experiments where the number of cells available was small, the volumes of indole-HCl mixture, perchloric acid extract, and chloroform were reduced to one-fifth or one-tenth of those described above. In these cases the intensity of the coloured solution was measured in the spectro-colorimeter.

RNA estimation

The orcinol method as described by Ashwell (1957) was modified and used for RNA determination.

Reagents

Ferric Chloride solution. Prepared by dissolving 0.2 g. of $FeCl_3.6H_2O$ in 1 litre of concentrated HCl.

Orcinol reagent. Freshly prepared each day by dissolving 0.6 g. of orcinol in 100 ml. of the ferric chloride solution.

Procedure

Equal volumes (2 ml.) of orcinol reagent and test solution were placed in a tube. This was immersed in a boiling water bath for 30 minutes, removed and cooled. The intensity of the coloured solution was measured at 665 mμ.

A solution of ribose (3.33 µg./ml.) was used to prepare standards by the same treatment used for test extracts. Standards were included with each series of estimations. The range of the method is 5-20 µg. of ribose and 10 µg. of ribose was taken as being equivalent to 4.13 µg. of RNA phosphorus.

Protein estimation

Protein was estimated according to the method of Lowry, Rosenbrough, Farr & Randall (1951) employing Folin-Ciocalteu's reagent.

Reagents

Sodium carbonate reagent. - 20 g./litre.

Copper sulphate reagent. - 10 g. $CuSO_4.5H_2O$/litre.

Potassium tartrate reagent. - 20 g./litre.

Folin-Ciocalteu's reagent. This was obtained commercially (B.D.H.) and diluted 1:2 before use.

NaOH. 1 N

Procedure

The cell residue was dissolved in N NaOH (1 ml./ 10^6 cells) so as to yield a solution containing approximately 300 µg. protein/ml. On rare occasions it was necessary to heat the mixture in a boiling water bath to promote solution of the precipitated protein. In such instances the standards were treated in the same manner.

A solution consisting of 100 ml. of the sodium carbonate reagent, 1 ml. of the copper sulphate reagent and 1 ml. of the

potassium tartrate reagent was freshly prepared each day. One ml. of this was added to a tube containing 0.1 ml. of the protein solution in N NaOH. The solutions were mixed well and allowed to stand for 10 minutes at room temperature. Diluted Folin-Ciocalteu's reagent (0.1 ml.) was then added and the solutions were immediately mixed. After 30 minutes the intensity of the blue colour produced was measured at 500 mμ in the spectro-colorimeter.

It was necessary to include standards for construction of a standard curve in each series since this varied among analyses. The standards were prepared using serum albumin. All estimates, tests and standards, were performed in triplicate and results given are the means.

The range of this method is 25-100 μg. protein.

Methods for Analysis of Growth Media

Growth medium was taken from test cultures and centrifuged to remove suspended cells or debris. It was then stored at -20°C until it was convenient to perform the analyses.

Deproteinization

For glucose estimation an aliquot of medium was diluted 1:10 with perchloric acid (0.55 N) to precipitate protein. For keto acid and lactate estimations deproteinization was accomplished by mixing 2 parts of medium with 4 parts of 0.62 M $ZnSO_4$ and 4 parts of 0.5 N NaOH.

The deproteinized medium for glucose estimation was

diluted 1:5 before analyses while that for keto acids and lactate was used without further dilution.

Glucose estimation

Principle

Glucose was estimated using glucose oxidase, peroxidase and o-dianisidine (Keilin & Hartree, 1945, 1948; Hugget & Nixon, 1957). The method involves the coupling of two enzymic reactions. First, glucose oxidation to gluconic acid is catalysed by glucose oxidase.

$$\text{glucose} + H_2O + O_2 \longrightarrow \text{gluconic acid} + H_2O_2$$

The hydrogen peroxide formed in this reaction is then estimated by another reaction which is catalysed by peroxidase. In the presence of this enzyme, hydrogen peroxide will oxidize a hydrogen donor o-dianisidine (DH_2) which by this reaction is converted to a brown dye (D).

$$H_2O_2 + DH_2 \longrightarrow 2H_2O + D$$

This is measured spectrophotometrically at 436 mμ. The reagents required were supplied in a "test combination" by C.F. Boehringer, Mannheim.

Reagents

Glucose oxidase-peroxidase mixture. The mixture contained peroxidase (40 μg./ml.) and glucose oxidase (250 μg./ml.) in 0.12M phosphate buffer at pH 7.

o-dianisidine. An aqueous solution containing 6.67 mg./ml.

Reaction mixture. Prepared just prior to use by adding 1 ml. of the o-dianisidine solution to 100 ml. of the buffer-enzyme mixture.

Procedure

To 5 ml. of the reaction mixture was added 0.2 ml. of the test solution. The solutions were mixed well and incubated at room temperature for 35 minutes. The intensity of the brown colour produced was measured at 436 mμ. Standards, prepared by adding 0.2 ml. of glucose solution (91 μg./ml.) instead of the test solution, were included in each series of estimations. The range of this method is 5-60 μg. glucose.

Keto acid estimation

Principle

The procedure described by Paul (1960) was adopted without modification. The method involves the colorimetric estimation of the ketone hydrazone formed by reaction of keto acids with 2, 4 dinitrophenylhydrazine.

Reagents

Dinitrophenylhydrazine hydrochloride. Prepared by dissolving 100 mg. of 2, 4 dinitrophenylhydrazine in 100 ml. of 2N HCl.

Trisodium phosphate. - 100 g. of $Na_3PO_4.12H_2O$/litre.

Chloroform.

NaOH - 1.5N

Procedure

One ml. of dinitrophenylhydrazine reagent was added to 4 ml. of deproteinized medium, the solutions were mixed and allowed to react for 10 minutes at 25°. The reaction mixture was then extracted successively with 4 ml., 3 ml. and 3 ml. of chloroform. The chloroform extracts were combined and extracted with 5 ml. of the sodium triphosphate solution. An aliquot (4 ml.) of this extract was removed and 2 ml. of 1.5N sodium hydroxide were added to it. The intensity of the brown colour produced was measured at 445 mμ.

Standard solutions were prepared from a stock solution (18.75 g./litre) of pyruvic acid. Standards, containing 15 or 30 μg. of pyruvic acid in the original 4 ml. used were included with each series of estimations. The range of the method is from 5 to 50 μg. of pyruvic acid. The results given are means of duplicate determinations.

Lactic acid estimation

Principle

The estimation of lactic acid involved its conversion to acetaldehyde by hot, concentrated sulphuric acid and the subsequent reaction of this with p-hydroxydiphenyl to yield a violet colour (Hullin & Noble, 1953).

Reagents

p-Hydroxydiphenyl reagent. p-Hydroxydiphenyl was

obtained from L. Light Co. Ltd. and was recrystallized from ethanol before use. The reagent was prepared by dissolving 1.5 g. in 10 ml. of 1.25N NaOH and diluting to 100 ml. with water.

Copper sulphate reagent (20%). 200 g. of $CuSO_4.5H_2O$/litre.

Copper sulphate reagent (12%). 120 g. of $CuSO_4.5H_2O$/litre.

Concentrated H_2SO_4. Ordinary grade (B.P.).

$Ca(OH)_2$. Solid.

Procedure

One ml. of the 20% copper sulphate solution was placed in a graduated centrifuge tube and 0.5 ml. of deproteinized medium was added. The volume was then made up to 10 ml. with water and approximately 1 g. of solid $Ca(OH)_2$ was added. The solutions and powder were well mixed, allowed to stand for 30 minutes and centrifuged.

One ml. aliquots of the supernatant were transferred to each of two ground glass-stoppered test tubes. The 12% copper sulphate solution (0.05 ml.) was added to each and they were held in the arm of a mechanical shaker. The lower ends of the tubes were immersed in an ice bath and 6 ml. of concentrated H_2SO_4 was added dropwise while shaking vigorously. The tubes were stoppered tightly and heated for 30 minutes in a water bath at $60^\circ \pm 1^\circ$. The tubes were cooled to 10-15° and 0.1 ml. of the p-hydroxydiphenyl reagent was added. The contents were thoroughly mixed and the colour was allowed to develop for 20 minutes at 28-30°. The tubes were then placed in a boiling

water bath for 90 seconds to destroy excess p-hydroxydiphenyl. The solution was cooled and the intensity of the colour produced was measured at 560 mu. The range of the method is from 2 to 10 ug. lactic acid in the final ml. to which the sulphuric acid is added.

A solution containing 200 ug. of lactic acid per ml. was used to prepare standards which were included in each series of estimations.

Methods of Analyses of Cell Extracts for Enzymes.

Preparation of extracts

Cells were harvested by trypsinization as described previously and were washed twice by suspension in 10 ml. of Hank's BSS (without phenol red) followed by centrifugation. An aliquot was taken during the second wash for estimation of cell number. The tube containing the cell pellet was drained as thoroughly as possible and deionized water (0.1 or 0.2 ml./ 10^7 cells) was added. Extracts were prepared by rapidly freezing the cell suspension to -70° (in alcohol-CO_2) followed by rapid thawing at 37°. This procedure disrupts cultured cells sufficiently to permit reproducible recovery of the enzymes studied (Fottrell, unpublished observations). The tube containing the disrupted cells was then centrifuged at 15,000 g. for 1 hour at 4° and the supernatant was used for enzyme assays.

For estimation of alkaline phosphatase activity in HeLa cells (strain A.P.) extracts were prepared using 2% (W/V) sodium deoxycholate (0.1 ml./10^6 cells) instead of water. Cox & MacLeod (1962) reported that deoxycholate extracts of cells

contained 12-30 times as much alkaline phosphatase activity as did extracts prepared by mechanical disruption.

Units of enzyme activity

Unless otherwise specified, a unit of enzyme activity was defined as the amount causing the removal of 1 µg. of substrate or yielding 1 µg. of product per minute. Activity was expressed as units per 10^6 or 10^7 cells or as units per mg. protein. Protein was estimated by the method of Lowry et al. (1951) described earlier. In this instance, however, since the protein was already in solution, it was added directly to 1 ml. of Na_2CO_3 solution, prepared by dissolving 20 g. Na_2CO_3 in 1 litre of 0.1N NaOH. In all other respects the procedure was identical to that described previously.

Esterase

Principle

The enzyme-catalysed hydrolysis of α-naphthyl acetate was followed by coupling the naphthol released with an azo dye, Fast blue B (Nachlas & Seligman, 1949). The coloured compound formed was extracted in ethyl acetate and the intensity of the solution was measured at 540 mµ.

Reagents

Buffered substrate. Prepared by adding 5 mg. of α Naphthyl acetate, in 1 ml. of acetone, to 100 ml. of 0.06M phosphate buffer at pH 6.3. This solution was freshly prepared for each series of estimations.

Fast blue B (T. Gurr, Ltd., London). An aqueous solution (4 mg./ml.) was prepared just prior to use and was placed in an ice bath, immediately.

Trichloracetic acid. 2N

Ethyl acetate.

Procedure

Buffered substrate (0.6 ml.) was added to a specimen tube (5 ml. capacity) and equilibrated for 5 minutes at 22°. Ten µl. of enzyme extract were added with mixing and the reaction was allowed to proceed for 30 minutes at the same temperature. The solution of azo dye (0.1 ml.) was then added and after 10 minutes 0.1 ml. of 2N trichloracetic acid was added. The colour was extracted by shaking the mixture with 1 ml. of ethyl acetate. After centrifugation to separate the two solutions, the intensity of the magenta colour of the supernatant was determined in the spectro-colorimeter. The amount of naphthol released was calculated from a calibration curve. This was constructed from results obtained by coupling 2-30 µg. of naphthol in 0.6 ml. of buffer with fast blue B. The colour was extracted with 1 ml. of ethyl acetate and the intensity was measured in the spectro-colorimeter.

β-glucuronidase

Principle

The enzyme catalysed hydrolysis of phenolphthalein-β-glucuronide was followed by colorimetric estimation of the phenol-

phthalein produced (Talalay, Fishman & Huggins, 1946).

Reagents

Buffered substrate. A stock solution of the sodium salt of phenolphthalein-β-glucuronide (0.003M) (L. Light & Co. Ltd.) was diluted with an equal volume of phthalate:NaOH buffer (0.1M) at pH 4.5.

Glycine buffer. 0.2M at pH 10.4.

Procedure

Buffered substrate (0.1 ml.) was added to a specimen tube (5 ml. capacity) and equilibrated at 37° for 5 minutes. Ten ul. of cell extract were added and the reaction was allowed to proceed for 30 minutes. Glycine buffer (0.5 ml.) was added and the intensity of the coloured solution was measured at 550 mu. in the spectro-colorimeter. The amount of phenolphthalein liberated was determined from a calibration curve. This was constructed by measuring the intensity of colour of solutions consisting of 2 parts phthalate buffer, 8 parts glycine buffer and containing 2-25 ug. of phenolphthalein per ml. The spectro-colorimeter was used.

Glucose-6-phosphatase

The liberation of glucose from glucose-6-phosphate was determined using the glucose oxidase method for estimating glucose which has been described previously.

Reagents

Buffered substrate. Prepared by dissolving 0.365 g. of the disodium salt of glucose-6-phosphate in 10 ml. of 0.1M citrate-sodium hydroxide buffer at pH 6.5.

$MgCl_2$ solution. 0.1M.

Procedure

A reaction mixture consisting of 2 volumes of buffered substrate and one part of magnesium chloride solution was prepared. Twenty ul. of this were added to a specimen tube (5 ml. capacity) and allowed to equilibrate at 37° for a few minutes. Ten ul. of cell extract were added and the reaction was allowed to proceed for 90 minutes. Five ml. of the glucose oxidase-peroxidase reaction mixture were then added and allowed to develop at room temperature for 35 minutes.

Extracts from HLM cells were found to contain considerable amounts of glucose. It was necessary, therefore, to estimate the glucose content of each cell extract after incubation without glucose-6-phosphate as well as the glucose present after incubation with glucose-6-phosphate. Glucose produced was calculated from the difference between the two estimations.

Glucose-6-phosphate dehydrogenase

Principle

Enzyme activity was determined by following the reduction of nicotinamide-adenine dinucleotide phosphate (NADP) in the

presence of glucose-6-phosphate (Glock & McLean, 1953). Estimations were performed immediately after preparation of the cell extracts.

Reagents

Substrate. An aqueous solution of the disodium salt of glucose-6-phosphate (0.02M) was prepared and stored at -20°.

NADP (0.001M). Stored at -20°.

Buffer. Tris : maleate : NaOH buffer was prepared by dissolving 3.48 g. of maleic acid and 3.63 g. of tris in 50 ml. of deionized water. The pH was adjusted to 7.5 with 0.3M NaOH and the volume was made to 100 ml.

$MgCl_2$ solution. 0.3M.

Procedure

A reaction mixture consisting of 21 ml. buffer, 1 ml. NADP solution, 1 ml. $MgCl_2$ solution and 1 ml. of substrate solution was placed in a water bath at 37°. A "blank" solution consisting of the same solutions but with water added instead of substrate was also prepared and brought to 37°.

The reaction mixture (2.4 ml.) or "blank" mixture (2.4 ml.) was added to 1 cm. quartz cuvettes and placed in a Beckman DB spectrophotometer with water jacket warmed to 37°. Cell extract (2 µl.) was added to the reaction mixture, the contents were mixed rapidly and the cuvette was replaced in the spectrophotometer. The change in the extinction value at 340 mµ. was recorded automatically. A unit of enzyme activity

was defined as an increase in optical density of 0.001/minute.

Lactic dehydrogenase

Principle

The activity of this enzyme was evaluated by measuring the disappearance of pyruvate in the presence of cell extract and reduced nicotinamide-adenine dinucleotide ($NADH_2$) (Cabaud & Wroblewski, 1958).

Reagents

Buffered substrate. Pyruvic acid (0.2 g.) and KH_2PO_4 (10 g.) were dissolved in 700 ml. deionized water. The pH was adjusted to 7.8 with N NaOH and the volume was made up to 1 litre. The solution was stable for at least 1 week if stored at 4°.

2, 4-Dinitrophenylhydrazine. A solution (1 g./litre) was prepared in 2N HCl.

$NADH_2$. An aqueous solution (10 mg./ml.) was freshly prepared for each series of determinations.

NAOH. 0.4 N.

Procedure.

Substrate (0.1 ml.) and $NADH_2$ (10 µl.) solutions were added to a specimen tube (5 ml. capacity) and placed in a water bath at 37°. Ten µl. of cell extract were then added and the reaction was allowed to proceed for 30 minutes. 2, 4 - Dinitrophenylhydrazine (0.1 ml.) was added and the solutions were left at room temperature for 20 minutes. One ml. of NAOH

(0.4 N) was then added and after a further 5-10 minutes the intensity of the coloured solution was measured at 550 mμ. in the spectro-colorimeter.

Reagent blanks provided estimates of the pyruvate concentration at the start of the incubation period. Substrate blanks, consisting of water instead of buffered substrate were also included. Disappearance of pyruvate was calculated by reference to a standard curve.

Hexokinase

Hexokinase activity was estimated by measuring the disappearance of glucose by the glucose oxidase-peroxidase method outlined previously. The reaction mixture was prepared according to Eagle, Barban, Levy & Schulze, (1958).

Reagents

Adenosine triphosphate (ATP) solution (0.05M). Prepared in 0.1M tris buffer and pH was adjusted to 7.7.

$MgCl_2$ solution. 0.1M.

NaF solution. 0.5M.

Tris buffer (0.1M). Prepared with pH adjusted to 7.7.

Substrate. An aqueous solution (0.02M) of glucose.

Procedure

A reaction mixture consisting of 2 parts ATP solution, 1 part $MgCl_2$ solution, 1 part NaF solution, 5 parts tris buffer and 5 parts substrate solution was prepared.

Reaction mixture (10 or 20 μl.) was added to specimen

tubes of 5 ml. capacity and equilibrated at 37° for 5 minutes. Cell extract (10 or 20 µl. respectively) was added and the reaction was allowed to proceed for 10 or 30 minutes. Five ml. of the glucose oxidase-peroxidase reaction mixture were then added and allowed to develop at room temperature for 35 minutes. Glucose was estimated as previously described.

Cell extracts from HLM cells were found to contain 2-8 µg. of glucose per 10^6 cells. The glucose level was not affected by addition of insulin or prednisolone to the growth medium. It was necessary, therefore, to estimate the glucose content of the combined cell extract and reaction mixture before incubation. This was done by adding 10 or 20 µl. of cell extract and 10 or 20 µl. of reaction mixture directly to the glucose oxidase-peroxidase reaction mixture. Disappearance of glucose was calculated by subtraction.

Acid and Alkaline Phosphatase

Principle

The activity of these enzymes was determined by estimating the amount of p-nitrophenol which was liberated from p-nitrophenyl phosphate (Fujita, 1939).

Reagents

Substrate. p-Nitrophenylphosphate (0.1 g.) was dissolved in 25 ml. of water. The solution was freshly prepared for each series of estimations.

Buffer solutions. Citrate buffer (0.1M) at pH 4.9

was used for alkaline phosphatase estimations.

Buffered substrate. Equal parts of substrate solution and the appropriate buffer solution were mixed.

NaOH. 0.02 N.

Procedure

Buffered substrate (1.6 ml.) was added to a test tube and allowed to equilibrate to 37° in a water bath. Cell extract (2 µl. if HLM cells; 0.1 ml. if HeLa cells) was added and the reaction was allowed to proceed for 30 minutes. The reaction was terminated by adding 16 ml. of 0.02N NaOH and the intensity of colour was measured at 410 mµ.

In the case of the deoxycholate extracts prepared from HeLa cells, strain A.P., 0.1 ml. of extract was added to 1.0 ml. of buffered substrate and after incubation 10 ml. of 0.02N NaOH was added. Extracts of cells grown in the presence of prednisolone were diluted 1:5 before use to estimate alkaline phosphatase activity.

The acid phosphatase estimations were performed by adding 2 µl. of aqueous cell extract to 0.1 ml. of buffered substrate at 37°. After 30 minutes 1.0 ml. of 0.02N NaOH was added and the intensity of colour produced was determined in the spectro-colorimeter. The amount of p-nitrophenol liberated was calculated from a reference standard prepared by adding 1 part of a stock standard to 10 parts of 0.02N NaOH. The stock standard was an aqueous solution containing 42 mg. of p-nitrophenol per litre.

DETERMINATION OF GLUCOSE TRANSPORT

In every glucose transport experiment HLM cells, which had been adapted to growth in Waymouth's medium prepared with tris-citrate BSS, were used. Cells from stock cultures were harvested by trypsinization and were suspended in Waymouth's medium prepared with tris-citrate BSS. The cell suspension, containing 10^5 to 2×10^5 cells per ml., was dispensed into 25 ml. conical flasks (5 ml./flask) using a Cornwall syringe. During this time the suspension was agitated continually with a magnetic stirrer. The mouths of the flasks were covered with a square of aluminium foil and the cultures were incubated at 37°.

In the majority of experiments the cultures used had been incubated for 3 days (3 day old cultures). Cultures were removed from the incubator and the overlying medium was discarded. Five ml. of tris-citrate BSS containing glucose (5.6 mMolar) was added, the flask was gently agitated and the wash was discarded. Five ml. of equilibration medium which had been warmed to 37°, was then added. Unless otherwise stated, the equilibration medium consisted of tris-citrate BSS containing glucose (5.6 mMolar) and had been adjusted to pH 7.4. The test medium, unless otherwise mentioned, was of the same composition as the equilibration medium but contained, in addition, labelled glucose. This was obtained from the Radiochemical Center, Amersham, Bucks. ^{14}C glucose was uniformly labelled while ^{3}H glucose was nominally

labelled on C_6. After 30-45 minutes equilibration at 37°, a culture was removed from the water bath and the equilibration medium was discarded. The vessel was inverted and shaken rapidly to remove as much of the residual fluid as possible. It was placed immediately in a shaking water bath and 2 ml. of test medium at 37° was added for precisely 1 minute. The test medium was removed with a pasteur pipette and the culture was immediately washed 4 times in succession by the addition of approximately 5 or 10 ml. of tris citrate BSS at 4° containing glucose (5.6 mM). It had been found that this procedure removed a high percentage of the residual labelled glucose, which was presumed to be extracellular (Table 1). After removing the final wash, 2 ml. of 0.2N perchloric acid at 4° were added. The culture was then placed in crushed ice for 30-45 minutes. The cold acid extract was removed with a pasteur pipette and added to a tube containing 0.5 ml. of N KOH to precipitate perchlorate. The extract was then kept aside for determination of radioactivity. Two ml. of 2N perchloric acid were then added to the culture flask and the vessel was placed in a shaking water bath at 70° for 20 minutes to extract DNA. Estimation of DNA was performed by the method described earlier.

Determination of radioactivity in the neutralized extract was accomplished, in experiments using ^{14}C glucose, through use of a Nuclear Chicago gas flow counter with a thin end-window and an automatic changing device. The extract (0.2 ml.)

TABLE 1

REMOVAL OF EXTRACELLULAR RADIOACTIVITY FROM HLM CELL CULTURES AFTER EXPOSURE TO LABELLED GLUCOSE

Experiment A: Three day old cultures were equilibrated and exposed to test medium containing ^{3}H glucose at a specific activity of 3.5 mCuries/mMole. After 1 minute exposure, the cultures were washed as indicated and extracted (see text).

Experiment B: The procedure was identical to that described for experiment A but the cultures were set up at a different time.

The figures given in the table indicate the radioactivity remaining in the cultures after washing as shown.

No. of washes	Counts/min./µg.DNAP	
	Wash Volume	
	5 ml.(Exp. A)	10 ml. (Exp. B)
1	2440	610
2	935	95
3	636	69, 94
4	570	51, 48
5	554	122, 97
6	400	92, 48
7		73
8		56

was plated out on a stainless steel planchet and was dried under infra red lamps. A 1:2 dilution (0.2 ml.) of the extract was also plated out and self absorption was minimal. At least 1000 counts were recorded for each sample.

When ^{3}H glucose was used, 1 ml. of the neutralized extract was added to 8 ml. of "Scintant" scintillator made up in dioxane. This was obtained from Nuclear Enterprises Ltd., Edinburgh and was designated NE572, size D. With experiments cited in tables 19, 22, 34, 42 and 43, 0.5 ml. of neutralized extract was added to 4.0 ml. of "Scinstant" scintillator and counted.

Counts were performed using the Packard Tricarb or Nuclear Chicago liquid scintillation spectrometers. The efficiency was determined by noting the ratio of counts obtained on 2 separate channels. Efficiency was approximately constant at 8% and no correction was ever necessary.

