Ph.D. Biochemistry

James W. Gurd

The Effects of Glucose on the Energy Metabolism of Novikoff Ascites Hepatoma Cells

ABSTRACT

Glucose addition to Novikoff ascites hepatoma cells causes a rapid depletion of ATP and a decrease in total adenine nucleotides. Re-establishment of the steady state concentrations of ATP requires 50 to 60 minutes. Decreased rates of protein synthesis, glycogen synthesis, ion transport, PRPP synthesis, and incorporation of adenine and hypoxanthine into the acid soluble fraction are associated with the decrease in ATP concentration. During the first 6 minutes following glucose addition fructose diphosphate accumulates and the concentration of inorganic phosphate decreases. Glycolysis and glucose utilization decrease in the interval between 1 and 6 minutes after the addition of glucose. After preincubation of the cells, addition of glucose does not cause large changes in ATP levels and the period of decreased metabolic activity is eliminated. The maximum rates of PRPP synthesis, glycolysis, and adenine incorporation are greater in non-preincubated than in preincubated cells. Partial replacement of sodium by potassium ions in the incubation medium causes a prolongation of the period of ATP recovery and of decreased metabolic activity. Possible factors involved in the regulation of the above events are discussed.

Short Title

ENERGY METABOLISM IN NOVIKOFF ASCITES HEPATOMA

Gurd J.

THE EFFECTS OF GLUCOSE ON THE ENERGY METABOLISM OF NOVIKOFF ASCITES HEPATOMA CELLS

by

James W. Gurd

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Department of Biochemistry
McGill University
Montreal

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LIST OF ABBREVIATIONS

AIB	∝ -aminoisobutyric acid
ACPC	1-aminocyclopentanecarboxylic acid
AMPS	adenylosuccinic acid
cyclic AMP	cyclic-3', 5'-adenosine monophosphate
DNP	2, 4-dinitrophenol
FDP	fructose diphosphate
K-103 medium	modified calcium-free Krebs-Ringer solution buffered with 10 mM sodium phosphate in which the final concentrations of Na^+ and K^+ are 59 and 103 mM respectively
PCA	perchloric acid
PEI-Cellulose	poly(ethyleneimine)-cellulose
PRPP	5-phosphoribosyl-1-pyrophosphate
7mnp	7 minute hydrolyzable nucleotide pyrophosphate
TCA	trichloroacetic acid
μatoms glucose-2-C^{14}	μatoms of radioactive carbon from glucose-2-C^{14}
UDPG	uridine diphosphate glucose

TABLE OF CONTENTS

Chapter | Page

Chapter Page

Chapter Page

CHAPTER I

INTRODUCTION

A continuing supply of energy is required for the maintenance of life and it is obtained by most organisms through the oxidation of organic materials, one of the most important groups of which is the carbohydrates. Much of the energy released upon the oxidation of these compounds is, through a series of carefully regulated enzymatic reactions, conserved in the form of high energy organic phosphate bonds (Lipmann, 1941). These are subsequently used to form the terminal phosphate groups of nucleoside triphosphates, such as ATP, and these compounds are generally the immediate source of utilizable energy within the cell. Experience has demonstrated that optimum conditions for in vitro studies of cell growth and function often necessitate the addition of an exogenous energy source to the extracellular medium. The ability of glucose to serve this function is perhaps best exemplified by the fact that it is a standard addition to most tissue culture media (Fisher et al., 1948; Eagle, 1956). The following discussion will deal largely with the contribution made by glucose to the overall pattern of energy metabolism within the cell, with particular emphasis

being placed on the production and utilization of glycolytically generated ATP.

In the work to be presented the addition of glucose was found to produce considerable changes in the intracellular pool of purine nucleotides and this introduction will therefore contain a section on the metabolism of these compounds. Changes in the extracellular concentration of potassium ions will be shown to result in significant alterations in the metabolism of glucose and in the general energy metabolism of the cell. Hence some selected aspects of the effects of potassium on cellular metabolism will also be included.