ORGAN CULTURE OF LIVER EXPLANTS AND LIVER CELL AGGREGATES

The ability to maintain liver tissue in vitro would obviously be a considerable advantage in a study involving insulin or prednisolone action in vitro. Trowell (1961) was able to maintain adult liver explants in culture for periods up to 6 days. The results were not consistent, however, and not all cultures within a given experiment survived. Attempts were made, therefore, to devise means of improving the culture method to promote consistent survival of liver tissue. These attempts met with limited success only, and owing to the inconsistency encountered, the method could not be used for critical experiments. Since some modifications of the organ culture technique showed promise, the methods and impressions gained are described below.

Culture Apparatus

The culture method was modified from that reported by Trowell (1959). Perspex boxes of approximate dimensions 7 x 4 x 2½ inches were used as culture chambers. In these were placed petri dishes, 5 cm. in diameter, containing the liver explants and open petri dishes containing water to humidify the atmosphere. The perspex box was sealed with cellotape and was gassed with a mixture of 95% O_2 and 5% CO_2 by inserting the delivering tube through a small hole in the lid. The hole was also sealed with cellotape after 10-15 minutes gassing.

Within each petri dish culture vessel was a grid made

by bending the ends of a strip (25 x 33 mm.) of stainless steel expanded metal. These bent ends formed legs about 4 mm. in length supporting the 25 mm. square grid. The petri dish was filled with medium so that it just came to the top of the grid. A square of lens paper was then placed on top of the grid so that it just became wet. The liver explants or cell aggregates were placed on the lens paper, the petri dish was covered and placed in the perspex culture chamber. This was then treated as described above and placed in the incubator.

Culture Technique

Animals were killed by exsanguination after stunning or anaesthetizing with ether. The liver was immediately removed and explants were prepared as rapidly as possible. Several methods of explant preparation were tried.

Conventional explants were prepared by cutting small portions of liver, especially from the edge of the papilliform lobe as advised by Trowell (1961). Explants were also prepared from liver which had been minced by pushing it through a fine wire mesh. The disaggregated liver tissue was placed directly on the culture grid so as to form an explant of about 1-2 mm. in diameter and 0.5 mm. thick. Alternatively the minced liver was washed by suspension in Hank's BSS and, after centrifugation, was placed on the culture grid as before. One other method of preparation of cell aggregates was tried. The minced liver was added to Hank's BSS containing agar (20 g./litre) at 43°.

Special agar "Noble" (Difco) was obtained through W. B. Nicolson, Ltd., Glasgow. The mixture was poured into a petri dish and allowed to solidify. "Explants" were then cut out of this with a cork borer and were placed on the culture grid.

Histological and Histochemical Technique

Haemalum - Eosin stain

Reagents

Bouin's fixative. A solution consisting of 25 parts formalin, 75 parts saturated picric acid solution and 5 parts glacial acetic acid.

Mayer's Haemalum. Prepared by dissolving 2 g. haematoxylin (T. Gurr, Ltd.), 100 g. potassium alum and 0.4 g. of sodium iodate in 2 litres of deionized water. This was allowed to stand overnight and 100 g. of chloral hydrate and 2 g. of citric acid were added. The solution was boiled and left overnight before filtering.

Ethyl eosin. Prepared by dissolving 0.5 g. of eosin in 100 ml. of 95% ethyl alcohol.

Mayer's albumin. Prepared by mixing 50 ml. of egg white with 50 ml. glycerol.

Ethyl alcohol. Absolute, 90%, 70%, 50%, 20% (v/v).

Xylene - Paraffin mixtures. 75%, 50%, 25% (v/v).

Xylene.

Paraffin (T. Gurr, Ltd.).

Depex (T. Gurr, Ltd.).

Procedure

Liver explants were removed from the culture vessels and placed directly into Bouin's fixative. After being left for 24 hours in this fixative, the explants were removed and placed in 70% alcohol. The alcohol was changed every 2-3 hours and the explants were left in fresh, absolute alcohol overnight. They were then placed in pure xylene for several hours and finally were taken through the xylene-paraffin mixtures to paraffin. Paraffin blocks were prepared for sectioning and sections (10-15 μ thick) were mounted on slides which had been dipped in Mayer's albumin solution and dried.

The slides were placed in xylene to remove paraffin and were then taken through the series of alcohol-water mixtures to water. The sections were then stained with Haemalum by immersion for 10 minutes. The slides were washed thoroughly in water and taken through the water-alcohol series to absolute alcohol. They were stained in ethyl eosin by immersion for 3 minutes. They were washed in absolute alcohol, given two rinses in xylene and mounted in Depex.

Periodic acid - Schiff reaction

Principle

The method involves the selective oxidation of 1, 2 - glycol groups with periodic acid followed by detection of the aldehyde groups formed by reaction with Schiff's reagent.

Reagents

Schiff's reagent. Purchased from T. Gurr, Ltd.

Periodic acid. Prepared by dissolving 0.5 g. of sodium periodate in 0.01 N HCl.

Sulphite rinse. Freshly prepared solution consisting of equal parts of 1% (W/V) potassium metabisulphite and 0.1 N HCl.

Haemalum. Preparation described earlier.

Procedure

This test for glycogen was performed on sections treated as described above to the stage for staining with Haemalum. The original fixative used was the same but fixation was carried out at 4° to minimize loss of glycogen. The method was used according to Casselman (1959).

The slides were placed in periodic acid for 10 minutes at room temperature. They were then washed for 5 minutes in running water and were rinsed in distilled water. After being immersed in Schiff's reagent for 10 minutes, the slides were washed in sulphite rinse. They were then immersed for 2 minutes in each of three successive changes of the above rinse and were washed in tap water for 5 minutes.

The sections were counterstained by immersion in Haemalum and were dehydrated and mounted as described earlier.

This reaction was also used to demonstrate the existence of polysaccharide in HLM cells. Cells, grown on coverslips in petri dishes, were washed in Hank's BSS, fixed in Bouin's

solution and treated in essentially the same manner as described above.

Impressions and Results

The survival of liver explants and aggregates under numerous different conditions was determined by histological examination. The presence of glycogen was studied by means of the periodic acid-Schiff reaction. Some progress was made in maintaining liver cell aggregates in culture, but inconsistency of survival was still apparent.

Preliminary studies indicated that explants prepared from the edge of the papilliform or larger lobes of mouse liver contained areas of necrosis as well as areas of apparently healthy cells. The conditions which were altered in an attempt to improve on this survival pattern and the impressions obtained are outlined below.

Media used

The media tried were 199, Eagle's medium, Waymouth's medium and Trowell's medium, both as described by Trowell in 1959 and with decreased bicarbonate concentration (to 20 mM) as he suggested in 1961. Of these media, Trowell's (T8) and Waymouth's promoted good survival for 2 day culture periods in several experiments; hence they were used for all subsequent culture trials.

Serum

Calf or human sera, used at concentrations from 5-20% (v/v)

were apparently deleterious in 4 experiments where serum was added.

Sodium lactate

Addition of lactate (3.9 mM) to the growth medium had no noticeable effect on survival.

Gaseous Phase

Air with 5% CO_2 permitted as consistent survival as did 95% O_2/5% CO_2, 100% O_2, 50% air/50% Nitrogen, or air alone. Media used to maintain cultures in the absence of CO_2 were buffered by tris-citrate BSS instead of Hank's bicarbonate buffer. The cultures set up in tris-citrate buffered medium survived as well as did those maintained by the bicarbonate buffer.

Method of preparation

Aggregates of liver cells embedded in Hank's BSS-agar medium or used directly after preparation of the liver-mince, survived as well and often better than did the conventional "intact"explants. However, the liver-mince cell aggregates survived only when used directly without washing in Hank's BSS.

Temperature

Cultures maintained at 30° or 35° on a few occasions showed less necrosis than did companion cultures at 37°.

Source of liver

Liver explants prepared from the mouse, rat and guinea pig were maintained equally well. An attempt at the maintainance of rabbit liver was completely unsuccessful.

Culture situation

Partially submerged cultures and totally submerged cultures which were continually oxygenated did not generally survive as well as did those maintained on wire platforms as described by Trowell.

Platform support

The use of filter paper or fibre glass pads instead of lens paper on the stainless steel grids permitted somewhat better survival on several occasions.

Survival time

Partial survival of cell aggregates and explants was achieved for 2 to 3 days in 10 out of 30 experiments and rarely for 6 day periods (3 experiments).

Glycogen storage

Histochemical and chemical analyses of 2 day cultures indicated that no glycogen was present (6 experiments). No effect of insulin (0.1 unit/ml.) or hydrocortisone (1.0 ugms./ml.) on this was ever demonstrated.

Selection of fat mice of mice fed on high carbohydrate diets for liver tissue did not yield any different results.

Nucleic acid content

Analyses of aggregates after 2, 4 and 6 days in culture indicated progressive decreases in the amount of DNA phosphorus as is shown overleaf.

Days Incubation	Insulin concn (Units/ml.)	Explant wt.(mg.)	DNA phosphorus ug. per 10 mg.
2	0	12.9	7.3
	1	12.5	5.7
4	0	10.7	4.7
	1	12.9	4.4
6	0	10.4	1.4
		8.2	1.8
	1	6.5	1.3
		9.7	0.2

The DNA present in these explants was determined as previously described after extraction with 2 ml. of N perchloric acid. The cultures were maintained in Waymouth's medium without serum and buffered with tris-citrate BSS. The incubation temperature was 37° and the cultures were gassed with oxygen.

EXPERIMENTAL PROCEDURES AND RESULTS

INFLUENCE OF INSULIN AND PREDNISOLONE ON GROWTH AND COMPOSITION OF CELLS IN CULTURE

Insulin and corticosteroids are known to induce changes in the composition of tissues in vivo and in vitro. Attempts were made, therefore, to determine the effects of insulin and prednisolone on the composition of cells in culture. The results are presented in Table 2.

Experiments 2, 13, and 28, were performed using Roux flasks which had been inoculated with 50 ml. of Waymouth's medium and 5×10^6 HLM cells. In experiment 2, 0.1 unit of insulin was added per ml. of medium where indicated. In all other experiments the concentration of insulin was 1 unit/ml. Prednisolone was added to the culture medium where indicated at a concentration of 2.5 µg./ml. The medium was renewed every 3-4 days and insulin or prednisolone was added to appropriate cultures. Cells were harvested by trypsinization after 8, 9 and 6 days of incubation for experiments 2, 13, and 28 respectively.

Experiments 18, 20, 22, 24 and 26 (Table 2) were performed to determine the effects of insulin and prednisolone on cells maintained in the absence of serum. In this group of experiments HLM cells were allowed to become established in culture vessels containing Waymouth's medium with serum added as usual. After 24 hours incubation this medium was replaced with a medium which consisted of Waymouth's medium supplemented with Bacto-peptone (0.5% W/V) instead of serum. This procedure was adopted in an attempt to eliminate possible variation owing to difference in the serum used. Insulin (1 unit/ml.)

TABLE 2

INFLUENCE OF INSULIN AND PREDNISOLONE ON HLM CELL GROWTH AND COMPOSITION

(For Explanation See Text)

Exp No.		Control	Mean	Insulin	Mean	Prednisolone	Mean
2	Total µg.DNAP	43; 56; 55	51	63; 47; 56	55	33; 30; 24	29
	µg.CBH/µg.DNAP	57; 47; 46	50	143; 123; 129	132	39; 46; 48	44
13	Total cell no. (10^6)	34; 35; 32	33	28; 30; 32	30	14; 12	13
	Total µg.DNAP	53; 56; 49	52	43; 44; 44	44	34; 32	33
	µg.CBH/µg.DNAP	65; 69; 75	70	109; 120; 119	116	123; 124	124
	µg.DNAP/10^6 cells	1.6; 1.6; 1.5	1.6	1.5; 1.5; 1.4	1.5	2.4; 2.7	2.6
18	Total µg.DNAP	2.0; 2.3	2.2	2.2; 2.1	2.2	1.0; 0.9	1.0
	µg.CBH/µg.DNAP	51; 54	52	85; 61	73	37; 30	34
20	µg.CBH/µg.DNAP	30; 19; 20	23	43; 47	45	11; 13	12
	µg.DNAP/10^6 cells	1.1; 1.5; 1.6	1.4	1.6; 1.7	1.7	2.0; 1.9	2.0

Table 2 (Continued)

Exp No.		Control	Mean	Insulin	Mean	Prednisolone	Mean
	µg.Lipid/µg.DNAP	69; 49; 53	57	59; 71	65	116; 96	106
22	µg.Lipid/10^6 cells	131; 105; 123	120	120; 100	110		
	µg.Protein/10^6 cells	265; 209; 213	229	252; 175	214		
24	Total cell no. (10^6)	6.2; 7.0; 6.3	6.5	5.7; 7.4; 5.8	6.3	3.2; 3.7; 4.0	3.6
	Total µg.DNAP	11.7; 14.2; 12.9	12.9	11.8; 13.5; 10.7	12.0	10.8; 10.4; 10.5	10.6
	µg.CBH/µg.DNAP	18; 14; 15	16	27; 32	30	5; 5; 3	4
	µg.Lipid/µg.DNAP	106; 60; 61	76	69; 65; 73	69	77; 72; 75	75
	µg.Protein/µg.DNAP	155; 137; 125	140	183; 184	184	152; 142; 144	146
	µg.DNAP/10^6 cells	1.9; 2.0; 2.0	2.0	2.1; 1.9; 1.8	1.9	3.4; 2.7; 2.6	2.9
26	µg.CBH/µg.DNAP	21; 18; 19	19	28; 28; 28	28		
	µg.Protein/µg.DNAP	188; 164	176	192; 161; 162	172		
28	Total µg.DNAP	5.0; 4.6	4.8			3.7; 3.3	3.5
	µg.RNAP/µg.DNAP	3.1; 3.1	3.1			3.6; 4.0	3.8
	µg.Protein/µg.DNAP	185; 200	192			190; 210	200

or prednisolone (2.5 µg./ml) was then added as indicated. Experiments 20, 22, and 24, were performed using baby feeding bottles which had been inoculated with 10 ml. of Waymouth's medium and 3×10^6, 3.2×10^6, or 2.9×10^6 HLM cells respectively. After 24 hours the medium was replaced with 30 ml. of the growth medium containing no serum. Insulin (1 unit/ml.) or prednisolone (2.5 µg./ml.) was added as indicated. The medium was renewed every 2-3 days and insulin or prednisolone was added to appropriate cultures. Cells were harvested after total incubation times (initial 24 hr. set up period and test period) of 7, 5, and 4, days for experiments 20, 22, and 24, respectively.

Experiment 18 was performed using roller tubes which had been inoculated with 2 ml. of Waymouth's medium containing 2×10^5 HLM cells. After a 24 hour period of incubation the medium was replaced with 2 ml. of the growth medium containing no serum but containing insulin (1 unit/ml.) or prednisolone (2.5 µg/ml.) as indicated. This medium was renewed daily and cells were harvested by trypsinization on the fourth day.

Experiment 26 was performed using 4 oz. perscription bottles which had been inoculated with 10 ml. of Waymouth's medium containing 10^6 HLM cells. After 24 hours of incubation the medium was replaced with 10 ml. of Waymouth's medium containing no serum. Insulin (1 unit/ml.) was added where indicated. After 24 hours incubation the medium was replaced with 10 ml. of Waymouth's medium prepared using tris-citrate BSS. This BSS

was used in an attempt to minimize differences in pH which were found to develop between control and insulin-containing cultures in less heavily buffered media. This medium contained no serum but 0.5% (W/V) Bacto-peptone had been added and insulin (1 unit/ml.) was added where indicated. The cells were harvested by trypsinization after an additional 3 days of incubation.

Effects of Insulin on Growth and Composition of HLM Cells

Composition

Addition of insulin to the growth medium consistently led to an increase in carbohydrate storage by HLM cells whether or not serum was present (Table 2). The effect of time of exposure to insulin on the composition of HLM cells is shown in Table 3. The increase in cellular carbohydrate was evident after 3-4 days of growth in the presence of insulin. As was found in previous experiments (Table 2), no effect on DNA phosphorus or protein content was observed.

The intracellular carbohydrate levels in both control and insulin-stimulated cultures varied considerably among experiments. Efforts were therefore directed towards further clarification of the factors which govern carbohydrate storage.

Table 4 shows results of an experiment indicating that different methods of harvesting cells do not influence their carbohydrate content.

Table 5 presents results indicating that the glucose concentration of the incubation medium does not affect the carbohydrate content at least over the range 3-44 mMolar and for

TABLE 3

INFLUENCE OF INSULIN ON THE
COMPOSITION OF HLM CELLS

Roux flasks were inoculated with 5×10^6 HLM cells and 50 ml. of Waymouth's medium with or without insulin. The medium was renewed daily and each day cells from sample cultures were harvested for analysis.

	Insulin concn. (units/ml.)	Days Incubation			
		1	2	3	4
µg.CBH/ µg.DNAP	0	33; 34	41; 68	61; 82	84; 92
	1	39; 37	69; 60	104; 89	113; 112
µg.Protein/ µg.DNAP	0	250; 220	250; 300	270; 230	300; 300
	1	250; 220	270; 200	240; 300	310; 290
µg.DNAP/ 10^6 cells	0	1.2; 1.3	1.4; 1.4	1.4; 1.3	1.7; 1.6
	1	1.3; 1.4	1.4; 1.4	1.5; 1.5	1.3; 1.2

TABLE 4

CARBOHYDRATE CONTENT OF HLM CELLS
AFTER VARIOUS HARVESTING PROCEDURES

Baby feeding bottles were inoculated with 10^6 HLM cells in 10 ml. Waymouth's medium. After six days incubation the cell monolayer was removed by scraping (S), treatment with trypsin (T) or treatment with versene and scraping (V). The cells were then washed in cold Hank's BSS, counted and analysed.

Sample	µg.CBH/10^6 cells	µg.CBH/µg.DNAP
S1	14.5	11.9
2	16.2	14.4
T1	16.5	16.3
2	15.6	16.0
V1	14.9	13.3
2	15.5	16.5

TABLE 5

EFFECTS OF GLUCOSE CONCENTRATION ON CARBOHYDRATE STORAGE BY HLM CELLS

Baby bottles were inoculated with 5.8×10^6 HLM cells. Waymouth's medium (30 ml.) at pH 7.4, containing the glucose concentrations indicated was added to the culture vessels. The osmotic pressure of the media had been adjusted to that of Waymouth's medium normally used. The culture media were renewed daily and on the third day the cells were harvested for analysis.

The inoculum contained 1.2 µg.DNAP/10^6 cells and 27 µg.CBH/µg.DNAP.

Glucose concn. in medium (mMolar)	Cell No. (10^6)	$\frac{\mu g.DNAP}{10^6 \text{ cells}}$	$\frac{\mu g.CBH}{\mu g.DNAP}$
3	8.2; 8.7; 10.6	1.7; 1.4; 1.3	57; 54; 59
11	8.6	1.5	64
44	11.2; 11.3	1.3; 1.3	65; 56

three day culture periods. In contrast, Wu (1959) reported that the carbohydrate content of HeLa cells could be doubled by increasing the glucose concentration in the incubation medium. In the experiments mentioned, cultures had been incubated for seven days before analysis.

The carbohydrate content of HLM cells grown in the presence of sodium lactate was slightly higher than that of cells grown in its absence (Table 6).

Raising the pH of the growth medium from 7.4 to 7.9 caused a marked reduction in the carbohydrate content whereas no difference was noted between cultures maintained at pH 7.4 or at 7.0 (Table 7).

Analyses of cell samples taken at stages during the growth cycle indicated that the carbohydrate content increased at least until the sixth or seventh day (Figure 1). Bruni, Gey & Svotelis (1961) reported that glycogen present in HeLa cells 24 hours after renewal of the medium disappears by 48 hours. It is possible therefore that depletion of glucose present in the medium may lead to utilization of carbohydrate stores. In the experiments cited in figure 1, therefore, excess medium was used and this was renewed frequently.

The relatively small effects of pH, lactate concentration and cell growth stage on the carbohydrate content of HLM cells probably would not explain the large variation among experiments. It is possible that the variation occurred owing to differences in the carbohydrate content of the inocula or to differences in the sera used.

TABLE 6

EFFECT OF LACTATE ON CARBOHYDRATE STORAGE BY HLM CELLS

Baby bottles were inoculated with 5×10^6 HLM cells. Waymouth's medium (30 ml.) at pH 7.4 containing sodium lactate as indicated was added. The osmotic pressure of Waymouth's medium containing lactate had been adjusted to that of Waymouth's medium normally used. The culture media were renewed daily and on the fifth day the cells were harvested for analysis.

	Sodium Lactate Concn. (mMolar)			
	0	1.1	5.5	11.1
µg.CBH/µg.DNAP	107; 105	127; 99	119; 136; 123	137; 137; 141
Total µg.DNAP	24; 29	25; 24	32; 31; 30	26; 26; 25

TABLE 7

EFFECT OF pH ON CARBOHYDRATE STORAGE BY HLM CELLS

Baby bottles were inoculated with 5×10^6 HLM cells. Waymouth's medium (30 ml.) at pH 7.0, 7.4 or 7.9 was added. The medium was renewed daily and on the fifth day cells were harvested for analysis.

	pH		
	7.0	7.4	7.9
µg.CBH/µg.DNAP	112; 100; 119	107; 105	64; 54; 77
Total µg.DNAP	17; 18; 19	24; 29	12; 12; 9

FIGURE 1

Experiment 7

Baby feeding bottles were inoculated with 5×10^6 HLM cells in 30 ml. Waymouth's medium. The medium was renewed every second day. Bottles were removed at intervals, the cells were trypsinized, washed in cold Hank's BSS, counted and analysed. The cell number increased from 5×10^6 to 15×10^6 per bottle. Points, shown as open circles, represent the means determined from 2 replicate cultures. The vertical lines give the total range of results.

Experiment 8

This was essentially similar to experiment 7 but 10^5 cells in 2 ml. medium were inoculated into culture tubes. Cells were harvested by treatment with 2 ml. trypsin solution. They were suspended in this and counted. Points shown as filled-in circles, represent the means determined from 2-3 replicate cultures. The vertical lines give the total range of results.

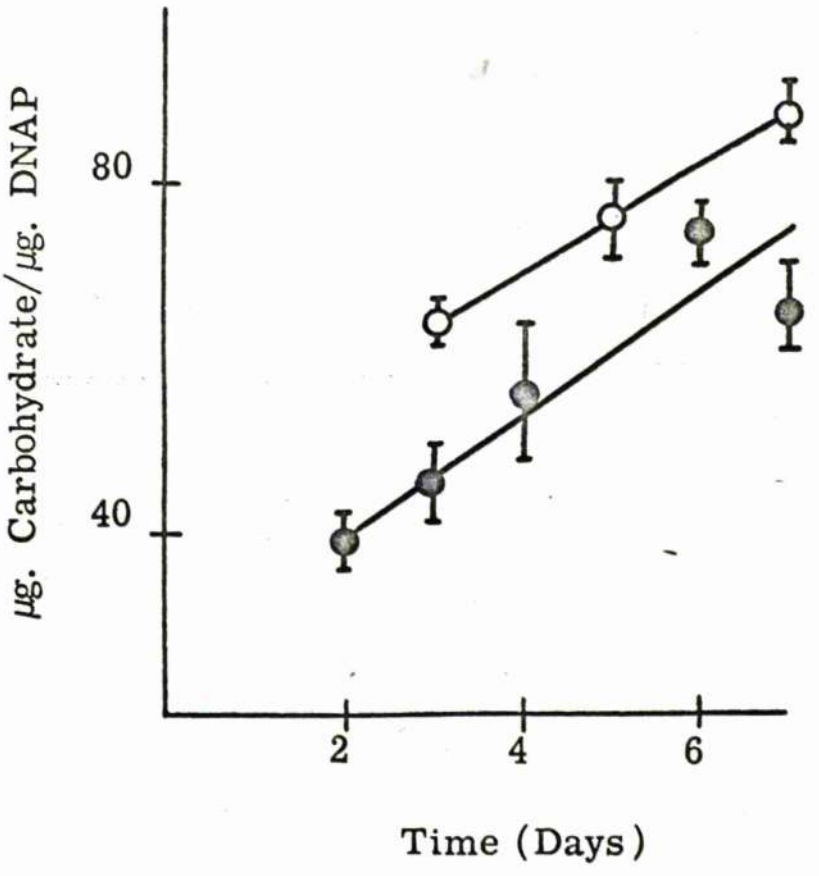

Fig. 1. Carbohydrate content of HLM cells at stages during growth

Growth

It is apparent from the results of Table 2 that no consistent effect of insulin on cell growth was found. Crockett & Leslie (1963), however, presented indirect evidence indicating that insulin has a stimulatory effect on the growth of HLM cells. These workers set up replicate cultures with cells which had been grown in the presence of insulin. They found that the omission of insulin from medium added to such cultures led to a decrease in growth. A slightly modified form of Waymouth's medium was used and this was supplemented with 4% human serum.