1-1. GLUCOSE AS AN ENERGY SOURCE

Glucose Oxidation and the Production of ATP

A summary of the reactions involved in the oxidation of glucose, and the attendant production of ATP may be found in any standard textbook of Biochemistry and need not be presented here. The regulation of glycolysis, and hence of glycolytic ATP production, will be the subject of later sections of this introduction. A number of reviews dealing with the reactions of the tricarboxylic acid cycle (Ochoa, 1954; Lowenstein, 1967) and the electron transport system (Ernster and Lee, 1965; Griffiths, 1965; King, 1966; Pullman and Schatz, 1967) are available.

The anaerobic conversion of 1 mole of glucose to 2 moles of

lactic acid is accompanied by the net formation of 2 moles of ATP. If glycogen serves as the original source of carbohydrate, the formation of hexose monophosphate does not require ATP, and the net yield is therefore increased from 2 to 3 moles of ATP per mole of glucose converted to lactate. Aerobically pyruvic acid may be completely oxidized to CO_2 and water by the reactions of the tricarboxylic acid cycle resulting in the formation of 15 moles of ATP per mole of pyruvic acid oxidized. 14 moles of ATP are produced as a consequence of the oxidation of 4 moles of NADH and 1 mole of succinic acid by the electron transport system, and the fifteenth is accounted for by the substrate level phosphorylation which accompanies the decarboxylation of α-ketoglutarate to succinate. In addition, the conversion of glucose to pyruvate is accompanied by the production of 2 moles each of NADH and ATP. The complete oxidation of 1 mole of glucose therefore gives a net yield of 38 moles of ATP and of the 688 kilocalories released approximately 56% are conserved in the high energy phosphate bonds of ATP (Ingraham and Pardee, 1967).

Glucose Utilization by Mammalian Tissues

In the higher animals glucose is made available through the digestion of carbohydrates. The various monosaccharides which are released into the blood stream from the gastrointestinal tract are stored as glycogen in the liver from which they are released as glucose when

required to meet the energy demands of other tissues (Cori, 1931).

The mammalian brain, in which ". . . almost the entire energy for . . . metabolic activities comes from the oxidative breakdown of glucose" (Quastel and Quastel, 1961) provides perhaps the best example of a tissue which depends almost exclusively on glucose as its source of energy. In contrast to the predominately oxidative metabolism of the brain a number of tissues appear to rely on glycolytically generated ATP for the performance of various cellular functions. Possibly the best understood and most well documented metabolic role for glycolysis is in the replenishment of the energy stores of skeletal muscle following contraction (Dubuisson, 1954). A role for aerobic glycolysis in providing the energy required for the generation of electrical impulses in mammalian retina has been proposed by Noell (1951). Similarly, the production of glycolytic energy is required for the normal functioning of mammalian spermatozoa (Mann, 1946) although the physiological substrate for these cells is probably fructose rather than glucose. The absence of the enzymes required for oxidative phosphorylation in mature erythrocytes (Rubenstein et al., 1956) indicates an important role for glycolytically generated ATP in these cells, and the intimate relationship between glycolysis and active cation transport which is observed in erythrocytes (Whittam and Ager, 1965) is of interest in this respect. Other tissues in which the aerobic production of glycolytic energy would appear to be of special significance include leukocytes (Beck and Valentine, 1952), placenta

(Greenstein, 1954), the synovial membrane (Bywaters, 1937), the renal medulla and the jejunal mucosa (Dickens and Weil-Malherbe, 1941).

Glucose Utilization by Malignant Tissues

On the basis of a series of observations on the fermentative behavior of tumor cells, both in vitro and in vivo, Warburg (1931 and 1956) proposed that the primary lesion in carcinogenesis affects the respiratory mechanism of the cell and that in order to produce the energy which is required for survival the tissue is forced to adapt to a fermentative type of metabolism. Later Greenstein (1954) also investigated the glycolytic capacity of a series of normal and malignant tissues and found that 10 of the 14 tumors investigated exhibited a $Q^{O_2}_{lactate}$ greater than 10, the value for the other 4 tumors falling between 6 and 9. In contrast, the aerobic glycolysis of normal tissues, with a few exceptions, yielded Q values of less than 3. More recent studies of the metabolism of minimal deviation hepatomas have indicated that a high rate of aerobic glycolysis need not always be associated with neoplasia (Aisenberg and Morris, 1963; Sweeny et al., 1963) blunting the original hypothesis of Warburg. It remains clear, however, that a positive correlation does exist between glycolysis and tumor growth rate (Weber et al., 1964) although the significance of this correlation is not understood.

The Utilization of Glycolytic vs Oxidative ATP

The preceding paragraphs have shown that a number of tissues exhibit high rates of aerobic lactic acid formation and it has been suggested that certain functions preferentially utilize glycolytically generated ATP. A number of investigations comparing the ability of the cell to use either oxidative or glycolytic energy for various activities have been reported.

The study of Quastel and Bickis (1959) demonstrated that tumor cells were able to use glycolytic ATP as efficiently as oxidative ATP for the incorporation of amino acids into protein. In contrast, some of the normal tissues investigated exhibited low capacities for anaerobic protein synthesis, in spite of relatively high rates of glycolysis. The ability of tumor cells to utilize glycolytically produced ATP for nucleic acid synthesis was demonstrated by Aisenberg (1961). Johnstone and Quastel (1960) provided evidence suggesting that glycolytic energy was utilized at least as efficiently as respiratory energy for the active accumulation of amino acids. The active transport of cations may be supported by either oxidatively or glycolytically generated ATP. Utilization of the latter energy source by tumor cells is demonstrated by the ability of glucose to reverse, or partially reverse, the inhibition of ion transport which occurs in the presence of inhibitors of oxidative phosphorylation (Maizels et al., 1958b; Hempling, 1966). Apparent variations in the ability to use oxidative and/or glycolytic energy for cation transport was demonstrated in a study in which the

addition of cyanide was found to produce varying degrees of inhibition of the uptake of potassium by rat livers from foetuses of different ages (Van Rossum, 1963). Although the residual uptake in the presence of cyanide was dependent on the generation of glycolytic ATP livers of different ages appeared to utilize this energy at different efficiencies, so that the amount of cyanide resistant transport did not correlate with glycolytic activity.

In contrast to the above metabolic processes there is some evidence which indicates that aerobic energy production is required for glycogen synthesis. Thus, inhibitors of oxidative phosphorylation prevent both the incorporation of glucose and glucose-6-phosphate into glycogen and the conversion of glucose-1-phosphate to UDPG by pigeon liver homogenates (Nigam and Freidland, 1964; Zancan and Hers, 1965). Neither ATP nor UTP could replace the aerobic energy supply in these systems. The conversion of glucose phosphates, but not of UDPG to glycogen was inhibited by the addition of DNP to Novikoff ascites cells which had been previously swollen by maintenance at $5^{o}C$ (Nigam, 1967b). Similarly, in the absence of a large change in the cellular ATP content, the glycogen synthesizing ability of the Novikoff tumor was almost totally abolished by DNP (Nigam, 1967a). The observation by Racker (1965) that uridine is phosphorylated more efficiently under aerobic, than under anaerobic conditions is perhaps relevant to the above findings.

High Energy Nucleoside Triphosphates Other than ATP

Although the discussion thus far has emphasized the importance of ATP as the utilizable form of cellular energy the triphosphates of uridine, guanosine and cytidine also participate in anabolic cellular metabolism, as illustrated in Figure 1. In addition to their role as precursors for the nucleic acids these compounds have been found to be involved in carbohydrate metabolism (Leloir and Cardini, 1960), lipid biosynthesis (Kennedy, 1956 and 1960), and protein synthesis (Keller and Zamecnik, 1956; Utter, 1960).

In 1959 Strominger and co-workers described a group of enzymes which catalyse the reversible phosphorylation of the monophosphates of adenosine, guanosine, uridine, and cytidine using ATP as the phosphate donor and yielding the respective nucleoside diphosphates and ADP. A separate group of enzymes catalysing the transfer of a phosphate group between any nucleoside triphosphate and AMP was also described (Heppel et al., 1959). The AMP-ATP transphosphorylase activity was subsequently separated from the enzyme which utilizes guanosine triphosphate or inosine triphosphate as the phosphoryl donor (Chiga and Plaut, 1960; Chiga et al., 1961). Although a specific enzyme utilizing cytidine triphosphate or uridine triphosphate as the phosphoryl donor was not isolated, the existence of such an enzyme was inferred from the presence of this activity in the original extract (Chiga et al., 1961).