In the experiments cited in Table 2, in spite of frequent renewal of medium, the pH of the insulin-treated cultures was often somewhat lower than that of controls. The rate of multiplication of cells in culture is markedly influenced by pH (Paul, 1959). Therefore a stimulatory effect of insulin on the growth of HLM cells may have been masked by the decrease in pH. It is also possible that the difference in the results of this study and those of Crockett and Leslie is due to differences in experimental design or in the medium used.

Effects of Prednisolone on Growth and Composition of HLM cells

Composition

Prednisolone usually caused a decrease in the carbohydrate content of HLM cells and an apparent increase in DNA phosphorus per cell (Table 2). In this respect it was noted that cells grown in the presence of prednisolone were larger and contained larger nuclei than did cells of control cultures. The lipid, RNA phosphorus and protein contents of HLM cells were not consistently affected by addition of this steroid.

Growth

Prednisolone was obviously growth inhibitory as the total cell number and total DNA phosphorus per culture were decreased. The effect of this compound on cell multiplication during a growth cycle is shown in figure 2. It appears that the lag phase of the prednisolone containing cultures was increased and the rate of growth was slightly decreased.

Effects of Insulin and Prednisolone on Growth and Composition of KaLe Cells

Results of an experiment similar to those of Table 2 but with the KaLe cell strain are shown in Table 8. It can be seen that the response to insulin and prednisolone was essentially similar to that of HLM cells. A slight stimulatory-effect of insulin on RNA phosphorus and protein content of these cells was apparent.

FIGURE 2

Roller tubes were inoculated with 2 ml. Waymouth's medium containing 10^5 HLM cells. One half of the cultures were set up with medium containing 2.5 µg. prednisolone/ml. Medium on all cultures was renewed daily.

Each day cell counts were performed, after trypsinization, on two cultures containing prednisolone (filled-in circles) and two control cultures (open circles). The points shown are means of the duplicate counts.

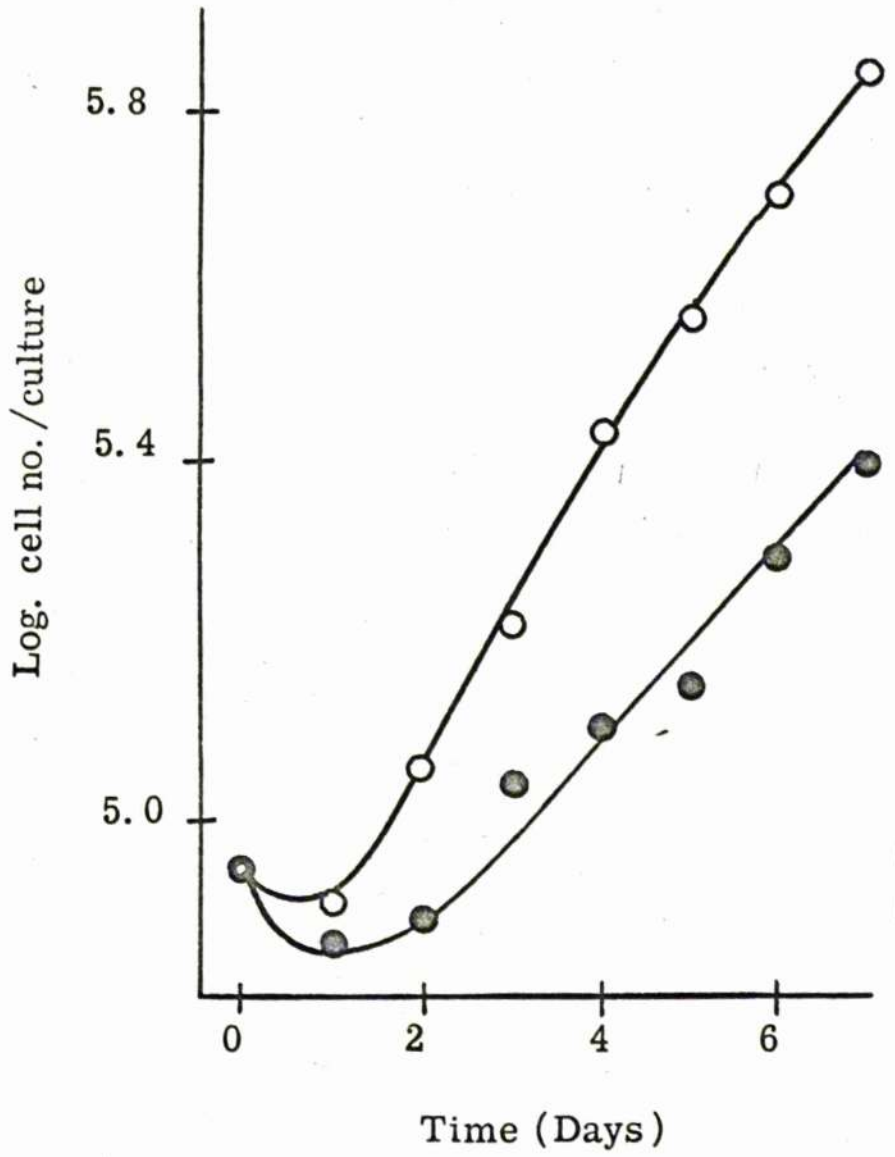

Fig. 2. Influence of prednisolone on growth of HLM cells

TABLE 8

INFLUENCE OF INSULIN AND PREDNISOLONE ON KaLe CELL GROWTH AND COMPOSITION

Prescription bottles (6 oz.) were inoculated with 1.2×10^6 KaLe cells. Waymouth's medium (30 ml.) at pH 7.4 with or without insulin or prednisolone was added. The culture medium was renewed every three days and on the tenth day the cells were harvested for analysis.

Hormone Addition	Cell No. (10^6)	µg.DNAP/ 10^6 cells	µg.CBH/ 10^6 cells	µg.RNAP/ 10^6 cells	µg.Protein/ 10^6 cells
0	9.8;9.5	2.0;2.0	32;35	4.3;4.6	238;274
Insulin (1 unit/ml)	10.5;11.7; 10.4	2.0;2.1	40;40;47	5.0;5.0	290;328;332
Prednisolone (2.5 µg/ml.)	6.2;6.4; 8.0	2.2;2.3 2.2	23;21;19	5.3;5.3 5.0	282;294;299

Effects of Prednisolone on Growth and Composition of Other Cell Strains

HeLa cells (strain A.P.)

Since prednisolone is known to induce considerable increases of alkaline phosphatase activity in certain strains of HeLa cells (Cox & MacLeod, 1962), its effects on the composition and growth of these cells were investigated. The results, presented in Table 9 indicate that cell multiplication was again inhibited. No effect on cell protein or RNA phosphorus was found but a slight increase in DNA phosphorus per cell was noted after 14 days. However, this is perhaps questionable in view of the scatter of the results. In contrast to these results Cox & MacLeod reported increases in cell protein and RNA as well as slight increases in DNA. The medium used by Cox & MacLeod was somewhat different from that used in this study. This could perhaps account for the discrepancy.

LS cells

Results of an experiment using a strain of L cells which grows spontaneously in suspension indicated that growth of this cell type was also inhibited by prednisolone even at a concentration of 0.1 µg. per ml. Although no increases in DNA phosphorus or RNA phosphorus were found, a slight increase in protein and a considerable increase in lipid was noted (Table 10).

TABLE 9

EFFECTS OF PREDNISOLONE ON COMPOSITION OF HeLa CELLS (STRAIN A.P.).

Roux flasks were inoculated with 5 x 10^6 HeLa cells (strain A.P.) and 50 ml. Waymouth's medium with or without prednisolone. The medium was renewed every 3 days. Cells of sample cultures were harvested for analysis after times indicated. 5 x 10^6 cells from each eight day old culture were used to seed fresh Roux flasks containing 50 ml. Waymouth's medium with or without prednisolone respectively. These cultures were incubated for a further 6 days with renewal of the medium on the fourth day.

	Prednisolone concn. (µg./ml.)	Days Incubation			
		2	4	8	14
Total cells (10^6)	0	8;7	18;22	32;35	19;21
	1	7;6	16;13	28;28	11;13
µg.DNAP/ 10^6 cells	0	1.4;1.5	1.9;1.4	1.7;1.7	2.0;1.7
	1	1.8;1.7	1.6;1.7	2.0;1.8	3.2;2.2
µg.RNAP/ µg.DNAP	0	3.3;3.1	2.6;3.2	3.0;3.2	3.4;2.8
	1	1.4;2.1	3.3;3.5	3.6;3.4	2.7;3.6
µg.Protein/ µg.DNAP	0	190;150	150	195;190	170;130
	1	150;130	140;160	230;230	120;170

TABLE 10

INFLUENCE OF PREDNISOLONE ON THE GROWTH AND COMPOSITION OF LS CELLS

Baby feeding bottles were inoculated with 10^6 LS cells in 10 ml. of Eagle's medium. Prednisolone was added as indicated. On the third day of incubation 10 ml. of fresh medium was added to each culture. On the sixth day cells were harvested for analysis.

Prednisolone concn. µg./ml.	Total cell No. (10^6)	µg.DNAP/ 10^6 cells	µg.RNAP/ 10^6 cells	µg.Protein/ 10^6 cells	µg.Lipid/ 10^6 cells
0	8.4;8.8; 9.2	1.56;1.68; 1.81	0.71;0.69; 0.90	155;172 173	58;69 70
2.5	5.6;4.2	1.48;1.56	0.78;0.77	177;170	88;113
0.1	7.0;5.4	1.69;1.52	0.88;0.85	194;165	

INFLUENCE OF INSULIN AND PREDNISOLONE ON THE CARBOHYDRATE METABOLISM OF CELLS IN CULTURE

Insulin

Observations that insulin causes increased glycolysis in cell cultures (Paul & Pearson, 1960; Crockett & Leslie, 1963) were confirmed with this cell strain and medium used (Table 11). No difference in the keto acid content of media from insulin-treated as compared to control cultures was noted in this experiment. Paul & Pearson (1960) reported increased disappearance of keto acid from the medium of insulin-stimulated cultures.

Prednisolone

The media from prednisolone-treated cultures contained less lactate and more keto acid than did that from controls. Although it is probable that glucose utilization was decreased, the results were somewhat variable (Tables 11 and 12).

Increased keto acid levels in prednisolone-treated cultures of the alkaline phosphatase inducible strain of HeLa cell and in the LS cell were also noted (Tables 13 and 14). With the latter cell strain, however, this effect was apparent only if the keto acid produced was expressed on the basis of cell number present. Glucose utilization by these cell types was decreased but again the results were somewhat variable.

TABLE 11

EFFECT OF INSULIN AND PREDNISOLONE ON GLUCOSE DISAPPEARANCE FROM AND ORGANIC ACID CONTENT OF MEDIUM SUPPORTING GROWTH OF HLM CELLS

Baby feeding bottles were inoculated with 2.9×10^6 HLM cells in 10 ml. Waymouth's medium and allowed to establish themselves by incubation overnight. The medium was then replaced with 30 ml. Waymouth's medium containing no serum but supplement with 0.5% (W/V) bacto-peptone and with and without insulin or prednisolone. After 3 days samples of the medium were removed and analysed.

Hormone Addition	Final Cell No. (10^6)	Glucose Utilized (mg.)	Lactate Present (mg.)	Keto Acid Present (mg.)
0	6.2	15.9	6.1	1.4
	7.0	14.4	5.8	1.4
Insulin (1 unit/ml.)	5.7	26.1	10.0	1.3
	7.4	25.2	8.8	1.6
	5.8	25.2	8.8	1.6
Prednisolone (2.5 µg./ml.)	3.2	6.6	2.4	2.0
	3.7	11.4	3.1	2.1
	4.0	2.7	2.2	1.9

TABLE 12

EFFECT OF PREDNISOLONE ON GLUCOSE DISAPPEARANCE FROM AND KETO ACID LEVELS IN MEDIUM SUPPORTING HLM CELL GROWTH

Roux flasks were inoculated with 5 x 10^6 HLM cells in 50 ml. Waymouth's medium with or without prednisolone. After 4 days incubation the medium on all cultures was renewed. Following a further 2 days incubation samples of the medium were removed and analysed.

Prednisolone concn. (µg./ml.)	Final Cell No. (10^6)	Glucose Utilized (mg.)	Keto Acid Present (µg.)
0	13.4	24	490
	12.2	31	440
2.5	6.7	27	500
	7.3	17	500

TABLE 13

EFFECT OF PREDNISOLONE ON GLUCOSE DISAPPEARANCE FROM AND KETO ACID LEVELS IN MEDIUM SUPPORTING GROWTH OF HeLa CELLS (STRAIN A.P.).

Roux flasks were inoculated with 5×10^6 HeLa cells (strain A.P.) and 50 ml. Waymouth's medium with or without prednisolone. The medium was renewed every 3 days. After 8 days 5×10^6 cells from each culture were used to seed fresh Roux flasks containing 50 ml. Waymouth's medium with or without prednisolone respectively. After 4 days incubation the medium was renewed. After an additional 2 days incubation samples of the medium were taken and analysed.

Prednisolone concn. (µg./ml.)	Final Cell No. (10^6)	Glucose Utilized (mg.)	Keto Acid Present (µg.)
0	19.3	37.4	540
	21.5	46	540
1	10.8	34	770
	12.9	15	820

TABLE 14

EFFECT OF PREDNISOLONE ON GLUCOSE DISAPPEARANCE FROM AND KETO ACID LEVELS IN MEDIUM SUPPORTING GROWTH OF LS CELLS

Baby feeding bottles were inoculated with 10^6 LS cells in Eagle's medium with or without prednisolone. On the third day of incubation, 10 ml. of fresh medium was added to each culture. Samples of the medium were taken on the sixth day and analysed after cells had been removed by centrifugation. Cell counts were performed directly on cells suspended in known volumes of Hank's BSS.

Prednisolone concn. (µg./ml.)	Final Cell No. (10^6)	Glucose Utilized (mg.)	Keto Acid Present (µg.)
0	8.7	13.9	400
	8.8	15.8	460
	9.2	16.7	390
	8.7	16.1	500
2.5	5.6	12.9	360
	4.2	14.9	340

INFLUENCE OF INSULIN AND PREDNISOLONE ON THE ACTIVITIES OF ENZYMES IN CULTURED CELLS

Tables 15 and 16 present results of enzyme analyses on extracts from HLM and HeLa cells incubated in the presence or absence of insulin or prednisolone.

Insulin

No consistent effects of insulin were found with any of the enzymes studied. On occasions, however, increases in hexokinase activity were apparent with HLM and with HeLa cells grown in the presence of this hormone. The inconsistency may indicate the existence in HLM cells of two glucose phosphorylating enzymes as has been found in rat liver (Walker, 1962).

HLM cells were found to contain considerably greater amounts of glucose-6-phosphatase and alkaline phosphatase than HeLa cells.

Results of an experiment to determine whether insulin affected the hexokinase activity of HLM cells at stages during the growth cycle are presented in Table 17. In this experiment no significant increase in hexokinase level was noted although by the third or fourth day the intracellular carbohydrate was increased (see Table 3).

It should be emphasised that the reaction mixture in hexokinase estimations reported in this study contained initial glucose concentrations of 4 to 6 mMolar and final concentrations (after incubation) of 2 to 3 mMolar. Since the Km for rat liver

TABLE 15

Experiments 2 & 13: Roux flasks were inoculated with 50 ml. Waymouth's medium and 5×10^6 HLM cells. Insulin (0.1 unit/ml. in experiment 2; 1.0 unit/ml. in experiment 13) or prednisolone (2.5 µg./ml.) was added as indicated. Medium was renewed every 3-4 days. Cells of experiment 13 were harvested on the 9th day. With experiment 2 cells were harvested by trypsinisation on the 8th day and 5×10^6 cells from each flask were subcultured to flasks containing 50 ml. Waymouth's medium and the appropriate hormone. After a further 9 days the cells were harvested and the enzyme analyses were performed.

Experiments 17 & 22: Baby feeding bottles were inoculated with 10 ml. Waymouth's medium and 10^6 or 3.2×10^6 HLM cells for experiments 17 and 22 respectively. After 24 hours the medium was replaced with 30 ml. of Waymouth's medium without serum but supplemented with 0.5% (W/V) Bacto-peptone. Insulin (1 unit/ml.) or prednisolone (2.5 µg./ml.) was added as indicated. The medium was renewed every 2-3 days. Cells were harvested after total incubation times of 7 and 5 days for experiments 17 and 22 respectively. Enzyme units are expressed per 10^6 cells and per mg. protein. The latter are given in parentheses.

TABLE 15

EFFECTS OF INSULIN AND PREDNISOLONE ON ENZYME LEVELS IN HLM CELLS

Exp No.	Hormone Present	Hexokinase	Glucose-6-Phosphatase	Glucose-6-Phosphate Dehydrogenase	Lactic dehydrogenase	Alkaline phosphatase	Esterase	β-Glucuronidase
2	0			65;44;48 54;75;68 68;70;76	0.9;0.9;0.9 0.9;0.9;0.9 0.9;0.9;0.9	69;71;69 50;68;65 71;82;87	0.2;0.1;0.2 0.1;0.2;0.2 0.8;0.9;0.8	
	Insulin Prednis							
13	0	0.2;0.2;0.2 (3);(3);(3);	0.1;0.1;0.1 (2);(2);(2)	120;70;85 (2400);(1600); (1800)	0.4;0.4;0.4 (9);(10);(9)	9; 8; 9 (170);(175); (195)	0.2;0.1;0.2 (3);(3);(3)	0.1;0.1;0.1 (1);(2);(2)
	Insulin	0.2;0.2;0.2 (3);-;(4)	0.1;0.1;0.1 (2);(1);(2)	80;70;95 (1550);(1690)	0.4;0.4;0.5 (8);(8)	10;9 (185);(170)	0.2;0.1;0.2 (3);(4)	0.1;0.1;0.1 (1);(1);(1)
	Prednis	0.2;0.2 (4);(4)	0.03;0.03 (0.6);(0.5)	90;90 (1490);(1360)	0.5;0.5 (8);(7)	8;11 (130);(170)	0.4;0.4 (8);(7)	0.1;0.1 (2);(2)
17	0	0.03;0.05;0.01 (1);(2);(0.2)	0.1;0.1;0.1 (4);(2);(4)	2;2;4 (60);(60);(130)	0.1;0.1;0.1 (4);(4);(4)	5;4;4 (180);(130); (120)	0.1;0.1;0.1 (2);(3);(3)	0.03;0.03;0.0 (0.7);(0.6); (0.7)
	Insulin	0.1;0.1;0.1 (2);(2);(3)	0.2;0.1;0.1 (3);(2);(2)	25;29;18 (460);(560) (350)	0.2;0.2;0.2 (3);(3);(3)	6;5;4 (121);(95); (82)	0.2;0.1;0.2 (3);(3);(3)	0.03;0.03;0.0 (0.6);(0.6); (0.6)
	Prednis	0.1;0.03;0.05 (2);(1);(1)	0.1;0.1;0.1 (4);(3);(2)	17;12;12 (490);(380); (310)	0.1;0.2;0.2 4;5;4	4;3 (129);(111)	0.1;0.2;0.2 (4);(6);(5)	0.04;0.03 (1.1);(1.1)
22	0	0.2;0.2;0.2 (7);(4);(2)	0.02;0.03;0.13	165;155;235				
	Insulin	0.2;0.2;0.2	0.05;0.04;0.03	185;195;120				

TABLE 16

Roux flasks were inoculated with 50 ml. Waymouth's medium and 5×10^6 HLM or HeLa cells as indicated. Insulin (0.1 units/ml.) or prednisolone (2.5 µg./ml.) was added as sho Medium was renewed every 3-4 days. Cells were harvested by trypsinization on the 14th day and 5×10^6 cells from each flask were used to seed fresh flasks containing 50 ml. of Waymouth's medium and the appropriate hormone. The remaining cells were used for enzyme analyses. On the 18th day, the second series of cultures were treated with trypsin and enzyme analyses were performed. Enzyme units are expressed per 10^6 cells.

TABLE 16

EFFECTS OF INSULIN AND PREDNISOLONE ON ENZYME LEVELS IN HLM AND HeLa CELLS

Cell Type	Hormone Added	Hexokinase		Glucose-6-phosphate dehydrogenase		Lactic dehydrogenase		Glucose-6-phosphatase	Alkaline phosphatase		Esterase		β-Glucuronidase
	Days Incubation	14	18	14	18	14	18	18	14	18	14	18	18
HLM	0	0.2	0.3		40	0.4	0.4	0.3	26		0.3	0.2	
			0.3	84	43	0.4	0.4	0.7	27	20	0.4	0.2	0.05
			0.4	6	23	0.4	0.4		17	37	0.2	0.1	0.06
	Insulin			17	38	0.4	0.4	0.3	11	28	0.2	0.2	0.04
		0.4	0.4	54		0.4			17		0.2		
				24		0.4			5		0.2		
	Prednis.	0.3	0.3		44	0.4	0.4	0.3	4	34	0.4	0.5	0.06
		0.4	0.3	64	92	0.4	0.4	0.4	8	34	0.4	0.6	0.07
			0.3	84	80	0.5	0.4	0.5	4	35	0.6	0.6	0.06
HeLa	0	0.4	0.3	115	130	0.4	0.4	0.003	0.6	3.2	0.3	0.4	0.06
		0.2	0.4	86	118	0.4	0.4	0.004	0.5	2.0	0.2	0.2	0.10
			0.4			0.4	0.4	0	1.2	1.1	0.2		0.08
	Insulin	0.3	0.5	119	133	0.4	0.4	0	1.0	0.6	0.2	0.4	0.08
		0.2	0.6	95	124	0.4	0.4	0	0.7	0.5	0.2	0.3	0.12
			0.5	114	132	0.4	0.4	0	0.8	0.4	0.2	0.3	0.11
	Prednis.	0.2	0.4	87	75	0.4	0.4	0.005	1.0	2.1	0.4	0.5	0.13
		0.3	0.4	75	69	0.4	0.4	0.005	1.7	2.5	0.4	0.4	0.09
			0.4	69	94	0.4	0.4	0.004	1.2	2.1	0.4	0.5	0.12

TABLE 17

INFLUENCE OF INSULIN ON HEXOKINASE ACTIVITY IN HLM CELL EXTRACTS

Roux flasks were inoculated with 5×10^6 HLM cells and 50 ml. of Waymouth's medium with or without insulin. The medium was renewed daily and each day cells from sample cultures were harvested for preparation of enzyme extracts.

Insulin concn. (units/ml.)	Hexokinase Activity (units/10^6 cells)			
	Days Incubation			
	1	2	3	4
0	0.8; 1.0	0.6; 0.4	1.2; 1.1	1.2
1	0.7; 0.9	0.4; 0.5	1.2	0.9; 1.0

hexokinase is about 37μM (Walker, 1963), this change in glucose concentration should not have affected hexokinase assay, presuming the enzymes of liver and HLM cells are similar. However, if a glucokinase similar to that found in rat liver (Walker, 1962) exists in HLM cells, the range of glucose concentrations used for the hexokinase estimations could include assay of both enzymes. Furthermore, since the Km for rat liver glucokinase is about 10 mMolar (Walker, 1963; Vinuela, Salas & Sols, 1963) the changes in glucose concentration which occurred during incubation of the enzyme extract would influence considerably the final total activity measured.

Prednisolone.

A consistent effect of prednisolone in increasing the esterase activity of extracts from HLM and HeLa cells was observed (Tables 15 and 16). In one experiment of long duration a slight increase in the alkaline phosphatase content of HLM cells was also noted (Exp. 2, Table 15). The increments of esterase activity varied considerably from one experiment to another but the basal level was relatively constant.

None of the other enzymes studied varied consistently following addition of prednisolone.

The significance of the increase in esterase activity of extracts from prednisolone treated HLM cells is not known. Wellington & Moon (1961) by histochemical methods showed that the esterase activity of a strain of cells isolated from human

liver (Chang, 1954) increased during growth in the presence of hydrocortisone. In that case there were also increases in acid phosphatase and decreases in succinic dehydrogenase and glucose-6-phosphate dehydrogenase. The dose of hydrocortisone was, however, much higher (150 µg./ml.) than the prednisolone concentration used in this study.