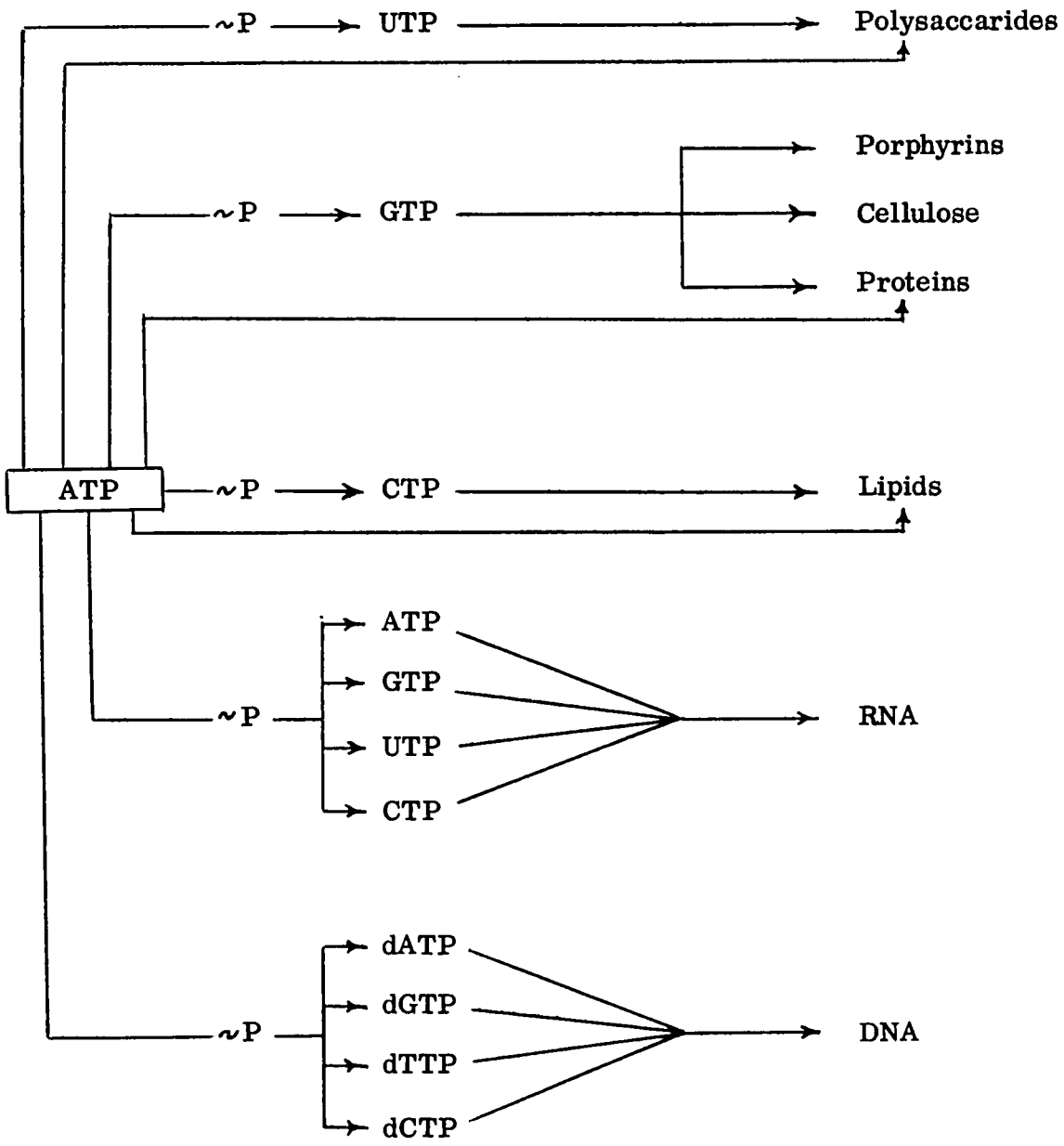

FIGURE 1. Channeling of phosphate bond energy of ATP into specific biosynthetic routes. (from Lehninger, A. L., 1965. Bioenergetics, New York, Amsterdam: W. A. Benjamin, Inc.)