Prednisolone induces large increases in the alkaline phosphatase activity of certain strains of HeLa cells (Cox & MacLeod, 1962). Therefore, the effect of this steroid on esterase, β-glucuronidase and alkaline and acid phosphatase levels in such a cell strain during stages of growth was determined (Table 18). While the alkaline phosphatase level was increased even after only two days, the levels of esterase, β-glucuronidase and acid phosphatase were unaffected after two weeks of growth in the presence of prednisolone.

EFFECT OF HORMONES ON GLUCOSE TRANSPORT IN HLM CELLS

Glucose utilization by muscle is accelerated by insulin owing to a stimulation of glucose transport. In cultured cells glucose utilization is also stimulated by insulin but the mechanism is not known.

Steroid hormones, in contrast to insulin, inhibit sugar utilization by certain mammalian cells and cells in culture. Sugar transport in the erythrocyte is inhibited by corticosteroids (Wilbrandt, 1954).

Experiments were undertaken, therefore, in an attempt

TABLE 18

INFLUENCE OF PREDNISOLONE ON ENZYME LEVELS IN EXTRACTS OF HeLa CELLS (STRAIN A.P.) AT STAGES DURING GROWTH

Roux flasks were inoculated with 5×10^6 HeLa cells (strain A.P.) and 50 ml. Waymouth's medium with or without prednisolone. The medium was renewed every 3 days. Cells of sample cultures were harvested for preparation of enzyme extracts after times indicated. 5×10^6 cells from each 8 day old cultures were used to seed fresh Roux flasks containing 50 ml. Waymouth's medium with or without prednisolone respectively. These cultures were incubated for a further 6 days, with renewal of the medium on the fourth day.

Enzyme	Prednisolone concn. (µg./ml.)	Enzyme Activity (Units/10^7 cells)			
		Days Incubation			
		2	4	8	14
Alkaline Phosphatase	0	1.4	2.0;2.0	2.4;3.3	2.9;3.0
	1	2.6;2.2	10.2;8.9	10.7;10;6	8.9;12.4
Esterase	0	0.6;0.6	0.6;0.6	0.7;0.8	0.5;0.5
	1	0.5	0.6;0.6	1.0;0.8	0.5;0.4
β-Glucuron--idase	0	0.9;1.1	0.7;0.6	0.8;0.9	0.8;0.7
	1	1.2;1.2	0.7;0.7	1.3;1.2	0.8;0.6
Acid Phosphatase	0	3.3;4.3	4.0;4.0	4.4;5.3	4.0;3.3
	1	4.0;3.6	3.3;3.9	8.0;6.9	4.6;3.0

to characterize the process of glucose transport in HLM cells and to determine whether insulin and prednisolone influence it.

Glucose Transport in HLM Cells in the Absence of Hormones

Influx and efflux of radioactive glucose

Figure 3 illustrates results of an experiment to determine the net influx of labelled glucose into HLM cells during a 30 minute exposure period. It is apparent that a plateau level of radioactivity was approached after 20 to 30 minutes.

Figure 4 presents results of an experiment to determine influx and efflux of radioactivity from labelled glucose. As would be predicted, the influx curve, representing net inward transport (entry minus exit), has distinctly less slope than does the efflux curve. The system apparently consisted of two types of labelled components. One of these was readily diffusible while the other did not diffuse out of the cell, even after a 35 minute efflux period.

Wilbrandt (1961) pointed out that measurements of sugar exit are preferable to those of sugar entrance in kinetic analyses of transport. The former can be determined under conditions where transport is virtually unidirectional. The latter measurement usually represents net inward transport under conditions in which transport out of and into the cell are occurring simultaneously. With the technique used in this study, however, it is difficult to take samples during the first few minutes of efflux. Furthermore, efflux measurement is complicated by

FIGURE 3

Three day old cultures were equilibrated for 30 minutes as usual. After the equilibration medium was removed, corresponding test medium containing ^{3}H glucose at a specific activity of 1.4 mCuries/mMole was added. Sample cultures were taken after various times. Points represent the means determined from 3 replicate cultures and the vertical lines give the total range of results.

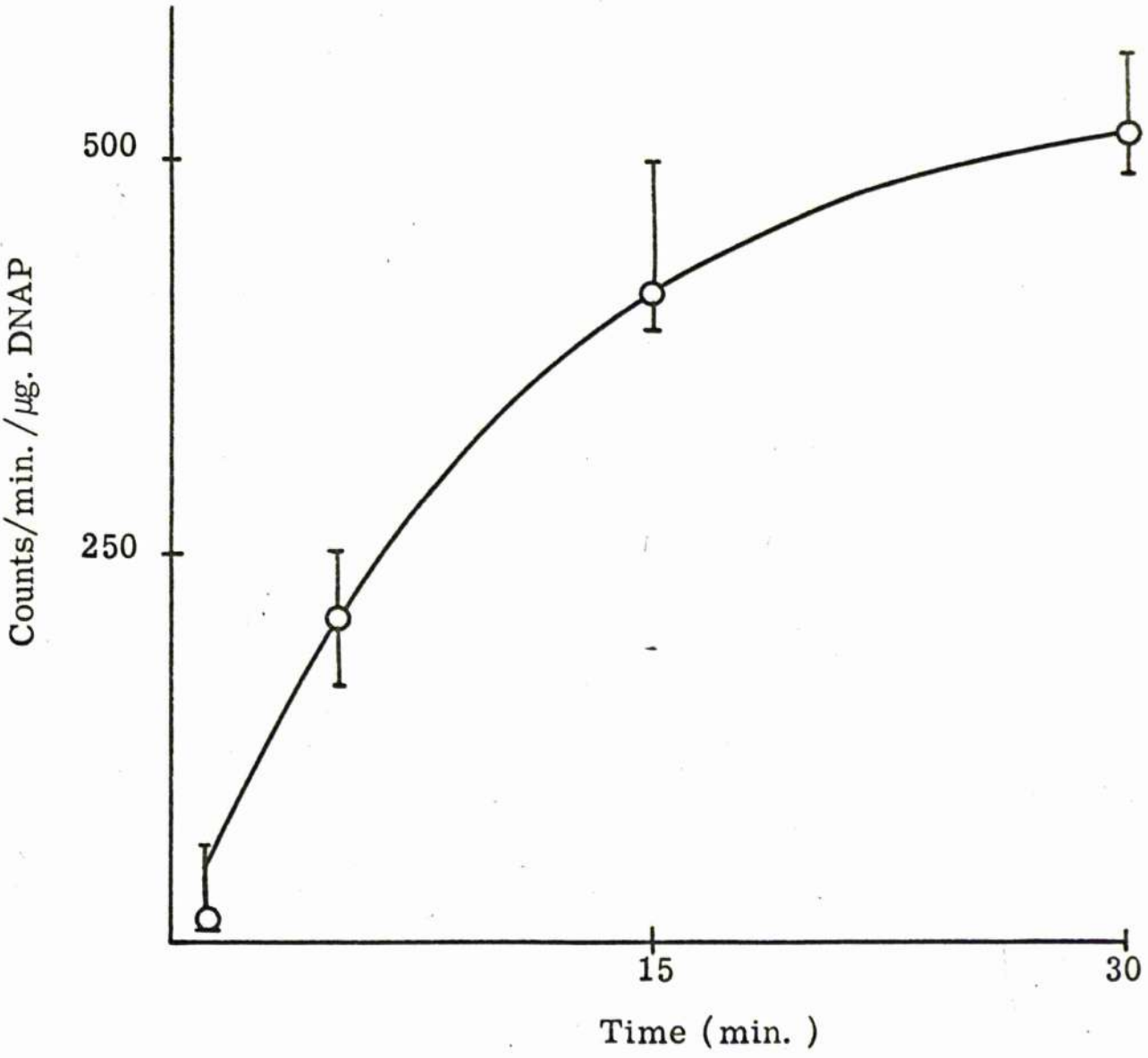

Fig. 3. Influx of glucose into HLM cells

FIGURE 4

Details of procedure in this experiment are similar to those for figure 3. In this case, however, the test medium contained glucose at a specific activity of 0.7 mCuries/mMole. After 25 minutes influx, test medium was removed and the cultures were washed 5 times with 5 ml. of equilibration medium at 37° and 5 ml. of this was added. After the times indicated sample cultures were removed, washed in cold tris-citrate BSS and extracted as usual. Points shown are the means determined from 3 replicate cultures. The vertical lines represent the total range of the results.

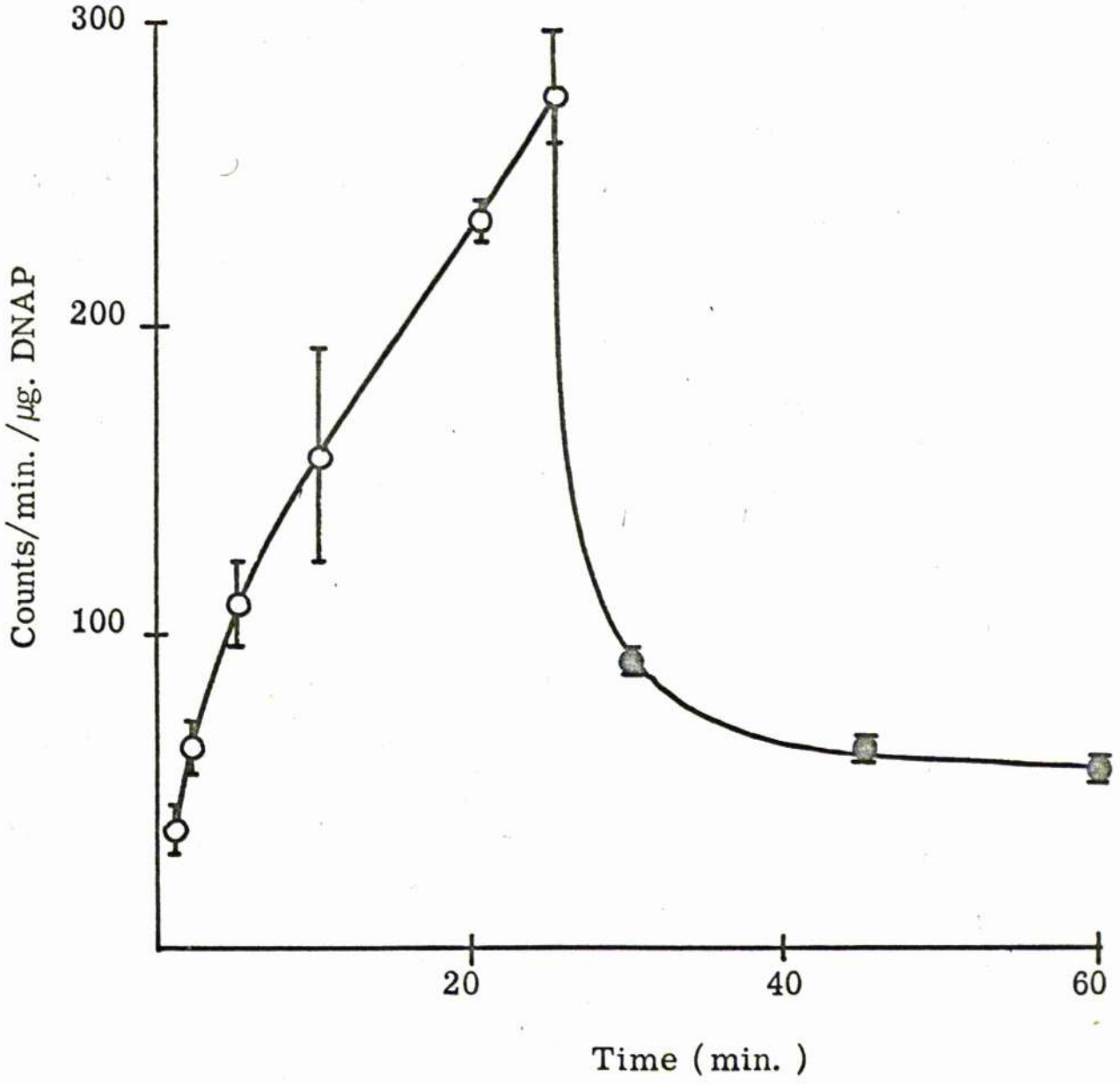

Fig. 4. Influx (○), and efflux (●) of glucose in HLM cells

the possibility that some of the radioactivity remaining within the cell represents products of glucose metabolism rather than residual glucose itself.

Estimates of rates of inward transport can be made by measuring the amount of labelled glucose entering the cell during very brief exposure periods. Errors introduced by back-flow would decrease with decreasing exposure times. In this study, therefore, the uptake by HLM cells of labelled glucose during a one minute exposure period was assumed to represent glucose entry. During this brief exposure it is probable that error due to back-flow was slight.

Cultures were equilibrated for 30 minutes (see methods) before addition of labelled glucose. From figure 3 it is apparent that during this time the intracellular/extracellular glucose exchange approached equilibration. It is also apparent that labelled glucose entering during the one minute period post equilibration would be diluted out considerably in the intra-cellular, non-radioactive glucose pool. Aqueous extracts (see hexokinase assay method) of HLM cells harvested by trypsinization contained 2-8 µg. glucose per 10^6 cells. Therefore only a small fraction of the labelled glucose would be phosphorylated and otherwise transformed during the one minute period. This is in support of the argument that glucose transport as opposed to glucose metabolism was the process under study.

Rate of uptake at various glucose concentrations

Sugar uptake by the perfused rat heart has been shown to conform to Michael-Menten kinetics (Park et al. 1959) and an approximate Km value of 157 mg. per 100 ml. has been calculated (Morgan, Henderson, Regen & Park, 1961). Erythrocytes also conform to this formulation and Km values of a similar magnitude have been found (Widdas, 195-).

Considerably lower Km values for glucose (1 mMolar) and galactose (0.5 mMolar) entry was reported for the L cell (Maio & Rickenberg, 1962). Crane, Field & Cori, (1957) found that the entry of a number of sugars into Ehrlich ascites tumor cells was too rapid at 37°C to permit Km calculation. At lower temperatures, however, it was possible to demonstrate Michaelis-Menten type kinetics.

Results of studies of the effect of varying the glucose concentration on rate of uptake by HLM cells are illustrated in figures 5 and 6. The specific activity of glucose used in these cases was the same at all glucose concentrations.

It is apparent that the rate of glucose entrance into HLM cells did not increase linearly with increasing glucose concentration. Although a plateau level of radioactivity was suggested in results of Figure 6 at about 44.4 mMolar (8 gm./litre) much higher counts were obtained at the 88.8 mMolar (16 gm./litre) substrate level. The non-linearity argues against an hypothesis that glucose enters these cells by simple diffusion or by pinocytosis.

FIGURES 5 & 6

Three day old cultures were equilibrated in tris-citrate BSS containing glucose at the concentrations shown. Test glucose contained the same glucose concentrations with ^{3}H glucose at a specific activity of 3.5 mCuries/mMole. Test medium was added to cultures for one minute only, prior to washing and extracting as usual. The points shown in figure 5 represent means determined from results of 3 replicate cultures. The vertical lines give the total range of results.

The points plotted in figure 6 represent means determined from results of 6 replicate cultures. The vertical lines represent 2 standard deviations of the means.

The dotted lines represent plots from the means corrected for the effect of increased osmotic pressure (see Figure 10).

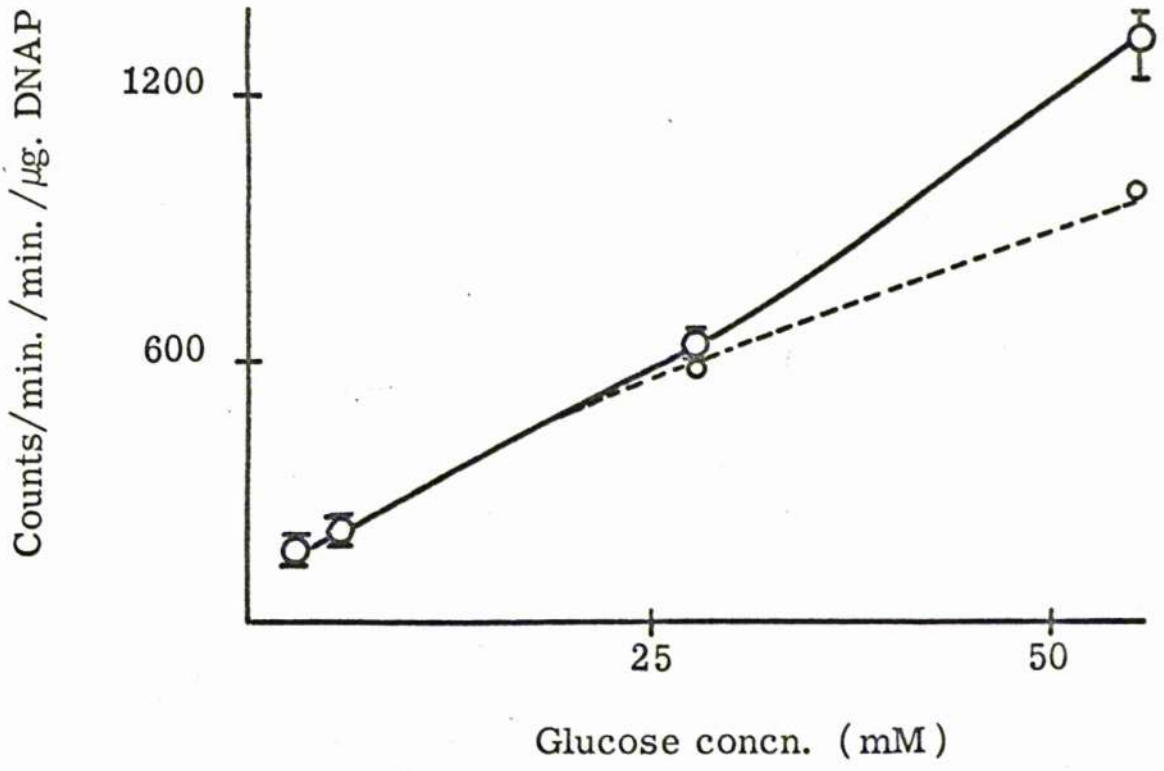

Fig. 5. Effect of glucose concentration on the rate of glucose uptake by HLM cells

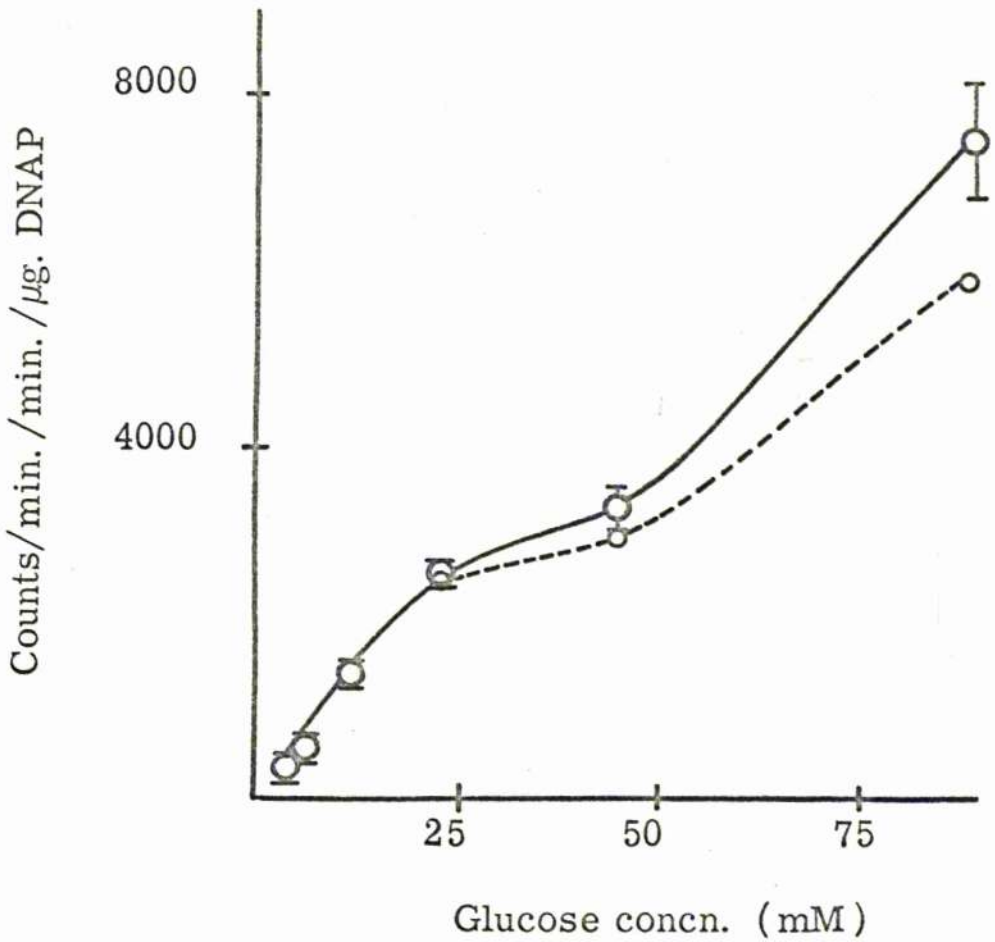

Fig. 6. Effect of glucose concentration on the rate of glucose uptake by HLM cells

Influence of pH and fluoride on the rate of glucose uptake

Rate of entry of glucose into HLM cells was not effected to any great extent by pH changes from 6.8 to 7.8 (Table 19). Paul (1959) reported that pH markedly influences glycolysis in cultured cells. This effect was noted over an extended period of time and may reflect other metabolic systems primarily.

Since fluoride is known to increase glucose transport in rat diaphragm (Newsholme & Randle, 1961) its effect on this process in HLM cells was determined (Table 19). A very marked stimulatory effect was noted at pH 6.8 and this decreased with increasing pH.

Influence of temperature on the rate of glucose uptake

Effects of temperature on glucose uptake vary widely according to the cell type under study and the temperature range used. The transport of sugars into erythrocytes was found to have a Q_{10} of about 2.5 (Le Fevre, 1948, 1954; Park et al. 1956). This process in Ehrlich ascites tumor cells, however, has a $Q_{10\ (20\text{-}30^{\circ})}$ of about 4 (Crane et al. 1957).

The influence of temperature on the rate of uptake by HLM cells is shown in Table 20. An approximate $Q_{10\ (27\text{-}37^{\circ})}$ of 1.7 was calculated by extrapolation from the results shown.

TABLE 19

EFFECT OF pH AND FLUORIDE ON THE
RATE OF GLUCOSE UPTAKE BY HLM CELLS

Three day old cultures were equilibrated at pH 6.8-7.8 in the presence or absence of sodium fluoride (10 mMolar). Test media at the appropriate pH and with or without fluoride contained ^{3}H glucose at a specific activity of 7.0 mCuries/mMole.

pH	Rate of Glucose Uptake (Counts/min./min./µg.DNAP)			
	Without Fluoride.	Mean	With Fluoride.	Mean
6.8	366; 415; 486	422	1624; 1874; 1671	1723
7.0	425; 403; 427	418	1254; 1204; 1147	1201
7.2	462; 342; 456	420	1131; 1246; 1300	1225
7.4	432; 365; 332	376	840; 781; 860	827
7.6	420; 384; 459	421	842; 581; 739	721
7.8	354; 301; 363	339	353; 463; 521	446

TABLE 20

EFFECT OF TEMPERATURE ON THE RATE OF GLUCOSE UPTAKE BY HLM CELLS

Two day old cultures were equilibrated and exposed to test medium at the temperatures indicated. Test medium contained ^{3}H glucose at a specific activity of 3.5 mCuries/mMole.

Temp.°	Rate of Glucose Uptake (counts/min./min./µg.DNAP)	Mean
37	610; 530; 480	540
33.5	370; 480; 490	447
30	340; 330; 330	333
25	230; 310; 360	300
20	200; 180; 180	187

Competitive inhibition of glucose uptake by other sugars

The well known existence of competition among hexoses and pentoses for the glucose transport system of red blood cells (Le Fevre, 1961), muscle tissue (Randle & Young, 1962), Ehrlich ascites tumor cells (Crane et al. 1957) and L cells (Maio & Rickenberg, 1962) prompted investigations to determine if a similar phenomenon exists in HLM cells.

A preliminary experiment (Table 21) suggested the possibility that galactose but not ribose acted as a competitive inhibitor of glucose uptake. This could not, however, be verified in subsequent experiments (see Table 35) involving larger sample numbers. Thus no inhibition of the rate of glucose uptake could be shown when D galactose or D or L arabinose were used.

The possibility that glactose might effect the net uptake of radioactivity from glucose by HLM cells was determined by incubating the cultures with labelled glucose and with or without galactose for a period of 30 minutes. No effect of galactose on the intracellular content of radioactivity could be demonstrated (Table 22).

It should be mentioned that no attempt was made to adjust the osmotic pressure of media containing galactose or arabinose to that of control medium. The effect of osmotic pressure on glucose transport is shown in a later section and may account for the apparent absence of competitive inhibition shown here.

TABLE 21

EFFECT OF OTHER SUGARS ON THE RATE OF GLUCOSE UPTAKE BY HLM CELLS

Three day old cultures were equilibrated in tris-citrate BSS containing glucose (5.6 mMolar) and other sugars as indicated. Test media contained the same sugar concentrations with ^{3}H glucose at a specific activity of 7.0 mCuries/mMole glucose.

Concn. of other sugar added (mMolar)	Rate of Glucose Uptake (counts/min./min./µg.DNAP)	Mean
0	1260; 920	1090
Galactose (5.6)	1110; 1150; 870	1043
Galactose (27.8)	935; 870; 1010	938
Ribose (6.7)	980; 875; 1040	965
Ribose (33.4)	1190; 965	1078

TABLE 22

EFFECT OF GALACTOSE ON THE UPTAKE OF GLUCOSE BY HLM CELLS

Three day old cultures were equilibrated for 30 minutes as usual. Test media with or without galactose and containing glucose (5.6 mMolar) and ^{3}H glucose at a specific activity of 1.4 mCuries/mMole were added for an additional 30 minute interval.

Concn. of Galactose (mMolar)	Intracellular Radioactivity (counts/min./µg.DNAP)
	Mean ± S.D.
0	338 ± 69 (6)
27.8	469 ± 122 (6)

Effect of serum on the rate of glucose uptake

Large differences in the rate of glucose uptake were noted among different experiments in spite of attempts to set these up in an identical manner. One possible reason for this variation could have been differences in the serum used in the growth medium. Pituitary and adrenal factors, present in serum, may affect glucose transport or the action of insulin on the perfused heart (Henderson et al., 1961a,b). The presence of such factors in the serum used to cultivate cells might influence glucose transport and metabolism.

HLM cells cannot be grown in serum-free medium from the trypsinized stage. It was possible, however, to use serum-free medium if the cells were first allowed to adhere to the glass in the medium containing serum.

Cells which had been allowed to establish themselves for a 24 hour period were washed with Hank's BSS and growth medium containing serum was added to some cultures while medium containing no serum was added to others. After a further 24 hour incubation period, the cultures were used in the normal manner to determine the rate of glucose uptake.

The results, shown in Table 23, indicated that maintenance in the absence of serum did not appreciably alter the rate of glucose uptake.

Unfortunately this does not completely rule out the possibility that variation among experiments was due to differences in serum used. It is possible that longer periods of exposure to

TABLE 23

EFFECT OF MAINTENANCE IN THE ABSENCE OF SERUM ON THE RATE OF GLUCOSE UPTAKE BY HLM CELLS

Two day old cultures maintained in the presence or absence of serum for the second day of incubation were used (see text). Test medium contained ^{3}H glucose at a specific activity of 3.5 mCuries/mMole.

Serum Concn. ml./100 ml. Medium	Rate of Glucose Uptake (Counts/min./min./µg.DNAP)
0	1560; 1060
7	1330; 1200

serum containing some compound inhibitory to glucose uptake may affect the glucose transport system for a considerable time after removal of serum.

In this respect, it is notable that Wu (1959) found that glycolysis in HeLa cells varied depending on the source of serum used in the growth medium. Such an effect might be due to an action on glucose transport.

Rate of uptake of glucose at stages in the growth cycle

Another possible explanation for the variation among experiments would be that cells at different stages in the growth cycle exhibit different rates of glucose uptake. All of the foregoing experiments were performed on cultures which were either two or three days old.

Table 24 presents results of an experiment performed to answer this question. Although there was apparently a slight difference in the rate of uptake over a four day growth period, this was not extensive enough to explain the wide variation noted among previous experiments. Cell monolayers of four day old cultures showed a marked tendency to fragment and peel off the glass during washing and equilibration procedures. This may explain the variability in results of this group. Most experiments were therefore performed on cultures which were two or three days old.

TABLE 24

RATE OF GLUCOSE UPTAKE BY HLM CELLS AT VARIOUS TIMES DURING GROWTH

Cultures were set up in conical flasks as usual. Sample cultures were taken each day for determination of the rate of glucose uptake. Test medium contained ^{14}C glucose at a specific activity of 0.72 mCuries/mMole.

Days Growth	Rate of Glucose Uptake (Counts/min./min./µg.DNAP)	Mean
1	1410	1410
2	1650; 2200; 1970	1940
3	1160; 1110	1135
4	1465; 2020; 1345	1606

Effect of Insulin on Glucose Transport in HLM Cells

A preliminary experiment, the results of which are presented in Table 25, showed that addition of insulin to the equilibration medium stimulated the rate of glucose transport. Experiments were therefore performed to further characterise the glucose transport process in HLM cells and the stimulatory effect of insulin on it.

Time of exposure to insulin for maximal stimulation

In the foregoing experiment cells were exposed to insulin during the entire thirty minute equilibration period. Table 26 presents results of experiments planned to determine the minimum exposure time necessary for a maximal response to insulin under these conditions. In experiment 40, maximal stimulation was achieved after 30 minutes exposure to insulin judging from the ratio of the rate of glucose uptake with insulin to the rate without insulin. This effect apparently decreased with increasing exposure time.

In experiments 41 and 44 after 10 minutes exposure to insulin the effect on glucose influx was already marked, and further exposure did not appear to increase the effect significantly. A pronounced decrease in stimulation was apparent at 90 minutes in experiment 41 and at 1020 minutes in experiment 44. It is notable that the rate of glucose uptake in experiment 41 was much lower than that in experiments 40 and 44.

Persistence of the effect of insulin after a ten minute exposure period

In order to determine how long this effect of insulin persisted, cell cultures were equilibrated for the usual 30 minutes, exposed to insulin for 10 minutes and washed three times to remove insulin. The cultures were then incubated in equilibration medium without insulin and uptake of labelled glucose was determined after various intervals.

The results (Table 27) indicate that the insulin effect

TABLE 25

EFFECT OF INSULIN ON THE RATE
OF GLUCOSE UPTAKE BY HLM CELLS

Two day old cultures were equilibrated in the presence or absence of insulin (1 Unit/ml.). Test medium containing ^{14}C glucose at a specific activity of 0.72 mCuries/mMole and with or without insulin was used as indicated. The number of observations is given in parentheses.

Rate of Glucose Uptake(Counts/min./min./µg.DNAP)	
Without Insulin. Mean ± S.D.	With Insulin. Mean ± S.D.
268 ± 54 (4)	510 ± 85 (6)

TABLE 26

EFFECT OF TIME OF EXPOSURE TO INSULIN ON THE RATE OF GLUCOSE UPTAKE BY HLM CELLS

Three day old cultures were equilibrated for 30 minutes in tris-citrate BSS containing glucose (5.6 mMolar) as usual. The equilibration medium was replaced with fresh medium of the same composition but with or without insulin (1 Unit/ml.). After incubation times indicated the second medium was removed and test medium containing ^{14}C glucose at a specific activity of 0.72 mCuries/mMole with or without insulin was added.

Exp. No.	Time of Exposure to Insulin (minutes)	Rate of Glucose Uptake (counts/min./min./µg.DNAP) Without Insulin	Mean	With Insulin	Mean
40	01	1800;1800;720	1440	1100;1440;1490	1343
	30	1430;1460;1320	1403	2400;2440;2710	2517
	120	2100;1980;1520	1867	3000;2380;2560	2647
	240	1680;1115;1680	1492	1620;1970;2200	1930
	720	1170;1200;1560	1310	1350;1610;1500	1487
41	1	238;275;212	242	175;282;274	244
	10	209;186	198	324;318;249	297
	30	186;228;204	206	351;282;310	314
	90	246	246	260;263	261
	180	179;206	193	280;292;265	279
44	10	1155;1110	1132	2080;2280	2180
	120	1310;1375	1342	2670;2080	2375
	240	775;1170	972	1800;1890	1845
	1020	1570;1175	1372	1520;1590	1555

TABLE 27

PERSISTANCE OF THE INCREASE IN GLUCOSE UPTAKE AFTER TEN MINUTES EXPOSURE TO INSULIN

Two day old HLM cell cultures were equilibrated as usual but insulin (1 Unit/ml.) was added where indicated for the last 10 minutes. Cultures were then washed 3 times with 5 ml. of equilibration medium to remove remaining insulin and were incubated in 5 ml. equilibration medium for the times shown. After removal of this, test medium containing ^{14}C glucose at a specific activity of 0.72 mCuries/mMole glucose was added.

Time after 10 minutes Exposure to Insulin (minutes)	Rate of Glucose Uptake (counts/min./min./µg.DNAP)			
	No Exposure to Insulin	Mean	With Exposure to Insulin	Mean
15	1150;1025;1120	1098	2100;2120;2320	2180
45	1200;1250;1150	1200	2060;1850	1955
75	1240;1150;950	1113	2180;1570;1750	1833
125	1010;1470	1240	1440;1560	1500
270	810;875;775	820	1020;975;825	940

persisted for about two hours during which time it decreased progressively. Bleenham & Fisher (1954) reported that the maximum stimulation of glucose uptake by the perfused rat heart was achieved after 10 minutes exposure to insulin. This effect, in contrast to the effect on HLM cells, disappeared within 10 minutes after removal of insulin.

Influence of pH on the rate of glucose uptake and the effect of insulin

Several experiments were performed to determine whether pH influences the effect of insulin on glucose uptake. It should perhaps be emphasized that the pH difference was introduced only at the equilibration and test stages of the experiment, not during the entire course of cell culture growth.

Results of such an experiment are presented in Table 28. No clear difference in the rate of glucose uptake or the effect of insulin over the pH range 6.8-7.6 could be ascertained.

Table 29 presents results of a similar experiment but with larger sample numbers. The rate of uptake by control and insulin-stimulated cultures at pH 7.8 appeared to be less than that at pH 7.0. The rate of uptake did not vary significantly, however, over the intermediate pH range 7.2-7.6 in which all other experiments were performed.

TABLE 28

EFFECT OF pH ON THE RATE OF GLUCOSE UPTAKE BY HLM CELLS AND ON THE EFFECT OF INSULIN

Four day old cultures were equilibrated at pH 6.8-7.6 as shown. Insulin (1 Unit/ml.) was added where indicated for the last 10 minutes of equilibration. Test media at the appropriate pH and containing ^{3}H glucose at a specific activity of 0.72 mCuries/mMole were used.

pH	Rate of Glucose Uptake (Counts/min./min./ug.DNAP)			
	Insulin not Added.	Mean	Insulin Added.	Mean
6.8	108;93;-	100	175;173;144	164
7.0	76;119;142	112	164;222;208	198
7.4	101;159;112	124	190;211;145	182
7.6	87;130;146	121	177;175;286	213

TABLE 29

EFFECT OF pH ON THE RATE OF GLUCOSE UPTAKE BY HLM CELLS AND ON THE EFFECT OF INSULIN

Three day old cultures were equilibrated at pH 7.0-7.8 as shown. Insulin (1 Unit/ml.) was added where indicated during the last 10 minutes of equilibration. Test media at the appropriate pH and containing ^{3}H glucose at 3.5 mCuries/mMole were used. The number of observations is given in parentheses.

pH	Rate of Glucose Uptake (Counts/min./min./ug.DNAP			
	Insulin Not Added. Mean ± S.D.		Insulin Added. Mean - S.D.	
7.0	429 ± 85	(4)	704 ± 16	(4)
7.2	340 ± 61	(5)	545 ± 130	(5)
7.4	323 ± 50	(5)	525 ± 39	(5)
7.6	426 ± 63	(5)	570 ± 44	(5)
7.8	245 ± 37	(5)	410 ± 65	(5)

Insulin concentration and the stimulation of the rate of glucose uptake

The effect of reducing the insulin concentration used during the usual ten minute exposure period following equilibration is shown in Table 30. The effect of insulin diminished at concentrations below 10^{-2} units per ml. and was not detectable below 5×10^{-4} units per ml. If the exposure period was increased to one hour (no equilibration period allowed) the effect was maximal from 10^{0} to 10^{-3} units per ml. and was detectable at 10^{-4} units per ml. or perhaps even lower (Table 31).

Thus this effect was shown to occur at concentrations of insulin which could be expected in vivo (Randle & Taylor, 1960). The sensitivity was at least as high as that of the perfused rat heart (Fisher & Lindsay, 1956) or the rat diaphragm (Randle & Taylor, 1960).

Influence of temperature on the rate of glucose uptake and the effect of insulin

To determine the effect of temperature on both the rate of glucose uptake and the action of insulin, cell cultures were equilibrated at various temperatures and exposed to insulin for ten minutes. The results, shown in figure 7, indicated a Q_{10} for control cultures of about 1.7. The effect of temperature on glucose uptake by insulin-stimulated cultures was not linear however. Below 30° there was virtually no effect of insulin on the rate of glucose uptake but above this temperature insulin had an increasing stimulatory effect. A Q_{10} $(27\text{-}37^{\circ})$ of about

TABLE 30

EFFECT OF INSULIN CONCENTRATION ON STIMULATION OF THE RATE OF GLUCOSE UPTAKE BY HLM CELLS

Two day old cultures were equilibrated for 30 minutes with insulin added at the concentrations indicated for the last 10 minutes. Test medium contained ^{3}H glucose at a specific activity of 3.5 mCuries/mMole.

Percent stimulation was calculated by subtracting the mean value for intracellular radioactivity of control cultures from that of cultures exposed to insulin, dividing by the former value and multiplying by 100.

Insulin concn. (Units/ml.)	Rate of Glucose Uptake (Counts/min./min./µg.DNAP)				% Stimulation
	Insulin Not Added.	Mean	Insulin Added.	Mean	
10^{-2}	356; 434; 440	410	735; 745	740	80
5×10^{-3}	427; 341	384	580; 450; 478	503	31
10^{-3}	430		540; 595	568	32
5×10^{-4}	392; 402	397	478; 630; 615	574	45
10^{-4}	398; 392	395	378; 410	394	0

TABLE 31

EFFECT OF INSULIN CONCENTRATION ON THE STIMULATION OF THE RATE OF GLUCOSE UPTAKE BY HLM CELLS

Three day old cultures were equilibrated for one hour with or without insulin added at the concentrations indicated. Test medium contained ^{3}H glucose at a specific activity of 3.5 mCuries/mMole.

Percent stimulation was calculated by subtracting the mean value for intracellular radioactivity of control cultures from that of cultures exposed to insulin, dividing by the former value and multiplying by 100. The number of observations is given in parentheses.

Insulin concn. (Units/ml.)	Rate of Glucose Uptake (Counts/min./min./ug.DNAP)		% Stimulation
	Without Insulin. Mean ± S.D.	With Insulin. Mean ± S.D.	
10^{-6}	629 ± 92 (5)	597 ± 91 (5)	-5
10^{-5}	595 ± 97 (5)	673 ± 144 (5)	13
10^{-4}	562 ± 108 (5)	731 ± 209 (5)	30
10^{-3}	606 ± 120 (5)	1114 ± 144 (5)	84
10^{-2}	652 ± 86 (5)	1326 ± 147 (5)	103
10^{0}	595; 575	1125; 1080	88

FIGURE 7

Two day old cultures were equilibrated for 30 minutes at the temperatures indicated with or without insulin (1 unit/ml.) added during the last 10 minutes. Test medium contained ^{3}H glucose at a specific activity of 3.5 mCuries/mMole.

The points plotted are means from results of 3 replicate cultures. The vertical lines represent the total range of results. The open circles represent uptake by control cultures while the filled-in circles represent uptake by insulin-stimulated cultures.

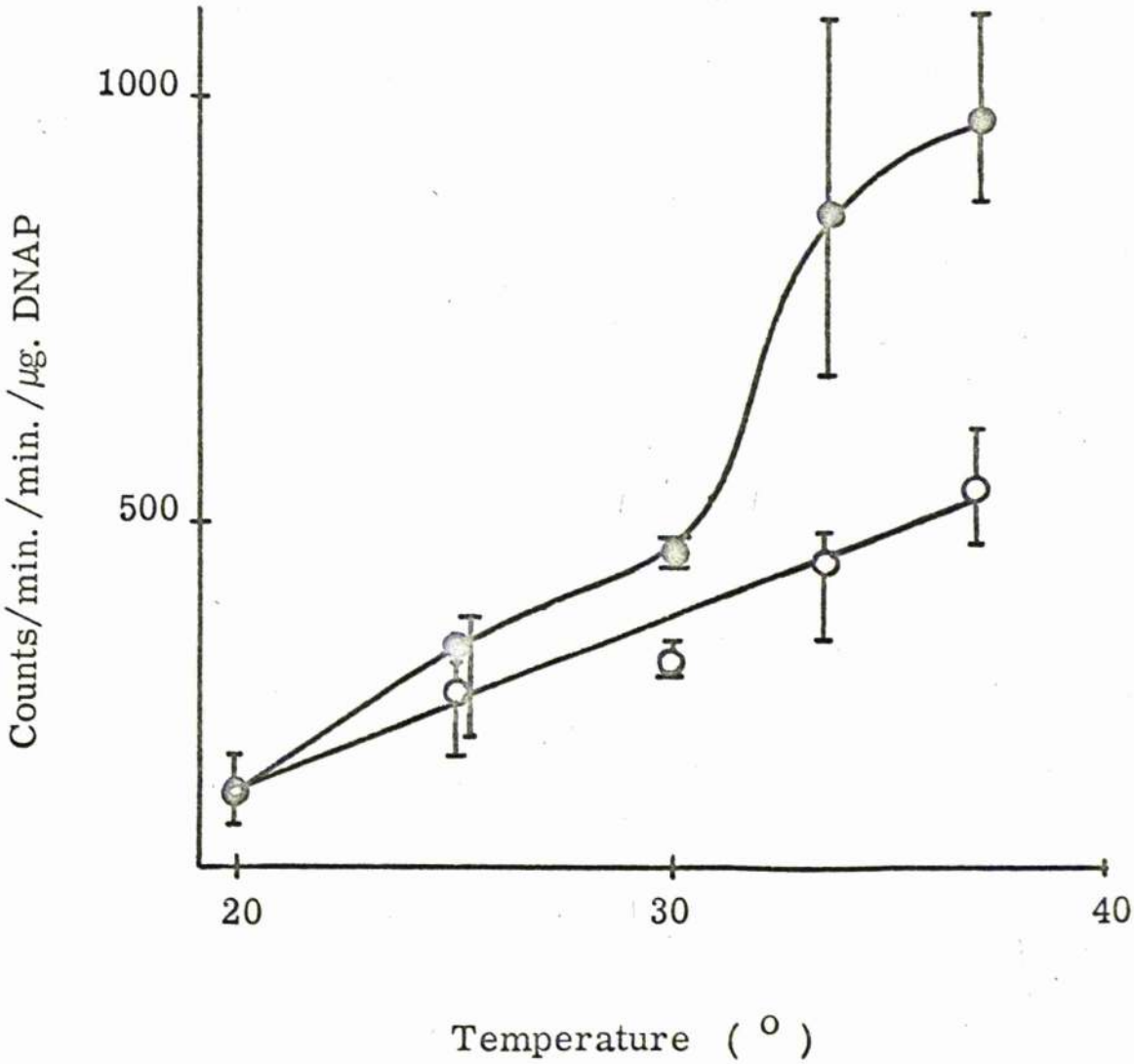

Fig. 7. Influence of temperature on the rate of glucose uptake by HLM cells and on the effect of insulin

2.4 for the rate of uptake by insulin-stimulated cultures can be calculated if the absence of linearity is ignored.

Other workers have reported widely differing Q_{10} values for glucose transport and for the insulin effect depending on procedure and cell type used. Levine & Goldstein (1955) found a Q_{10} of 1.0-1.2 for the insulin effect in the perfused dog preparation. Nahara, Ozand & Cori (1960) reported a $Q_{10\ (19-29^{\circ})}$ of 1.7 for glucose transport in insulin-stimulated frog muscle while Helmreich & Cori (1957) found a $Q_{10\ (27-37^{\circ})}$ of about 2 for pentose accumulation in rat skeletal muscles stimulated with insulin.

Some workers have argued that low Q_{10} values provide evidence against the existence of a sugar transport mechanism (Kipnis & Cori, 1959). Randle (1961) points out, however, that it is doubtful whether the effect of temperature on the transport process per se was measured in these experiments. Since an interrelationship between energy metabolism and glucose transport is probable (Randle & Smith, 1958b) the effect may be a result of influence of temperature on both processes.

Rate of glucose uptake and the effect of insulin at different glucose concentrations

Results of experiments to determine the uptake of glucose and insulin action at different glucose concentrations are presented in Tables 32 and 33. The rate of glucose entry did not increase linearly with increasing glucose concentration. It is also notable that the effect of insulin was diminished at the highest glucose concentrations used.

The means, determined from the results found in the above experiments, are plotted in the reciprocal form of Linweaver and Burk (Figures 8 and 9). Insulin apparently caused a decrease in the ratio Km/Vmax in both experiments. This suggests that the hormone may act to increase the affinity of the sugar carrier for glucose or to increase the availability of sugar carriers themselves.

The effect of insulin on glucose uptake at very low glucose concentrations is shown in Table 34. The specific activity was the same at both the glucose concentrations used.

It is apparent that insulin exerted an equally stimulatory effect at these low glucose concentrations. It is also apparent that the rate of glucose uptake was not linear with increase in concentration.

TABLE 32

EFFECT OF GLUCOSE CONCENTRATION ON THE RATE OF GLUCOSE UPTAKE BY HLM CELLS AND ON THE EFFECT OF INSULIN

Three day old cultures were equilibrated in tris-citrate BSS containing glucose at the concentrations shown. Insulin (1Unit/ml.) was added where indicated during the last 10 minutes of equilibration. Test media contained the same glucose concentrations with ^{3}H glucose at a specific activity of 3.5 mCuries/mMole.

Glucose concn. (mMolar)	Rate of Glucose Uptake (Counts/min./min./µg.DNAP)			
	Insulin Not Added.	Mean	Insulin Added.	Mean
2.8	137; 170; 172	160	252; 262; 244	253
5.6	222; 202; 207	210	438; 415; 390	414
27.8	625; 625; 622	624	1290; 1242; 1130	1221
55.6	1242; 1365; 1390	1332	1450; 1660; 1310	1473

TABLE 33

EFFECT OF GLUCOSE CONCENTRATION ON THE RATE OF GLUCOSE UPTAKE BY HLM CELLS AND ON THE EFFECT OF INSULIN

Three day old cultures were equilibrated in tris-citrate BSS containing glucose at the concentrations shown. Insulin (1 Unit/ml.) was added where indicated during the last 10 minutes of equilibration. Test media contained the same glucose concentrations with ^{3}H glucose at a specific activity of 3.5 mCuries/mMole. The number of observations is given in parentheses.