The occurrence of an enzyme which catalyzes the formation of nucleoside triphosphate from the diphosphate derivative, with ATP as the specific donor, was observed in preparations of yeast and rabbit muscle (Berg and Joklik, 1954) and pigeon breast muscle (Krebs and Hems, 1953). A crystalline preparation of this enzyme from yeast catalyzes the phosphorylation of the diphosphate derivatives of quanosine, inosine and uridine, and utilizes either ATP or dATP as the phosphoryl donor (Ratliff et al., 1964).

Although the various nucleoside triphosphates are required for the normal function of the cell, these studies serve to emphasize the central role played by the adenine nucleotides in the maintenance of the cell's pool of utilizable energy forms.

The Interaction between Glycolysis and Respiration

Under aerobic conditions ATP may be produced by both the oxidative and the glycolytic pathways and interaction between these two systems has been well documented. In 1876 Pasteur observed that the utilization of glucose and the production of lactic acid by yeast cells was very much decreased when oxygen was added to the system. Conversely, Crabtree (1929) found that the addition of glucose to aerobic tumor cells produced a decrease in respiratory activity. Subsequent studies have indicated that the Pasteur effect is of widespread occurrence (Burk, 1939) while the Crabtree effect

is confined to neoplastic tissues and a few normal tissues which possess a high rate of aerobic glycolysis (Ibsen, 1961).

It was suggested in 1941 that the Pasteur effect might be the result of a competition between the transphosphorylating enzymes of the mitochondrial and glycolytic systems for a common cofactor, such as inorganic phosphate or phosphate acceptor (Johnson, 1941; Lynen, 1941). The observation that uncouplers of oxidative phosphorylation reverse both the Pasteur and Crabtree effects lends credence to the concept that a competition for a common intermediate is the cause of these phenomena (Dodds and Greville, 1934; Judah and Williams-Ashman, 1951; Racker, 1956; Wu and Racker, 1959b).

Racker and his collaborators investigated the Pasteur and Crabtree effects in cell-free preparations and in intact cells. They found that both of these phenomena can be reproduced in reconstructed cell-free systems by varying the relative amounts of glycolytic and mitochondrial enzymes present or by making either inorganic phosphate or ADP limiting (Gatt and Racker, 1959a and b). It was also demonstrated that a partial reversal of the Pasteur effect in the intact cell can be obtained by elevating the level of inorganic phosphate. In these experiments, however, increases in the intracellular ADP content altered neither the rate of lactic acid production nor the rate of respiration (Wu and Racker, 1959 a and b). The results of these experiments were interpreted as demonstrating that under aerobic

conditions in the intact cell glycolysis is limited by the availability of inorganic phosphate, and that competition for this compound is responsible for the Pasteur effect. The role of inorganic phosphate in the production of the Crabtree effect was less clearly defined, and elevated levels of intracellular inorganic phosphate stimulated respiration in the presence of glucose in only one of the two experiments reported (Wu and Racker, 1959b).

Other seemingly contradictory results on the role of phosphate in the Crabtree effect have been reported. Brin and McKee (1956) for example, obtained a partial reversal of the glucose induced inhibition of respiration by increasing the level of inorganic phosphate, but Kvamme (1958) and Block-Frankenthal and Ram (1959) found that similar treatment enhanced the Crabtree effect.

It should be noted that control by inorganic phosphate necessitates that phosphate entrance into the cell be strictly regulated and the report of Levinson (1966) indicates that this is in fact the case. Ibsen, Coe and McKee (1960), on the other hand, observed a rapid equilibration between extracellular and intracellular phosphate, and suggested that this ion may be of regulatory significance when its concentration is less than 5 mM but not when its concentration exceeds 15 mM.

Regulation by ADP rather than, or in addition to inorganic phosphate has been proposed (Ibsen et al., 1958; Chance and Hess, 1959b) and the recent report by Lo and collaborators (1968) supports this concept.