Glucose conen. (mMolar)	Rate of Glucose Uptake (Counts/min./min./µg.DNAP)	
	Insulin Not Added. Mean ± S.D.	Insulin Added. Mean ± S.D.
1.4	150 ± 8 (6)	302 ± 31 (6)
2.8	276 ± 33 (6)	522 ± 32 (6)
5.6	604 ± 35 (6)	1289 ± 90 (6)
11.1	1449 ± 106 (6)	2938 ± 220 (6)
22.2	2552 ± 119 (6)	4665 ± 234 (6)
44.4	3298 ± 188 (6)	5650 ± 575 (6)
88.8	7484 ± 675 (6)	8727 ± 742 (6)

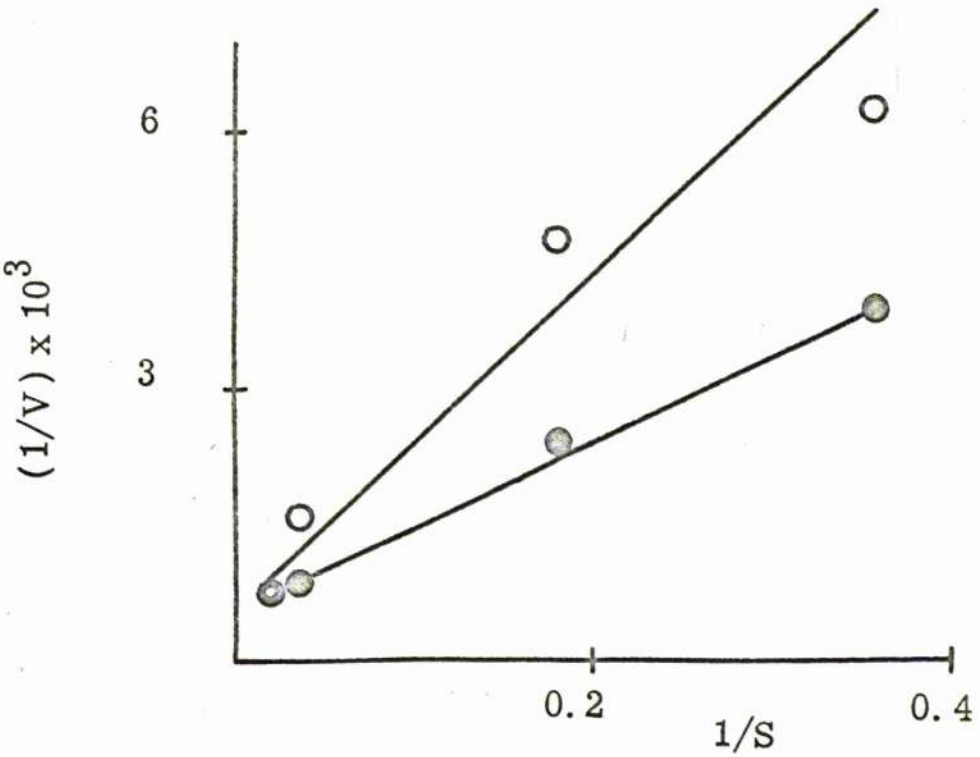

Fig. 8. Uptake of glucose; variation of 1/V with 1/S, where V is the rate of glucose uptake and S is the glucose concentration (mM). Derived from results of Table 32. Insulin not added (O); Insulin added (●).

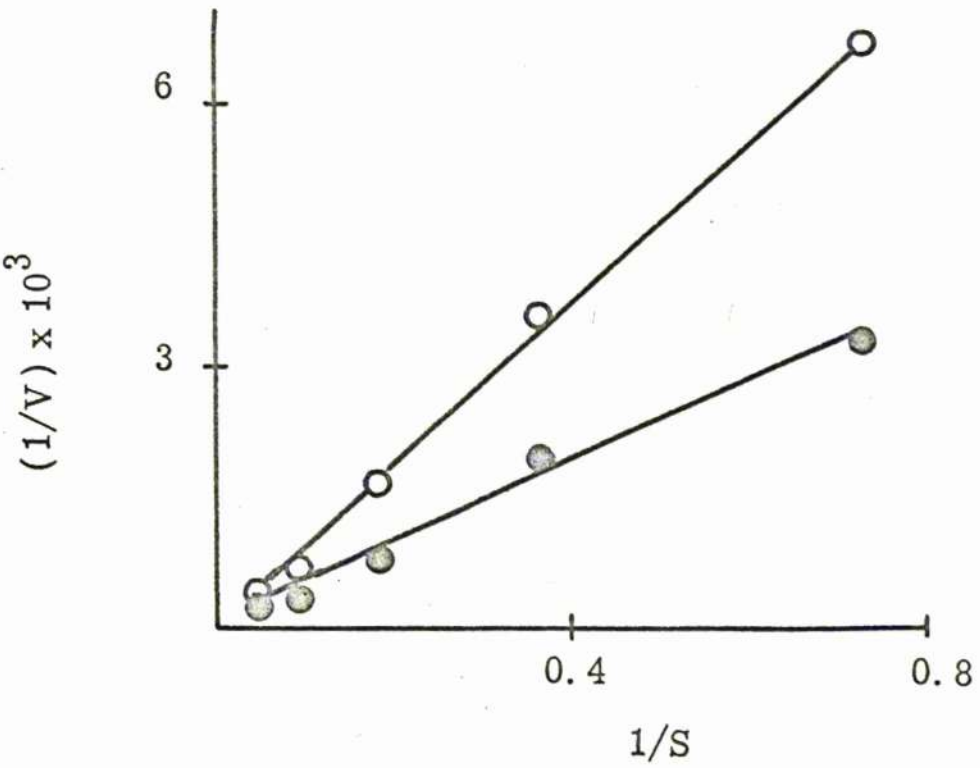

Fig. 9. Uptake of glucose; variation of 1/V with 1/S, where V is the rate of glucose uptake and S is the glucose concentration (mM). Derived from results of Table 33. Insulin not added (O); insulin added (●).

TABLE 34

EFFECT OF INSULIN ON THE RATE OF GLUCOSE UPTAKE AT LOW GLUCOSE CONCENTRATIONS

Three day old HEM cell cultures were equilibrated in tris-citrate BSS containing glucose at the concentrations shown. Insulin (1 Unit/ml.) was added where indicated during the last 10 minutes of equilibration. Test media contained the same glucose concentrations with ^{3}H glucose at a specific activity of 360 mCuries/mMole.

Glucose concn. (mMolar)	Rate of Glucose Uptake (Counts/min./min./µg.DNAP)			
	Insulin Not Added.	Mean	Insulin Added.	Mean
0.028	288; 241; 376	302	661; 654; 543	619
0.28	1859; 1430; 1451	1580	3589; 3186; 2894	3223

Influence of other sugars on the rate of glucose uptake and the effect of insulin

Results of experiments to determine whether or not D galactose and D or L arabinose influence either the rate of entry of glucose into HLM cells or the insulin effect on this, are shown in Table 35. Neither the basal rate of glucose uptake nor the rate of uptake by insulin-stimulated cell cultures was affected. As mentioned previously, the apparent lack of inhibition of glucose entry by other sugars may be due to a masking effect occurring as a result of increased osmotic pressure.

Osmolarity, the rate of glucose uptake and the effect of insulin

In foregoing experiments concentrations of sugars in the equilibrium and test media were altered over a wide range without adjustment of the NaCl concentration to maintain a constant osmotic pressure. Thus, changes in osmotic pressure, if in themselves affecting glucose uptake, could complicate interpretation of experiments of this kind.

The rate of glucose uptake of cells equilibrated in tris-citrate buffer at various osmolarities was determined and the results are given in Figure 10.

The rate of uptake was found to increase with increasing osmolarity to a maximum at around 450 milliosmolar.

TABLE 35

EFFECT OF OTHER SUGARS ON THE RATE OF GLUCOSE UPTAKE BY HLM CELLS AND ON THE EFFECT OF INSULIN

Three day old cultures were equilibrated in tris-citrate BSS containing glucose (5.6 mMolar) and other sugars as shown. Insulin (1 Unit/ml.) was added where indicated during the last 10 minutes of equilibration. Test media contained the same sugar concentrations with ^{3}H glucose at a specific activity of 3.5 mCuries/mMole. The number of observations is given in parentheses.

Exp. No.	Sugar Added (mMolar)	Rate of Glucose Uptake (Counts/min./min./µg.DNAP)	
		Insulin Not Added Mean ± S.D.	Insulin Added. Mean ± S.D.
58	0	549 ± 100 (5)	831 ± 197 (5)
	Galactose 5.5	503 ± 192 (5)	703 ± 122 (5)
	Galactose 55	445 ± 91 (5)	747 ± 211 (5)
65	0	124 ± 20 (5)	257 ± 65 (5)
	Darabinose 5.5	138 ± 10 (5)	344 ± 75 (5)
	Darabinose 55	162 ± 27 (5)	316 ± 81 (5)
	Larabinose 5.5	166 ± 17 (5)	365 ± 68 (5)
	Larabinose 55	167 ± 18 (5)	345 ± 124 (5)

FIGURE 10

Three day old cultures were equilibrated in tris-citrate BSS containing glucose (5.6 mM) but with adjusted osmolarity as indicated. The alteration in osmolarity was accomplished by increasing or decreasing the amount of Hank's BSS stock used to prepare the tris-citrate BSS. All other ingredients were added at the same concentration as usual.

Test media of comparable osmolarity contained ^{3}H glucose at a specific activity of 3.5 mCuries/mMole.

The points plotted are means determined from results of 2-3 replicate cultures. The vertical lines represent the total range of results. The open circles represent uptake by control cultures while the filled-in circles represent uptake by insulin-stimulated cultures.

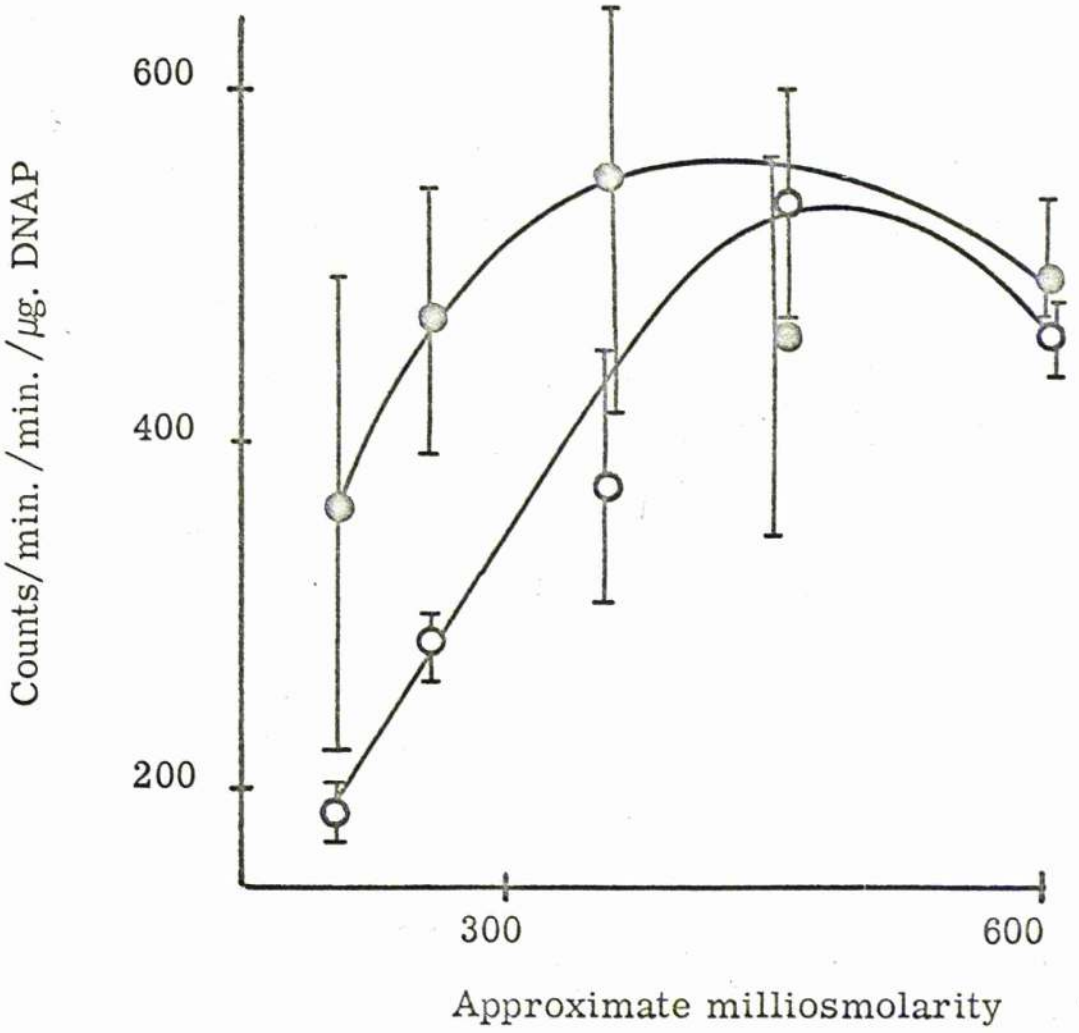

Fig. 10. Influence of osmotic pressure on the rate of glucose uptake by HLM cells and on the effect of insulin

It is also apparent that the effect of insulin was least at the highest osmotic pressures employed.

Vann et al. (1963) found similar results of the effect of osmolarity on glucose utilization by HeLa cells in the presence or absence of insulin. They reported that maximal stimulation by insulin occurred under slightly hypotonic conditions. In contrast, basal glucose utilization was maximal under somewhat hypertonic conditions. In view of the results presented here, it is possible that the effects observed by Vann et al. are secondary to effects of osmolarity on glucose transport.

Oxygen tension, the rate of glucose uptake and the effect of insulin

Since conditions of anoxia are known to accelerate glucose transport in muscle (Morgan, Randle & Regen, 1959; Randle & Smith, 1958b) an experiment was performed to determine whether similar effects on uptake would occur with HLM cells.

No difference in glucose uptake between cultures equilibrated in the presence of N_2 and those which were equilibrated

in the presence of air could be detected. Cultures equilibrated in the presence of O_2, however, showed increased uptake by control cultures to the same level as determined for insulin treated ones (Table 36). It has been found that the absence of oxygen induces marked increases of glycolysis in cell cultures (Paul, 1960, 1961). This could reflect an alteration in glucose uptake or in phosphorylation as has been shown for muscle (Morgan et al. 1959).

Influence of ions on the rate of glucose uptake and the effect of insulin

The effects, on the rate of uptake of glucose, of omitting magnesium or calcium salts or of altering the sodium to potassium ratio in the equilibration and test media are shown in Table 37. Although omission of either divalent cation had no considerable effect, reversing the molar ratio of sodium to potassium caused a marked stimulation in the rate of glucose uptake and a slight decrease in the effect of insulin. Experiments were therefore performed to determine the rate of uptake of glucose at several sodium to potassium ratios (Table 38). The effect of increasing or omitting phosphate was also determined.

The striking observation which arose from these experiments was the apparent disappearance of the insulin effect in the absence of potassium. Omission of this ion induced increases in the rate of uptake of glucose by control cultures to levels observed for insulin treated ones.

Other alterations in the sodium to potassium ratio

TABLE 36

EFFECT OF THE GAS PHASE ON THE RATE OF GLUCOSE UPTAKE BY HLM CELLS

Three day old cultures were equilibrated as usual but the gas phase was varied as shown. Cultures in conical flasks with rubber stoppers were used. The gases were added by inserting a delivery tube into the neck of the flask. Equilibration and test media were pre-equilibrated in the presence of the appropriate gas. Insulin was added where indicated for the last 10 minutes of equilibration. Test medium contained ^{3}H glucose at a specific activity of 7.0 mCuries/mMole.

Gas Phase	Rate of Glucose Uptake (Counts/min./min./µg.DNAP)			
	Control	Mean	Insulin	Mean
N_2	1320; 1165; 1560	1348	2040; 1960; 2210	2070
Air	1540; 1340; 1140	1340	2340; 2250; 2340	2310
O_2	1920; 1960	1940	2020; 1960; 1720	1900

TABLE 37

INFLUENCE OF THE COMPOSITION OF EQUILIBRATION AND TEST MEDIA ON THE RATE OF GLUCOSE UPTAKE AND THE EFFECT OF INSULIN

Three day old HLM cell cultures were equilibrated in Hank's BSS containing glucose (5.6 mMolar) with ionic composition altered as shown. Insulin was added where indicated during the last 10 minutes of equilibration. Test media of appropriate ionic composition and containing ^{3}H glucose at a specific activity of 7.0 mCuries/mMole was used.

Alteration to Hank's BSS	Rate of Glucose Uptake (Counts/min./min./µg.DNAP)				
	Insulin Not Added.	Mean	Insulin Added.		Mean
0	2600; 2115; 2345	2353	3975; 3145; 3200		3440
Mg^{++} Omitted	2290; 2550; 2000	2280	3735; 4220; 3975		3977
Ca^{++} Omitted	3110; 2335; 2700	2715	4420; 4150; 3575		4048
Molar Ratio K^{+}/Na^{+} 2:1	5770; 4570; 6520	5620	6970; 6150; 7420		6847

TABLE 38

INFLUENCE OF THE COMPOSITION OF EQUILIBRATION AND TEST MEDIA ON THE RATE OF GLUCOSE UPTAKE AN D THE EFFECT OF INSULIN

Three day old HLM Cell cultures were equilibrated in tris-citrate BSS containing glucose (5.6 mMolar) and with ionic composition adjusted as shown. Insulin was added where indicated for the last 10 minutes of equilibration. Test media of appropriate ionic composition and containing ^{3}H glucose at a specific activity of 3.5 mCuries/mMole was used. Standard deviations from means of six results are shown.

Molar Ratio	Rate of Glucose Uptake (Counts/min./min./µg.DNAP)	
$Na^{+}:K^{+}$	Insulin Not Added. Mean ± S.D.	Insulin Added. Mean ± S.D.
24:0	885 ± 48 (6)	1028 ± 93 (6)
2:1	428 ± 52 (5)	935 ± 71 (6)
1:2	602 ± 62 (6)	1205 ± 80 (6)
0:24	695 ± 52 (6)	1290 ± 80 (6)
24:0	1198 ± 268 (6)	1147 ± 317 (6)
24:1	415 ± 89 (6)	766 ± 148 (6)
1:2	333 ± 45 (6)	475 ± 79 (6)
0:24	392 ± 90 (6)	597 ± 124 (6)
24:1	280 ± 94 (5)	408 ± 53 (5)
24:1	266; 222	436; 505

No Phosphate

10 x usual Phosphate Conc.

and in the phosphate content of the equilibration and test medium did not appear to have any consistent effect.

Influence of pyruvate and lactate on the rate of glucose uptake and the effect of insulin

The intracellular glucose concentration of the isolated rat heart is markedly increased by addition of pyruvate and to a lesser extent by addition of lactate to the perfusion medium (Newsholme, Randle & Manchester, 1962). Zachariah (1961) found that addition of pyruvate to the perfusion medium led to a decrease in the accumulation of arabinose in the isolated heart.

Table 39 shows results of experiments performed to determine whether addition of pyruvate or lactate to the equilibration and test media would affect the rate of uptake of glucose by HLM cells.

Although very few figures are available, it is apparent that the addition of lactate had no effect on the rate of glucose uptake by control cultures. The addition of pyruvate, however, had a marked stimulatory effect on the rate of glucose uptake by both control and insulin-treated cultures. In addition, in the presence of pyruvate, insulin did not cause any further increase in the rate of glucose entry.

Influence of inhibitors on the rate of glucose uptake and the effect of insulin

Compounds which are known to react with sulfhydryl, amino and other groups likely to be found at the cell surface have been shown to inhibit glucose transport in erythrocytes (Le Fevre, 1961). On the other hand enzyme inhibitors such as fluoride, iodoacetate, azide and cyanide do not inhibit transport

TABLE 39

INFLUENCE OF PYRUVATE AND LACTATE ON THE RATE OF GLUCOSE UPTAKE BY HLM CELLS AND ON THE EFFECT OF INSULIN

Three day old cultures were equilibrated in the presence or absence of pyruvate or lactate as shown. Insulin (1 Unit/ml.) was added where indicated during the last 10 minutes of equilibration. Test medium with or without these compounds and containing ^{3}H glucose at a specific activity of 3.5 mCuries/mMole was used.

Concn. of Added Compound (mMolar)	Rate of Glucose Uptake (Counts/min./min./µg.DNAP)	
	Insulin Not Added	Insulin Added
0	338; 207	573; 526
Sodium Pyruvate (45 mMolar)	848; 858	800; 760
0	415	
Sodium Lactate (45 mMolar)	375; 465	

in these cells.

Anoxia and inhibitors of oxidative phosphorylation, however, stimulate sugar transport in muscle, as does sodium fluoride (Morgan et al. 1959; Randle & Smith 1958 b; Newsholme & Randle, 1961). This has been interpreted as indicating that products of oxidative phosphorylation somehow keep the glucose transport system inactive in muscle.

The effects of these and other inhibitors on the rate of glucose uptake by HLM cells (Tables 40 and 41) were somewhat different than those on muscle and red blood cells.

Mercuric chloride, iodoacetate and fluoride caused an increase in the rate of uptake of glucose by control cultures and masked or abolished the effect of insulin. Iodoacetate induced an increase in the rate of glucose uptake to levels above those shown by cultures treated with insulin.

Addition of azide or salicylate caused a slight increase in the rate of uptake of glucose by cultures equilibrated in the absence of insulin. Addition of dinitrophenol led to a decrease in the rate of glucose uptake by both insulin-treated and control cultures but did not alter the effect of insulin. Addition of phloridzin induced a decrease in the rate of uptake of glucose and abolished the effect of insulin. Cyanide addition had no apparent effect.

Because of the unusually large variation incurred with results of experiment 61 (Table 41) interpretation is made

TABLE 40

THE INFLUENCE OF VARIOUS COMPOUNDS ON THE RATE OF GLUCOSE UPTAKE BY HLM CELLS AND THE EFFECT OF INSULIN

Three day old cultures were equilibrated in the presence or absence of various compounds as shown. Insulin (1 Unit/ml.) was added where indicated for the last 10 minutes of equilibration. Test media containing the appropriate compounds and ^{3}H glucose at a specific activity of 4.3 mCuries/mMole were used.

Concn. of Compound Added (mMolar)	Rate of Glucose Uptake (Counts/min./min./µg.DNAP)			
	Insulin Not Added.	Mean	Insulin Added.	Mean
0	1100; 1300	1200	2325; 2295	2310
$HgCl_2$ 0.1	1900; 3020	2460	2450; 2420	2435
NaN_3 10	1560; 1435	1497	2375; 2300	2337
NaF 10	2335; 2160	2248	2860; 2615	2738
NaCN 1	1105; 1230	1168	2500; 2375	2437
SAL.* 5	1530; 1660	1595	2900; 2220	2560
DNP.* 0.25	825; 940	882	1570; 1555	1562
Phloridzin 3	750; 595	672	725; 625	675

*SAL. - Sodium Salicylate
DNP. - 2, 4 Dinitrophenol.

TABLE 41

THE INFLUENCE OF VARIOUS COMPOUNDS ON THE RATE OF GLUCOSE UPTAKE BY HLM CELLS AND ON THE EFFECT OF INSULIN

The procedure was essentially similar to that of the experiment shown in Table 40. Test media contained compounds as indicated and ^{3}H glucose at a specific activity of 3.5 mCuries/mMole. The number of observations is given in parentheses.

Exp No.	Concn. of Compound* Added (mMolar)	Rate of Glucose Uptake (Counts/min./min./µg.DNAP) Insulin Not Added. Mean ± S.D.	Insulin Added. Mean ± S.D.
60	0	623 ± 136 (5)	1159 ± 223 (5)
	PHL. 3	319 ± 42 (5)	384 ± 75 (5)
	IAC. 1	1974; 1773	1605; 2024; 1713
61	-	497 ± 167 (5)	864 ± 242 (5)
	OUB. 1	514 ± 94 (5)	889 ± 244 (5)
	pCMB 0.005	489 ± 75 (5)	464 ± 77 (5)
	NeM. 0.01	684 ± 100 (5)	1100 ± 360 (5)
	ST. 0.01	527 ± 136 (5)	634 ± 49 (5)
	IAM. 0.025	632 ± 280 (5)	499 ± 203 (5)

* PHL. - Phloridzin. IAC. - Sodium Iodoacetate

OUB. - Ouabain pCMB - p Chloromercuribenzoate

NeM. - N-ethyl Maleimide ST. - Stilbestrol.

IAM. - Iodoacetamide

difficult. It is apparent, however, that addition of p-chloromercuribenzoate abolished the effect of insulin but did not alter the basal rate of glucose uptake. This may also be true for addition of iodoacetamide or stilbestrol. Addition of N-ethylmaleimide did not appear to have any inhibitory effect at the low concentration used. Addition of ouabain also had no apparent effect.

The effect of phloridzin on HLM cells appears to be similar to its effect on other cell types. Phloridzin has been shown to inhibit sugar transport in erythrocytes (Le Fevre, 1959), rat heart (Morgan & Park, 1957; Morgan et al. 1959; Park et al. 1959), Ehrlich ascites tumor cells (Crane et al. 1957), L cells (Maio & Rickenberg, 1962) and others. Keller & Lotspeich (1959) reported that phloridzin inhibited the insulin-activated transport of galactose into skeletal muscle; a finding in accord with that reported here with the HLM cell.