These authors studied the amounts of glycolytic and respiratory ATP produced by cell extracts from liver and hepatomas of different growth rates. The results indicated that with rapidly growing hepatomas, the glycolytic transphosphorylating enzymes compete successfully with the mitochondria for the available ADP, whereas with slow growing tumors the respiratory enzymes apparently have a greater affinity for this compound. Experiments in which the soluble and particulate fractions from the different sources were mixed, further emphasized the role played by ADP competition in determining the relative amounts of respiratory and glycolytic phosphorylation demonstrated by the various tissue preparations. In the latter experiments, respiratory phosphorylation was increased at the expense of glycolytic phosphorylation when the particles of a rapidly glycolysing, slowly respiring tumor homogenate were replaced by those of a rapidly respiring tissue, indicating that the latter particles competed more successfully for the available ADP. Conversely, replacement of mitochondria from a rapidly respiring homogenate by those from a slowly respiring tissue was accompanied by an increase in glycolytic phosphorylation, at the expense of respiratory ATP production.

In terms of competition for ADP, some interesting observations with respect to the levels of the two glycolytic transphosphorylating enzymes, phosphoglycerate kinase and pyruvic kinase have been made. High levels of pyruvic kinase in hepatomas were reported by Shonk and collaborators (1965), and Farina and co-workers (1967) observed a more than tenfold increase in

the level of this enzyme in poorly differentiated as compared to well differentiated hepatomas. Similarly, high levels of both pyruvic kinase and phosphoglycerate kinase were found in extracts of Ehrlich ascites cells (Wu and Racker, 1959a). It has been suggested that the apparent excess of these enzymes in rapidly glycolysing tumors may play a role in increasing the "affinity" of the glycolytic system for ADP and hence in determining the high aerobic rate of glycolysis which is observed in these cells (Wu and Racker, 1959a; Racker, 1965).

The Crabtree Effect and Tumor Energy Metabolism

The effects of the decrease in oxidative energy production which occurs upon the addition of glucose to tumor cells on the energy levels and anabolic capacity of these cells was investigated by Quastel and Bickis (1959). These authors demonstrated that the loss of respiratory energy was balanced by the concomitant increase in the production of ATP by the glycolytic system. These findings were confirmed by Ibsen, Coe, and McKee (1959). The turnover of ATP in Ehrlich ascites cells was demonstrated by Creaser et al. (1959) to be similar in the presence and absence of glucose, further emphasizing that the production and utilization of ATP were not altered in the former instance.

The Regulation of Glycolysis

Measurement of the activity of the glycolytic enzymes from a

number of tissues has shown clearly that the glycolytic potential far exceeds the actual rate of lactate production which is observed in the intact cell (Racker, 1965; Scrutton and Utter, 1968). It is apparent from this discrepancy between the maximum potential enzyme activity and the actual glycolytic rate of the intact cell that strict regulatory mechanisms must be operative in the latter instance. Glycolytic control mechanisms in normal tissues (Scrutton and Utter, 1968) and tumor cells (Wenner, 1967) have been recently reviewed.

Although the levels of glycolytic intermediates are relatively constant under various steady state conditions, a number of transient changes in the amounts of these compounds occur during the transition from one steady state to another. By determining the levels of the intermediates at different times and applying the "cross-over" theorem, as used by Chance (1958) in his studies of electron transport, an indication of those reactions which are limiting at a particular time may be obtained. Alternatively, a comparison of the mass action ratios of the various enzymes under in vivo conditions with the known equilibrium constants of the reactions may be useful in indicating regulatory enzymes. Using this method, for example, Williamson (1965) and Rolleston and Newsholme (1967) found that hexokinase, phosphofructokinase, pyruvic kinase and glyceraldehyde-3-phosphate dehydrogenase were all potential regulatory enzymes in muscle and brain. Hess (1961) found that the mass action ratio of phosphofructokinase in ascites

tumor cells was 0.65 in vivo as compared to the equilibrium constant of 1.2 x 10^3 for the isolated enzyme, thus implicating this reaction as a possible control site. Some of the limitations of these approaches as applied to glycolysis are discussed by Scrutton and Utter (1968).