Addition of fluoride or iodoacetate, known inhibitors of glycolysis accelerated the rate of uptake of glucose by control cultures and masked or abolished the effect of insulin. This effect of fluoride on sugar transport has also been observed with rat diaphragm (Newsholme & Randle, 1961). It is notable in the case of HLM cells that the addition of substances known to interfere with oxidative phosphorylation did not accelerate transport as it does in muscle (Morgan et al. 1959; Randle & Smith 1958 b). The effect of 2, 4 dinitrophenol in inhibiting

the rate of uptake of glucose by HLM cells was also reported for galactose uptake by L cells (Maio & Rickenberg, 1962). Azide (5 mMolar) had no effect on galactose uptake by the L cell but the addition of iodoacetate (5 mMolar) inhibited uptake by 14%.

Comparison of the influence of insulin or fluoride on the influx and efflux of glucose

Since the addition of fluoride enhanced the rate of uptake of glucose by HLM cells, the effect of its addition on influx of glucose over a longer period of time and on efflux of glucose was compared with the effect of insulin (Tables 42 and 43).

It is apparent that the addition of fluoride caused an even greater increase in the rate of uptake than did addition of insulin. The efflux of radioactivity was very rapid in both cases.

Results reported earlier (see Figure 4) suggested that the system under study in this type of experiment consisted of two types of labelled components. One of these was readily diffusible while the other did not diffuse out of the cell. If allowance for a two component system was made, the results for control cultures (Table 43) indicated that the efflux of radioactivity occurred as a typical decay reaction (Figure 11).

TABLE 42

EFFECT OF INSULIN AND FLUORIDE ON THE INFLUX OF GLUCOSE INTO HLM CELLS

Three day old cultures were equilibrated in the presence or absence of insulin (1 Unit/ml.) or sodium fluoride (10 mMolar). After equilibration media were removed, corresponding test media containing ^{3}H glucose at a specific activity of 1.4 mCuries/mMole were added. Sample cultures were taken after the times indicated.

Influx Time (Min.)	Intracellular Radioactivity (Counts/min./µg.DNAP)									
	No Addition			Mean	Insulin Added			Mean	Fluoride Added			Mean
1	14;	63;	28	35	175;	203;	162	180	204;	172;	197	191
5	166;	230;	252	216	364;	393;	402	386	422;	508;	433	454
15	498;	392;	390	427	600;	587;	637	608	695;	658;	713	689
30	492;	569;	562	541	713;	748;	764	742	819;	851;	890	853

TABLE 43

EFFECT OF INSULIN AND FLUORIDE ON
EFFLUX OF GLUCOSE FROM HLM CELLS

Preliminary details of this experiment are identical to those described in Table 42 but cultures were exposed to test medium for 30 minutes. After this time sample cultures (Efflux time = 0) were removed for determination of total intracellular radioactivity as usual. Cultures remaining were washed 5 times with 10 ml. of the appropriate equilibration medium at 37° and 5 ml. of this was added. After the time indicated sample cultures were removed, washed in cold tris-citrate BSS and extracted as usual.

Efflux Time (Min.)	Intracellular Radioactivity (Counts/min./µg.DNAP)					
	No Addition	Mean	Insulin Added	Mean	Fluoride Added	Mean
0	289; 272; 350	304	559; 440; 389	463	493; 369; 594	485
5	169; 147; 174	163	177; 152; 175	168	235; 236; 184	218
15	80; 102	91	123; 147; 135	135	111; 127; 90	109
30	90; 64	77	103; 98	100	86; 73	80

FIGURE 11

The points plotted are derived from the means shown in Table 43. Radioactivity of the "non-diffusible" component was taken as being that which remained in the cells after a 35 minute efflux period. This was subtracted from the total radioactivity present at times during efflux to give the radioactivity due to the diffusible component. The solid line indicates efflux of the diffusible component while the dotted line represents results obtained if the non-diffusible component is ignored.

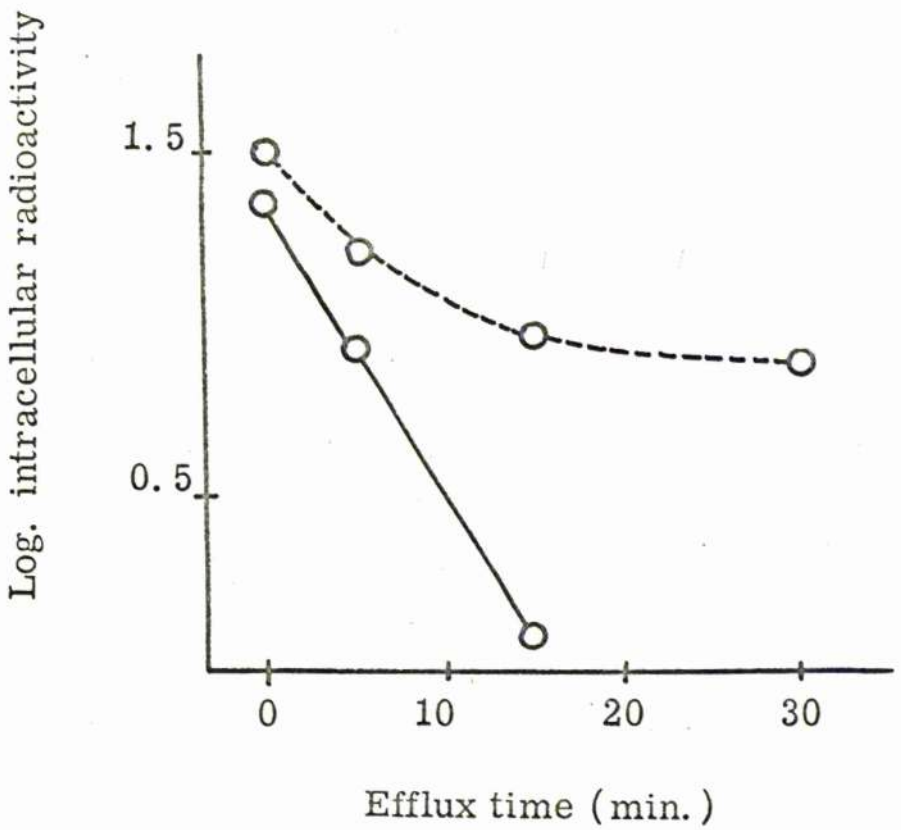

Fig. 11. Efflux of radioactivity from HLM cells. Derived from results of Table 43.

Influence of Prednisolone on Glucose Transport and on the Effect of Insulin

Wilbrandt (1954) stated that corticosteroids inhibit sugar transport in erythrocytes. Henderson et al. (1961 b) reported that administration of hydrocortisone to hypophysectomized rats reduced the sensitivity of the glucose transport system of perfused heart preparations to insulin stimulation.

Nirenberg & Hogg (1958) found that fructolysis but not glucolysis by Gardener Lymphosarcoma ascites tumor cells was inhibited by corticosteroids. It was suggested that this represented an effect on transport of fructose into these cells.

Table 44 presents results of an experiment to determine whether addition of prednisolone during growth of HLM cells affects the rate of glucose transport. It is apparent that the rate of entry of glucose into cells grown in the presence of the steroid was markedly reduced. The insulin stimulation of glucose entry was not affected.

TABLE 44

THE INFLUENCE OF PREDNISOLONE ON THE RATE OF GLUCOSE UPTAKE BY HLM CELLS AND ON THE EFFECT OF INSULIN

Three day old cultures were equilibrated in the presence or absence of prednisolone. This steroid had been added to the one group of cultures 24 hours prior to their use. Insulin was added to cultures where indicated for the last 10 minutes of equilibration. Test media with and without insulin and/or prednisolone and containing ^{3}H glucose at a specific activity of 4.3 mCuries/mMole was used.

Prednisolone concn. (µg./ml.)	Rate of Glucose Uptake (Counts/min./min./µg.DNAP)			
	Insulin Not Added.	Mean	Insulin Added.	Mean
0	1100; 1300	1200	2325; 2295	2310
2.5	687; 717	702	1290; 1410	1350

DISCUSSION

The contention has often been made that the use of organized adult tissue in culture is preferable to the use of cell strains for the study of hormone action (Lasnitzki, 1958). The organ culture technique permits selection of particular target cells or tissues whereas this is not usually possible with cell strains. Negative results obtained through the use of cell strains probably indicate that the particular "trigger mechanism" sensitive to hormonal stimulation is not present in the cell type selected. Also inherent in the above argument is the belief that the particular geometry of organized tissue, including similar and dissimilar cells, is required if a typical hormonal response is to be obtained.

The ability to maintain adult liver explants in characteristic organized form in vitro would seem, therefore, to be a considerable advantage in an investigation of the mechanism of action of hormones which influence the metabolism of this organ.

Trowell (1959) reported that rat liver explants could not be successfully maintained by his technique. He found later (Trowell, 1961) that excellent but inconsistent survival did occur. Hillis & Bang (1959, 1962) reported that they were able to culture parenchymal cells from liver of the adult rhesus monkey and from human embryonic liver for extended periods of time. Ingram (1962), however, was unable to maintain monkey liver cells for periods of more than 2 days, although 10 day

survival was noted on some occasions.

The results of Ingram are somewhat similar to those obtained in this study even though different liver sources were used. Both the inconsistency of survival and the inability to distinguish healthy from unhealthy explants, before analysis, render the technique unsuitable for biochemical studies. Improvements in this culture technique or adaptation of techniques to select healthy explants would overcome these difficulties and permit use of the liver culture method in biochemical studies.

There are probably numerous explanations for the relatively poor survival of liver tissue in vitro. One possibility could be the leakage of metabolites and enzymes from liver cells following liver extirpation and explant cutting. In this connection, Zimmerman (1960) reported that isolated rat liver cells could not convert glucose to lactic acid due to losses of aldolase, glycerophosphate dehydrogenase and lactic dehydrogenase. This "leakiness" might also explain the finding that liver mince aggregates could be maintained only if they were placed directly on the culture grids. Prior washing of this material with balanced salt solution resulted in complete necrosis in these cultures within 24 hours.

Owing to the difficulty in maintaining liver tissue in a satisfactorily healthy state for an investigation of cell-hormone interactions, the study of hormonal influence on cell strains was initiated. At the outset, it was recognized that

there might be difficulty in selecting a strain which would respond to hormonal stimulation. Nevertheless, the results clearly indicate that responses to insulin and prednisolone which are at least qualitatively similar to responses known in vivo, do occur in cultured cells. The hormones had characteristic effects and can be discussed separately.

EFFECTS OF INSULIN

Insulin consistently caused an increase in carbohydrate storage, glycolysis and in apparent glucose transport. Qualitatively, these effects are similar to those observed in muscle and other tissues in vivo and in vitro.

Possible Mechanisms Involved in the Increase in Carbohydrate Storage

Polysaccharide, demonstrated by the periodic acid-Schiff base reaction, was found to be present in HLM cells, especially in certain areas of the monolayer. Wu (1959) isolated glycogen from HeLa cells and Pieck & Kuyper (1961) reported that glycogen is present in KaLe cells. No difference could be detected in the glucose content of aqueous extracts from HLM cells grown in the presence or absence of insulin (see hexokinase assay method). Therefore, the increase in reducing substance in perchloric acid extracts of HLM cells grown in the presence of insulin was probably due, at least in part, to glycogen.

Work done with mammalian tissues can provide clues to the locus at which insulin may act in this cell strain to increase the carbohydrate content.

Gemmil & Hamman (1941) found that 58-92 percent of the "extra" glucose taken up by rat diaphragm following stimulation by insulin could be accounted for as glycogen. Beloff-Chain et al. (1955) similarly reported that almost all the radioactivity of C^{14} glucose taken up by rat diaphragm under the influence of insulin was incorporated into glycogen or oligosaccharides. They postulated that insulin has a directive influence on glucose conversion to glycogen in muscle. In support of this it was shown that increases of glucose uptake induced by increases in the glucose concentration did not stimulate glycogen synthesis as markedly as did similar increases caused by insulin.

Bessman (1960) and Norman et al. (1959) also showed a stimulatory effect of insulin on glycogen synthesis in rat diaphragm preparations. These had been cut to such an extent that no effect of insulin on permeability could be shown.

Larner et al. (1959, 1960) and Villar-Palasi & Larner (1960 a, 1961) studied the effects of insulin on the activities of phosphoglucomutase, uridine diphosphate glucose (UDPG) pyrophosphorylase, glycogen synthetase and phosphorylase in isolated, cut rat diaphragms. The activity of glycogen synthetase was shown to be rate-limiting in glycogen synthesis (Villar-Palasi & Larner, 1960 b). Furthermore the activity of this enzyme when measured in diaphragm homogenates in the absence of added glucose-6-phosphate was found to be higher in preparations from

diaphragms which had been stimulated by insulin (Villar-Palasi & Larner 1960 a). The addition of glucose-6-phosphate, known to activate glycogen synthetase (Leloir, Olavarria, Goldemberg & Carminatti, 1959), caused a fourfold increase in activity. It also abolished the difference between homogenates from insulin-stimulated diaphragms and those from controls.

It had been shown (Larner et al. 1959) that the intracellular concentration of glucose-6-phosphate increased in rat diaphragm preparations stimulated by insulin. It was possible, therefore, that the increase in glycogen synthetase activity in insulin-stimulated diaphragm could have been due to an activation of this enzyme by the increased level of glucose-6-phosphate. It was shown, however, that the enhanced activity of this enzyme extracted from insulin-stimulated diaphragm persisted even after precipitation of the enzyme preparation with ammonium sulphate and dialysis of the extract. Furthermore, it was found that the increase in glycogen synthetase activity occurred even if the diaphragms were incubated in the absence of glucose (Villar-Palasi & Larner 1961). Villar-Palasi & Larner (1960 a) suggested that two species of glycogen synthetase exist in diaphragm muscle, one dependent for activity on the presence of glucose-6-phosphate and the other active in the absence of glucose-6-phosphate. Insulin could act by converting the glucose-6-phosphate dependent species into the glucose-6-phosphate independent enzyme.

More recent studies (Rosell-Perez, Villar-Palasi &

Larner, 1962; Friedman & Larner, 1963) have yielded further proof of the existence of two forms of glycogen synthetase and information concerning the mechanism of interconversion of one form to the other. It was found that the glucose-6-phosphate independent glycogen synthetase was converted to the dependent form by a process, presumably enzymic, requiring ATP and Mg^{2+}. The conversion of the ^{32}P labelled dependent form to the independent form was accompanied by a release of ^{32}P and was therefore presumed to be catalysed by a phosphatase. Further support for the assumption that these interconversion processes were enzymic was also reported. The 600-800 fold purified preparations did not exhibit these changes presumably because the necessary enzymes had been removed in the process.

Friedman & Larner (1963) drew attention to the similarities between the glycogen synthetase interconversion and the phosphorylase interconversion (Sutherland & Rall, 1960). Like the phosphorylase conversion reactions, those of glycogen synthetase appear to involve phosphorylation and dephosphorylation. Unlike that system, however, the conversion of the glycogen synthetase to the less active or inactive form is the ATP - and Mg^{2+} - dependent phosphorylation reaction. In the case of phosphorylase, the kinase reaction leads to the formation of the active enzyme form.

The authors speculated as to the significance of these enzymic interconversions in terms of control mechanisms. They

suggested that both phosphorylase and glycogen synthetase could be regulated either through a common kinase (or phosphatase) or through a common mechanism which could act simultaneously on two separate kinases (or phosphatases). Thus, as one of the enzymes was activated, the other would be inactivated and vice versa. The glycogen synthetase system is thought to be the rate limiting step for glycogen synthesis and the phosphorylase reaction is principally involved in glycogenolysis in vivo. The above scheme would imply, therefore, that activation of glycogenesis would occur concomitantly with inactivation of glycogenolysis and vice versa. In this respect the authors referred to Belocopitow (1961) who reported that cyclic adenylate inhibited glycogen synthetase activity but activated phosphorylase. This result would concur with an hypothesis that this nucleotide could be the activator of a common kinase or two separate kinases concerned with the activation of phosphorylase and "inactivation" of glycogen synthetase.

Steiner, Rauda & Williams, (1961) reported a marked increase in the glycogen synthetase activity of liver of diabetic rats following insulin administration. It is possible, therefore, that a similar phenomenon occurs in liver as has been shown in rat muscle. It is conceivable that such a mechanism of enzymic interconversion could explain the observations (Krahl, 1961; de Duve, 1960; Weinhouse et al. 1963) that insulin leads to a stimulation of glycogen synthesis in the liver under certain

conditions.

Such a mechanism could also account for the increase in glycogen of HLM cells cultured in the presence of insulin.

The possibility that cyclic adenylate is the mediator of this and other actions of insulin on mammalian tissues and on cultured cells should also be emphasized.

No evidence is available to suggest that insulin has a directive influence on glycogen synthesis in HLM cells. Therefore, it is not essential to postulate that this hormone activates an enzyme involved in glycogen synthesis. An increased availability of glucose or glucose-phosphates for synthesis of UDPG might also be expected to increase the cellular glycogen content. Madsen (1961) found that UDPG inhibits phosphorylase of Agrobacterium tumefaciens or of rabbit muscle. If such an inhibition also occurs with phosphorylase of HLM cells, the intracellular concentration of UDPG might regulate the rate of glycogen synthesis and breakdown. A high intracellular content of this compound would be expected to inhibit glycogen breakdown. When the intracellular level decreased, the inhibition of phosphorylase would be released and glycogenolysis could occur.

Either or both of these mechanisms for the regulation of glycogen synthesis may exist in this cell strain. The effect of insulin to increase the cellular carbohydrate content could be explained equally well in each case.

Possible Mechanisms Involved in the Increase in Glycolysis

Insulin-stimulation of glycolysis is well documented for both isolated tissues and cultured cells. In diaphragm and heart muscle this has been attributed not only to an increased rate of transport of glucose into the cell, but also to an increased rate of conversion of intracellular glucose to lactic acid (Newsholme & Randle, 1961).

At least two major enzymic reactions involved in the conversion of glucose to lactate have been implicated as possible loci of insulin action on this process. Either or both of these enzymic steps could be stimulated in cultured cells grown in the presence of insulin and could account for the effects observed.

Cori (1946) reported that insulin stimulated hexokinase activity when added to cell free preparations. These were prepared from muscle of diabetic rats or from the brain of normal rats and a combination of pituitary and adrenal extracts was added. It was postulated that the pituitary or adrenal hormones act to inhibit hexokinase activity and that insulin acts to release this inhibition. Although other investigators also reported occasional observations of response to insulin in cell-free extracts these were found to be very inconsistent (see Krahl, 1961).

Recently evidence has been presented that two enzymes capable of phosphorylating glucose exist in mammalian liver (Walker, 1962, 1963; Vinuela et al. 1963). One of these is a typical animal hexokinase in that it has a comparatively low

K_m for glucose, is strongly inhibited by glucose-6-phosphate or an analogue 1, 5 - anhydroglucitol-6-phosphate and is moderately competitively inhibited by N-acetyl glucosamine. The other glucose-phosphorylating enzyme, however, has a high K_m for glucose, is insensitive to glucose-6-phosphate or the analogue and is highly sensitive to competitive inhibition by N-acetyl glucosamine. An argument is presented indicating that this second enzyme, glucokinase, is adequate as the enzyme catalysing the first step in glycogen synthesis in the liver. This glucokinase activity was found to disappear in fasted animals and in diabetic animals. Further characterization of the glucokinase revealed that insulin or refeeding produced an increase in its activity within a few hours (Salas, Vinuela & Sols 1963). Furthermore, its reappearance was inhibited by ethionine or p-fluorophenylalanine and this effect was largely reversed by methionine and phenylalanine respectively. This suggests that the formation of new enzyme protein was involved. Actinomycin D also inhibited glucokinase formation following insulin administration suggesting that the formation of messenger RNA may be involved. Since no evidence of accumulation of an activator or an inhibitor of this enzyme could be found in liver, the authors concluded that insulin stimulates de novo synthesis of glucokinase by a direct or an indirect means.

The findings of Vester (1963) and of Vester & Reino (1963) however cast some doubt as to whether this effect is

an enzyme activation or one of enzyme formation. These workers reported a direct stimulatory effect of insulin on glucokinase activity in particle free extracts of liver from normal but not from alloxan diabetic rats. Insulin concentrations used varied from thirty to three hundred milliunits per ml. with increasing stimulation of glucokinase activity. The effect was apparently specific for insulin since neither bovine serum nor inactivated insulin produced any stimulation.

No consistent increases in hexokinase activity could be found in HEM cells grown in the presence of insulin. This does not, however, preclude the possibility that insulin acts to induce increases in glucokinase which may also be present in these cells. Such an induction, whether by enzyme activation or by increased enzyme synthesis, could possibly account for the increased glycolytic rate.

Another possible locus of action of insulin to increase glycolysis in these cells may be the conversion of fructose-6-phosphate to the diphosphate. Newsholme & Randle (1961) presented indirect evidence indicating that insulin may have a stimulatory effect on the conversion of fructose-6-phosphate to fructose-1, 6-diphosphate in muscle. They concluded that this conversion may limit the rate of glycolysis under aerobic conditions. Since fructose-1, 6-diphosphatase activity is very low in muscle and is not influenced by insulin (Newsholme & Randle, 1962), the enzyme involved must be phosphofructokinase.

It is noteworthy that anoxia, which accelerates monosaccharide transport and glucose phosphorylation in rat heart (Morgan, Henderson, Regen & Park 1961) also accelerates conversion of fructose-6-phosphate to fructose-1, 6-diphosphate (Newsholme & Randle 1961). Newsholme & Randle (1961) suggested that the acceleration of glucose phosphorylation is secondary to the acceleration of the phosphorylation of fructose-6-phosphate. This was supported by the argument that acceleration of fructose-6-phosphate phosphorylation would lower the intracellular glucose-6-phosphate concentration. Since the latter compound competitively inhibits hexokinase, a decrease in its concentration would be expected to increase hexokinase activity.

The mechanism by which insulin could stimulate phosphofructokinase is not known. It is interesting to note, however, that phosphofructokinase from rat skeletal muscle is activated by cyclic adenylate (Passonneau & Lowry, 1962 a, b). Similarly, Mansour, Le Rouge & Mansour (1961) have found that phosphofructokinase of the liver fluke Fasciola hepatica, can be activated in soluble fractions of homogenates by cyclic adenylate, ATP and Mg^{2+}. It is conceivable that this could represent another possible locus of insulin action, mediated by cyclic adenylate, although there is no direct supportive evidence for such an hypothesis at present.

Glucose Transport in HLM Cells and Hypotheses Concerning the Effect of Insulin

The demonstration of sturation kinetics, competitive inhibition and stereospecificity in the entrance of sugars into various cell types has been taken as good evidence for the participation of specific transport sites in this process (Le Fevre, 1961; Wilbrandt, 1961). Transport of one sugar from intracellular to extracellular space, against a concentration gradient, during inward movement of a second sugar has been shown with the rabbit erythrocyte (Park et al. 1956) and the perfused heart (Park et al. 1959). This has been suggested as conclusive evidence against simple diffusion and in favor of a mobile carrier-mediated diffusion process.

In HLM cell cultures it was possible to demonstrate saturation of the glucose transport mechanism especially if the effects of increasing osmotic pressure were taken into consideration.

Inhibition of glucose entry by galactose can probably be inferred by suitable correction for the influence of osmotic pressure change. The addition of 55 mMolar galactose would be expected to increase the rate of glucose entry due to increased osmotic pressure by about 17% (Figure 10). Thus the marginal inhibition of glucose uptake shown in Table 35 becomes an inhibition of 30% by correction for osmotic change. It will be necessary of course to repeat these experiments under conditions of constant osmotic pressure in order to answer this

question conclusively.

Maio & Rickenberg (1962) reported that the affinity of sugar "carriers" in L cells for galactose is slightly greater than that for glucose (Km values of 5×10^{-4} Molar and 1×10^{-3} Molar respectively). In HLM cells, however, the affinity of the carriers for glucose would have to be considerably higher than that for galactose. This can be inferred from the finding that galactose inhibited glucose entry by only 30% when the molar ratio of galactose to glucose was about 10 : 1. Nirenberg & Hogg (1958) presented evidence suggesting that the affinity of sugar carriers of the Gardener Lymphosarcoma ascites tumor cell for glucose is much higher than that for galactose or fructose. Similarly, differences in the affinity of transport sites for various sugars exist among tissues of different mammals and even among tissues of the same species.

The above considerations strongly indicate that transport of glucose into HLM cells is brought about by a process similar to that shown for other cell types. The possibility remains, however, that some of the intracellular radioactivity may have resided in intermediates of glucose metabolism, even in the cells which had been exposed to the labelled glucose for only a short time. In this connection, Maio & Rickenberg (1962) found that radioactive galactose-1-phosphate and uridine diphosphate galactose left L cells very rapidly and that all of this was released into the medium as free galactose. It

is therefore not possible to conclude that the rapid loss of radioactivity from HLM cells, shown in the efflux experiments, could not have been due to a similar loss of radioactivity from glucose intermediates.