The metabolic events which occur upon the addition of glucose to aerobically metabolizing tumor cells demonstrate the controls which are exerted on the glycolytic system in the intact cell. Typically, there occurs an initial period of rapid glucose phosphorylation, lactic acid production and oxygen uptake. This is followed by a brief period prior to the establishment of the steady state during which these three activities are markedly reduced. There also occurs during the first minutes following glucose addition an accumulation of hexose phosphates, a transitory decrease in ATP and an increase in ADP (Ibsen et al., 1958; Lonberg-Holme, 1959; Wu and Racker, 1959b; Hess and Chance, 1961; Wu, 1965a; Wu et al., 1965; Coe, 1966). Regulation of these events has been variously attributed to such factors as intracellular compartmentation, inorganic phosphate, adenine nucleotides and product inhibition. An example of the latter is provided by the studies of Rose (1965) and Wenner (1965) in which it was shown that there can be an inverse relationship between glucose utilization and intracellular levels of glucose-6-phosphate which is an inhibitor of hexokinase activity (Crane and Sols, 1953). Control of such events by inorganic phosphate has been emphasized by the studies of Wu and his co-workers (Wu, 1965a; Wu et al.,

1965), whereas the work of Coe (1966) has stressed the importance of the adenine nucleotides in regulating the flow of intermediates through the glycolytic system during the initial minutes following glucose addition. Chance and Hess (1959b) originally proposed a scheme of regulation based largely on mitochondrial trapping of adenine nucleotides. Later work, however, led to the conclusion that the metabolic events which occur upon the addition of glucose are the consequence of multisite control of the glycolytic sequence, although the need for some compartmentation of ADP and/or inorganic phosphate was still emphasized (Maitra and Chance, 1965).

The necessity for invoking compartmentation arises from the discrepancy between the kinetic constants of the various enzymes or enzyme systems for inorganic phosphate and ADP and the apparent concentrations at which these compounds are thought to exert control in the intact cell. For example, transition of respiratory control from state 3 to state 4 was attributed to a drop in ADP from 2.4 to 1.8 μmoles per gram fresh weight (Maitra and Chance, 1965), although the K_m of the respiration of isolated mitochondria for ADP was reported to be only 20 to 30 μmolar (Chance and Williams, 1955). Similarly, Wu and Racker (1959b) found that inorganic phosphate concentrations of between 3 and 9 mM appeared to be regulatory in Ehrlich ascites cells, although the K_m of glyceraldehyde-3-phosphate dehydrogenase for inorganic phosphate was determined to be only 1.5 mM.

The following pages will describe some of the properties of

hexokinase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, and pyruvic kinase which are thought to contribute to the regulatory role played by these enzymes. Enzymes concerned with gluconeogenesis will not be considered since these have generally been found to be decreased or absent in tumor cells (Weber et al., 1964; Boxer and Shonk, 1966).

Hexokinase

The phosphorylation of glucose by ATP to yield glucose-6-phosphate and ADP is catalyzed by the enzyme hexokinase. This enzyme catalyzes the phosphorylation of a number of hexoses in addition to glucose, but glucose demonstrates the highest phosphorylation coefficient (Sols and Crane, 1954a). Another more specific glucose phosphorylating enzyme has been described (Viñuela et al., 1963) but this enzyme, the high K_m glucokinase, has been found to be decreased or absent in hepatomas (Shatton et al., 1962; Manjeshwar et al., 1965; Nigam, 1967a).

Inhibition of hexokinase by glucose-6-phosphate was reported in 1951 by Weil-Malherbe and Bone and subsequently confirmed by Crane and Sols (1953). In contrast to the low specificity which the enzyme demonstrates with respect to substrate, inhibition by hexose phosphate is relatively specific, only six of twenty-five compounds tested by Crane and Sols (1954) exhibiting inhibitory properties. The inhibition of hexokinase which is produced by glucose-6-phosphate is antagonized by inorganic phosphate (Tiedemann and

Born, 1959; Rose et al., 1964). Inhibition by ADP, the other reaction product was reported by Sols and Crane (1954b) for the particulate hexokinase from brain. Subsequent studies have described inhibitory actions of both adenosine mono- and diphosphates for the enzyme derived from several other tissues, including heart (England and Randle, 1967), muscle (Hanson and From, 1965 and 1967) and ascites tumor cells (Uyeda and Racker, 1965a; Kosow and Rose, 1968).