It is also possible that labelled glucose could have been metabolized to pyruvate during the one minute exposure and could have escaped from the cell in that form. However, in the presence of fluoride, which inhibits pyruvate formation, accumulated radioactivity left almost as rapidly as did that of control cultures.

This uncertainty as to the nature of the metabolites which become labelled during the one minute exposure to radioactive glucose renders interpretation of the effects of insulin and inhibitors more difficult. Because of this uncertainty it is not possible to distinguish clearly between glucose transport and glucose metabolism. Nevertheless, the considerable quantities of glucose found in HLM cells would be expected to dilute extensively the radioactive glucose which would enter in one minute. This would presumably decrease the amount of label which would be converted to glucose derivatives during that short exposure time.

The pronounced stimulatory effect of insulin on this process can therefore be interpreted in at least two ways. First, it could represent an increase in transport of glucose across the cell membrane probably by a mechanism similar to

that postulated for muscle (Randle & Young, 1962) and other insulin sensitive tissues. A second but perhaps less probable possibility is that it represents an increase in the rate of transformation of labelled intracellular glucose to glucose derivatives.

In view of the well established action of insulin to increase the glycolytic rate, this latter interpretation deserves some attention. The mechanism by which the rate of uptake of labelled glucose is stimulated could, according to this, be identical with any of those proposed for the stimulation of glycolysis. It should be emphasized, however, that an hypothesis to explain this action of insulin on the basis of the induction of a glucokinase type of enzyme would not be consistent with the evidence presented. It was shown that insulin stimulated the uptake of labelled glucose over a total range of external concentrations from 0.028 mM. to 88.8 mM. The lowest of these is below the Km reported for animal hexokinase while the highest is well above the Km reported for liver glucokinase. These findings do not support the hypothesis that the stimulation of the rate of glucose uptake or of glycolysis is due to increases in glucose phosphorylating enzyme.

No evidence to support or refute the theory that an increase in phosphofructokinase could account for the insulin stimulation of glucose uptake in HLM cells is available.

Fluoride and iodoacetate markedly increased the rate

of glucose uptake in HLM cells as did pyruvate which is also known to inhibit glycolysis, probably by indirect inhibition of phosphofructokinase (Newsholme et al. 1962). These findings render the second interpretation even more unlikely.

The assumption that the process under study was glucose transport can be more readily reconciled with the results observed here and by other workers using other, insulin sensitive, cell types. Randle & Smith (1957, 1960) suggested that the glucose carrier mechanism is normally kept in an inhibited state in muscle. This is presumed to involve an ATP-dependent phosphorylation reaction. Conditions which decrease the supply of ATP decrease the rate of carrier inactivation and hence increase the transport rate. Insulin is thought to act by inhibiting the carrier phosphorylation reaction, which is dependent on ATP, or by accelerating its dephosphorylation. Such an hypothesis could also explain the insulin effect on glucose transport in HLM cells. To adapt this hypothesis, however, a distinct difference with regard to ATP availability must be postulated to exist between muscle and HLM cell types.

Sugar transport in muscle is accelerated by fluoride, 2, 4 dinitrophenol, salicylate, cyanide and anoxia (Randle & Smith, 1958 b; Morgan et al. 1959; Newsholme & Randle, 1961). This has been attributed to the action of these compounds to limit the availability of ATP for inactivation of the membrane transport system. In HLM cells, however, only fluoride and

other substances which could be expected to inhibit glycolysis had a stimulatory effect on glucose transport. Inhibitors of respiration or respiratory chain phosphorylation, such as cyanide, 2, 4 dinitrophenol and salicylate, did not have this effect.

It is possible that this reflects a fundamental difference in the importance of oxidative phosphorylation as an energy supplying mechanism for glucose transport inactivation in these cells. Leslie & Sinclair (1959) pointed out that a major part of glucose taken up by this and other cell strains is accounted for as organic acid released into the medium. It is possible that glycolysis serves a major role, while the citric acid cycle plays a minor role, in the provision of energy in this and other cell strains. In muscle, however, the reverse would be expected. Such a difference between these cell types could explain the difference in the relative sensitivities of their glucose transport systems to the two groups of inhibitors.

The hypothesis of Randle & Smith (1960) that substances limiting ATP production thereby decrease the rate of inactivation of the glucose carrier has also been taken up by Levine (1961) and by Tepperman & Tepperman (1960). It must be emphasized, however, that other interpretations are possible. Increases in the availability of adenosine monophosphate (AMP) or of cyclic adenylate might activate the glucose transport system (Haynes et al. 1960). Both of these nucleotides have been shown to activate phosphofructokinases (Passonneau & Lowry, 1962 a, b;

Mansour et al. 1962). Therefore an increase in either nucleotide might explain both increased transport and increased glycolytic rate. Wu (1959) found that the content of AMP in HeLa cells was markedly increased by fluoride although the total concentration of AMP, adenosine diphosphate and ATP was decreased. Such an action in HLM cells might account for the stimulation of glucose transport by fluoride.

Certain other interpretations of cell permeability to sugars and other substances and of the influence of insulin on this deserve mention.

Mitchell (1961, 1963) developed a concept that an anisotropic arrangement of diffusion catalysts and/or enzymes in or around the cell membrane could bring about "translocation" of organic molecules, groups or ions. For "downhill" transport of sugars the diffusion catalyst could be capable of translational or rotational movement in the membrane. Model systems were presented to explain transport against a concentration gradient based on "group translocation" of substrates during reaction with such anisotropically arranged enzymes bound to the cell membrane. Insulin or lack of high energy phosphates might be envisaged to alter the orientation of the diffusion catalyst and hence the rate of translocation as well.

It seems improbable that the transport of glucose in HLM cells occurs against a gradient. Such "active" transport is known to occur in cells of the small intestine and proximal

kidney tubule (Hokin & Hokin, 1963; Wilbrandt, 1963). The process requires energy and is not accelerated by insulin. Translocation of glucose into HIM cells however was accelerated by insulin. Furthermore, it is not apparently dependent on a supply of energy since inhibitors of oxidative metabolism had little effect and inhibitors of glycolysis accelerated rather than diminished translocation rate.

Although insulin is known to induce an increase in potassium uptake by muscle and liver it is not known whether a similar phenomenon exists with cultured cells. Manchester (1961) suggested that the action of insulin to hyperpolarize the cell membrane and induce K^+ movement might represent a primary action since amino acid transport, also stimulated by insulin, and potassium movement are thought to be connected. If some relationship between hyperpolarization of the membrane and sugar uptake exists, it might explain the effect of reversal of the molar ratio of Na^+/K^+ on this process in HIM cells. This would, however, have to apply only to a certain degree of polarization since only one particular ratio of Na^+/K^+ appeared to have an effect.

Bhattacharya (1959 a, b, 1961) studied the effects of ions on glucose utilization by rat hemidiaphragms. He reported that the basal uptake of glucose was not greatly influenced by the presence or absence of ions but that the maximal response to insulin was dependent on the presence of both Mg^{2+} and an

alkali metal ion such as Na^+. Although no such dependence on Mg^{2+} was noted in HLM cells, it must be emphasized that Bhattachrya measured glucose disappearance and did not distinguish between glucose transport and glucose metabolism.

Bhattachrya also found that the basal rate of glucose uptake and accumulation of xylose was highest with Lithium and decreased as follows - $Li^+ > Na^+ > K^+ = Rb^+ > Ce^+$. These results, with respect to Na^+ and K^+, are similar at least qualitatively with those reported for HLM cells (Table 38).

No insulin effect on the rate of glucose entry into HLM cells could be observed in the absence of K^+. Under these conditions the uptake of glucose by control cultures was increased to that of insulin stimulated ones. This finding emphasizes the probability of a relationship among glucose transport, presence of ions and insulin action.

Troshin (1961) reiterated the hypothesis that cell permeability and its functional changes are determined by properties of the protoplasm itself rather than by the membrane. This "sorption theory" of permeability regards the intracellular water as existing in an organized or bound form. Therefore the solubility of substances in it is lower than in free water. The accumulation of some substances to higher concentrations intracellularly as compared to the extracellular concentration is thought to indicate that these exist in a bound state in the protoplasm.

In the case of sugar transport, proponents of this theory could argue that insulin or lack of ATP alter the sorption properties of the cytoplasm. This alteration would presumably lead to an increased rate of sugar entry.

EFFECTS OF PREDNISOLONE

Possible Events Leading to Decreased Carbohydrate Storage

The decrease in carbohydrate stored in HLM cells grown in the presence of prednisolone is probably a secondary result of effects of this steroid in inhibiting the rate of glucose phosphorylation and/or transport. These effects could lead to a decrease in the intracellular glucose-6-phosphate availability. Since glycogen synthetase is activated by glucose-6-phosphate (Leloir et al. 1959), a lower intracellular availability of this would presumably decrease the rate of glycogen synthesis. A decrease in intracellular glucose-6-phosphate might also lead to a drop in UDPG and hence both increase the phosphorylase activity (Madsen, 1961) and decrease the rate of glycogen synthesis.

Eisenstein (1962) reported that cortisol administration to rats led to an activation of liver phosphorylase. Although no results are available to suggest a similar action of prednisolone on HLM cell phosphorylase, such a mechanism would be consistent with the decrease of intracellular carbohydrate observed. It is also possible that the decrease in glucose utilization, observed in prednisolone treated HLM cell cultures, would reduce the availability of high energy phosphate compounds

required for glycogen synthesis.

Possible Interrelationships Involved in the Inhibition of Glycolysis and Increase in Keto Acids

The decrease in glucose utilization by prednisolone-treated cell cultures may be an indirect result of the increase in keto acids present in the medium. Newsholme et al. (1962), using the perfused rat heart preparation, have shown that pyruvate decreases glucose utilization probably by inhibiting phosphofructokinase. The mechanism of this inhibitory effect is not known but Newsholme et al. suggested that it may be due to increased oxidation of this compound.

The mechanism by which pyruvate could be increased in cell cultures treated with prednisolone is also not readily apparent. It should be noted, however, that the blood pyruvate level is increased in patients treated with corticosteroids and the pyruvate tolerance is impaired (Hermes, Wajchenberg, Fajans & Conn, 1957). In this respect, Weil, Altszuler, & Kessler (1961) suggested that enhanced oxidation of fatty acids may impair pyruvate oxidation by competition for coenzyme A. If prednisolone were to increase fatty acid oxidation, therefore, both the decrease in glucose utilization and the increase in the keto acid content of the medium might be explainable. It could also be suggested that the induction by prednisolone of esterase activity might account for an increased availability of fatty acids for oxidation.

Weber et al. (1961, 1962) reported that administration of cortisone to adrenalectomized rats in-

duced increases in enzymes present in liver such as lactic dehydrogenase and glucose-6-phosphatase. No increase in these enzymes was found in HLM cells grown in the presence of prednisolone. This suggests either that the in vivo response is indirect or that HLM cells do not respond to these steroids as do liver cells.

Hypotheses Concerning the Inhibition of Glucose Transport

No evidence is avilable to indicate whether or not direct addition of corticosteroids to the equilibration and test medium inhibits glucose transport in HLM cells. The decreased rate of glucose transport in HLM cells grown in the presence of prednisolone may be due to an action on the glucose transport system following direct addition or to an effect induced during growth. Wilbrandt (1954) claimed that corticosteroids decrease glucose transport in erythrocytes following direct addition. Maio & Rickenberg (1962) reported that stilbestrol competitively inhibits the uptake of galactose by strain L cells. They suggested, therefore, that this compound acts by reversibly combining with the sugar carrier to inhibit formation of the galactose-carrier complex. Le Fevre (1961) suggested, however, that this and other steroid-like compounds combine with the cell surface at a point adjacent to the sugar transport sites and thereby interfere with sugar absorption.

Either of these hypotheses might account for the inhibitory action of prednisolone on glucose transport in HLM cells.

It has thus been established that hormones do act on certain cells in culture and that some of the effects produced bear a clear resemblence to those which occur in isolated tissues. The action of insulin on HLM cells in increasing carbohydrate storage, glucose transport and glycolysis is explainable on the basis of presently existing hypotheses for its action on other cell systems. The primary mechanism by which these responses are elicited, either in HLM cells or in tissues taken from animals, remains to be determined.

The action of prednisolone on HLM cells in decreasing glucose transport and glucose utilization may be primary to other effects; namely, decrease in cell multiplication and carbohydrate storage. In this case, also, the precise mechanism by which the responses are elicited has yet to be clearly established.

The criteria suggested by Hechter (1955) for evaluating the potential physiological significance of hormone action in vitro are closely met in this system, especially with respect to insulin. It has been demonstrated that hormones act on this system to produce effects which are similar in essential details to effects which occur in vivo. An action of insulin has been shown which can occur at concentrations to be anticipated in vivo. Furthermore, the time course of events with regard to development and recession of the hormonal response, in this system is probably similar to that of the response in vivo. The cell culture method and in particular the cell strain HLM

would therefore appear to be excellent tools for a frontal attack on the problem of the primary actions of these hormones at the cellular level.

SUMMARY

1. Attempts were made to maintain adult mammalian liver explants or cell aggregates in vitro by modifications of the Trowell technique.

2. Tissue preparations survived for periods up to 6 days but results were not consistent enough to permit biochemical study of hormone action. Advantages of the use of the organ culture method in studies of hormone action are mentioned and a possible reason for the difficulty in maintaining liver in vitro is suggested.

3. Owing to the numerous advantages in the use of cell strains for metabolic studies and investigations into the mechanisms of hormone action, these were employed for subsequent experiments. Investigations into the effects of adding of insulin or prednisolone to the culture medium were undertaken.

4. Addition of insulin was found to induce an increase in the carbohydrate content of cell strains isolated from foetal human (HIM) and foetal calf (KaLe) liver. No marked effect of this hormone on the cellular content of DNA, RNA, protein or lipid was observed but a slight increase in cellular RNA and protein was noted with the KaLe strain. No stimulatory effect of insulin on cell multiplication could be detected.

5. Possible mechanisms for the insulin-induced increase in cellular carbohydrate are discussed. These have been evolved by other workers to explain the effect of insulin on carbohydrate storage in muscle.

6. Addition of prednisolone was found to promote a decrease in the cellular carbohydrate content. It is suggested that this may be a secondary event to other effects observed. The presence of this steroid also led to a marked decrease in cell multiplication with all cell strains studied. With HLM cells, prednisolone induced an increase in the cellular DNA content. This steroid also led to an increase in the lipid content of a variant of a mouse fibroblastic cell strain.

7. The effects of addition of insulin or prednisolone on glucose utilization by cells in culture and on the organic acid content of the growth medium were also determined.

8. Insulin induced a marked increase in glycolysis in cultures of HLM cells. No change in the keto acid concentration of the medium was noted. Possible mechanisms involved in the insulin-induced increase in glycolysis are suggested. These are based on experimental evidence obtained by other workers explaining the effects of insulin on isolated muscle and liver. Insulin may act by increasing the activity of an enzyme catalysing the phosphorylation of glucose and/or by increasing the rate of conversion of fructose-6-phosphate to fructose-1,6-diphosphate.

9. Addition of prednisolone led to an inhibition of glucose utilization with all cell strains studied. The growth medium of prednisolone-containing cultures contained a higher keto acid content than that of controls when expressed on the basis of cell number present. It is suggested that

this latter effect of prednisolone might be primary to the former, since pyruvate is known to inhibit glycolysis in isolated muscle.

10. The effects of addition of insulin or prednisolone on enzyme levels in strains of cultured cells was also investigated.

11. Addition of insulin did not alter the levels of any enzyme consistently but increases in hexokinase activity were found on 2 out of 5 occasions. The induction of hexokinase or glucokinase by insulin could explain the increase in glycolysis observed with cells grown in the presence of this hormone.

12. Addition of prednisolone caused a consistent increase in the esterase levels in HLM cells. The levels of other enzymes such as glucose-6-phosphatase, glucose-6-phosphate dehydrogenase, lactic dehydrogenase and hexokinase were not consistently affected.

13. A technique for the study of glucose transport into HLM cells was developed. This involved the measurement of the uptake of labelled glucose during a one minute exposure period. Experiments to determine the influx of labelled glucose and efflux of radioactivity over longer periods were also performed.

14. It was found that the level of intracellular radioactivity approached a plateau after 20-30 minutes of exposure to labelled glucose. The efflux of radioactivity, following a 30 minute influx period, was more rapid than influx. Evidence is presented suggesting that two components can be distinguished.

during measurement of efflux of radioactivity. One of these was readily diffusible but the other remained within the cell even after a 30 minute efflux period.

15. The rate of transport of glucose was found to obey Michaelis-Menten kinetics and it is emphasized that this finding argues against an hypothesis that glucose enters the cell by simple diffusion or pinocytosis.

16. The influence of physical factors on the rate of glucose transport was investigated. No marked effect of pH was noted over the range pH 6.8-7.8. The rate of glucose transport increased linearly with temperature from 20° to 37°. An approximate Q_{10} (27°-37°) of 1.7 was calculated.

17. No inhibition of glucose transport by D galactose, D or L arabinose or D ribose was detected. It is suggested that an inhibitory effect of these sugars may have been masked by an increase in osmotic pressure on addition of the test sugar.

18. The rate of transport of glucose into cells grown in the absence of serum was not altered as compared to that of cells grown in medium containing serum.

19. The rate of transport of glucose into cells in different stages of growth was relatively constant.

20. Exposure of cells to insulin for periods as little as 10 minutes brought about a 2-fold increase in the rate of entry of glucose. This effect persisted for about 2 hours after removal of insulin, during which time it decreased progressively.

21. No clear effect of pH on the action of insulin was noted over the range pH 6.8-7.6. Temperature markedly influenced the rate of glucose transport into insulin-stimulated cells. Below 30° the effect was absent but above this temperature insulin had an increasing stimulatory effect. A Q_{10} (27°-37°) of about 2.4 was calculated.

22. The maximal effect of insulin was found to occur at a concentration as low as 10^{-3} units/ml. A less pronounced stimulatory effect occurred at 10^{-4} units/ml.

23. The rate of glucose transport into insulin-treated cells was found to obey Michaelis-Menten kinetics. Insulin apparently caused a decrease in the ratio K_m/V_{max} suggesting that it acts to increase the affinity of the glucose carrier for glucose or to increase the availability of the carriers themselves. Insulin was found to approximately double the rate of entry of glucose over a range of external glucose concentrations from 0.028 mM - 44.4 mM.

24. D galactose or D or L arabinose could not be shown to exert any inhibitory effect on the rate of glucose transport into insulin-stimulated cells.

25. The rate of entry of glucose was increased in solutions of increased osmolarity in both control and insulin-treated cell cultures. It is suggested that this effect might explain the lack of competitive inhibition of glucose entry on addition of D galactose and other sugars.

26. Incubation of cell cultures for 30 minutes under reduced oxygen tension had no apparent effect on the rate of glucose transport or on the effect of insulin. Incubation at high oxygen tension, however, led to an increase in the rate of transport into control cells and masked or abolished the effect of insulin.

27. Omission of Ca^{2+} or Mg^{2+} did not affect the rate of glucose transport nor the effect of insulin. Omission of K^{+}, however, led to an increase in the rate of transport of glucose into control cell cultures to levels observed for insulin-treated ones. Reversing the molar ratio of Na^{+} to K^{+} led to a marked increase in the rate of glucose transport and a decrease in the effect of insulin. No change in the rate of transport of glucose was noted on omission of phosphate or on increasing the phosphate concentration by a factor of 10.

28. Addition of pyruvate led to a stimulation in the rate of glucose transport in both control and insulin-treated cultures and the effect of insulin was masked or abolished. Addition of lactate had no apparent effect on the rate of glucose uptake by control cultures.

29. Substances which inhibit glycolysis, such as fluoride and iodoacetate, induced an increase in the rate of glucose transport and masked or abolished the effect of insulin. It is suggested that this concurs with current concepts involving regulation of glucose transport in other insulin-sensitive cell types. Similarities and differences between this insulin-sensitive cell type and others are discussed.

30. Other inhibitors such as p-chloromercuribenzoate and iodoacetamide did not alter the basal rate of glucose transport but abolished the effect of insulin. Phloridzin abolished the effect of insulin and inhibited the basal rate of transport of glucose. Other inhibitors had slight effects and ouabain and cyanide had no apparent effect.

31. The rate of transport of glucose into cells grown in the presence of prednisolone was found to be decreased. It is suggested that this might explain the decrease in glucose utilization, carbohydrate storage and cell multiplication observed in prednisolone treated cultures. Insulin had an equally stimulatory effect on cells grown in the presence of prednisolone as on cells grown in its absence.

APPENDIX

HANKS' BALANCED SALT SOLUTION
(Hanks & Wallace, 1949)

	Concn. g./litre
NaCl	8.00
KCl	0.40
$CaCl_2$	0.14
$MgSO_4 \cdot 7H_2O$	0.10
$MgCl_2 \cdot 6H_2O$	0.10
$Na_2HPO_4 \cdot 2H_2O$	0.06
KH_2PO_4	0.06
Phenol red	0.02

The ingredients were made up in a stock at 10 times the above concentration in glass distilled water. The $CaCl_2$ was dissolved in 100 ml. water and added to a solution (approximately 800 ml.) containing the other components. The volume was then made to 1 litre, and the solution was sterilized by autoclaving at 15 lbs. pressure for 15-30 minutes.

TRIS-CITRATE BUFFERED BSS

Stock Solutions	ml./litre
Tris (0.2M)	80
Citric acid (0.1M)	50
$MgCl_2$ (1.23M)	10
Hanks' BSS (Stock)	85

The above solutions were combined and made to 1 litre with glass distilled water. The solution was sterilized by autoclaving at 15 lbs. pressure for 15-30 minutes.

TRYPSIN SOLUTION

	concn. g./litre
Trypsin	2.5
Sodium citrate	3.0
NaCl	6.0
Phenol red	0.02

The above ingredients were dissolved in 1 litre of glass distilled water. The pH was brought to 7.8 and the solution was sterilized by seitz filtration. Trypsin 1:250 (Difco) was obtained through W. B. Nicolson, Ltd., Glasgow.

WAYMOUTH'S MEDIUM MB 752/1 (1959)

	Concn. mg./litre	Equivalent in millimoles
Glycine	50	0.66
L-Phenylalanine	50	0.30
L-Glutamic acid	150	1.02
L-Aspartic acid	60	0.46
L-Lysine. HCl	240	1.42
L-Methionine	50	0.34
L-Threonine	74	0.63
L-Valine	64	0.54
L-Isoleucine	26	0.20
L-Leucine	50	0.38
L-Arginine. HCl	76	0.36
L-Tryptophan	40	0.20
L-Histidine. HCl	150	0.80

A stock (A) was made up at 25 times the above concentration in Hanks' BSS.

L-Cystine	15	0.06
L-Tyrosine	40	0.22

A stock (B) was made up at 25 times the above concentration in 0.1N HCl.

Glucose	5000	27.8
Ascorbic acid	16	0.09
Choline. HCl	250	1.8
Cysteine. HCl	90	0.57
Glutathione	16	0.05
Hypoxanthine	25	0.18
Glutamine	350	2.38

A stock (C) was made up at 25 times the above concentration in Hanks' BSS.

	Concn. mg./litre	Equivalent in millimoles
Thiamine	10	0.03
CaPantothenate	1	0.003
Pyridoxine. HCl	1	0.003
m-Inositol. $2H_2O$	1	0.005
Nicotinamide	1	0.008
Vitamin B_{12}	0.2	0.00015
Folic acid	4	0.008
Biotin	0.2	0.0008
Riboflavin	1	0.003

A stock (D) was made up at 10 times the above concentration in Hanks' BSS.

A working stock was prepared by combining 4 parts each of stocks A, B and C to 1 part of stock D. The solution was brought to pH 7.4 with NaOH and sterilized by seitz or millipore filtration. All stocks were stored at -20°.

Medium was made by adding 13 ml. of working stock and sera, if required, to the appropriate volume of Hanks' BSS or tris-citrate BSS to make 100 ml. The pH was adjusted to 7.4 with a saturated solution of $NaHCO_3$ if Hanks' BSS was used or with 0.1N NaOH if tris-citrate BSS was used. Sodium Penicillin G, obtained from J. W. Miller, Ltd., Glasgow, was added at a final concentration of 50 units/ml.

The composition of other media used occasionally in this study was as given by Paul (1960).

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