Hexokinase is present in both the particulate and soluble cell fractions of a number of tissues and differences in the kinetic properties of these two forms of the enzyme have been described. The soluble enzyme has been reported to have a lower affinity for ATP (Li and Ch'ien, 1965; Karpatkin, 1967; Schwartz and Basford, 1967) and to be more sensitive to glucose-6-phosphate inhibition (Kosow and Rose, 1968; Newsholme et al., 1968) than the particulate enzyme. Glucose-6-phosphate and ATP promote solubilization of the bound enzyme, and inorganic phosphate antagonizes this process (Kosow and Rose, 1968; Wilson, 1968). On the basis of these observations, Wilson (1968) has proposed that hexokinase activity in vivo may be partially controlled by the relative distribution between soluble and particulate forms, the latter being more active.

Phosphofructokinase

Phosphofructokinase, the second phosphorylating enzyme in the

glycolytic sequence is the only enzyme unique to this pathway. In 1914 Harden and Young discovered the formation of hexose diphosphate in fermenting extracts of yeast, but several years elapsed before it was shown that this compound was formed by the phosphorylation of hexose monophosphate by ATP (Dische, 1935; Ostern et al., 1936). In 1941 Cori suggested that this reaction might play a regulatory role in glycolysis. Since these early observations phosphofructokinase has been the object of extensive study and it has been implicated as a regulatory enzyme under a variety of conditions and for a number of different tissues (Mansour, 1962; Lowry and Passonneau, 1964a; Maitra et al., 1964; Salas et al., 1965; Tsuboi and Fukunaga, 1965; Wu, 1965a; Williamson, 1966). The properties of phosphofructokinase have recently been reviewed by Lardy (1962) and by Stadtman (1966).

In 1956 Lardy and Parks reported that the phosphorylation of fructose-6-phosphate is inhibited by ATP. Subsequent studies confirmed this observation and demonstrated that ATP inhibition is counteracted by increasing the concentration of fructose-6-phosphate (Passonneau and Lowry, 1962; Underwood and Newsholme, 1965). Citric acid also inhibits the reaction, and the two inhibitors, ATP and citrate, behave synergistically (Parmigianni and Bowman, 1963; Garland et al., 1963; Passonneau and Lowry, 1963).

Mansour and Mansour (1962) observed that cyclic AMP activates the ATP inhibited enzyme from the liver fluke Fasciola hepatica. In the same year inhibition of the enzyme from guinea pig heart was shown to be counter-

acted by cyclic AMP, AMP, and ADP (Mansour, 1963), and Passonneau and Lowry (1964) added inorganic phosphate and fructose diphosphate to the list of activators. In addition to the above compounds ammonium ions, and to a lessor extent potassium ions also activate the enzyme (Muntz and Hurwitz, 1951; Passonneau and Lowry, 1964; Lowry and Passonneau, 1966). The activators are synergistic in their action. For example, in the presence of inhibitory concentrations of ATP the enzyme from brain was stimulated approximately 30, 90, and 500 fold by addition of AMP, AMP plus inorganic phosphate, and AMP plus inorganic phosphate plus ammonium ions respectively (Lowry and Passonneau, 1966). On the basis of these observations Lowry and Passonneau (1966) have proposed a model involving not less than seven, and possibly as many as twelve substrate, inhibitor, and activator sites. The properties of the enzyme from several different rat tissues were compared by Lowry and Passonneau (1964b). Although all the preparations were inhibited by ATP, the sensitivity showed marked variations. Thus, 0.3 mM ATP produced a 50% inhibition of the enzyme from brain and blood, but 2 mM ATP was required to give a similar inhibition of the heart enzyme. Phosphofructokinase from all the tissues tested was activated by AMP, ADP, cyclic AMP, and inorganic phosphate although, similar to inhibition by ATP, some tissue variation was observed.

Wu (1965c and 1966) studied the enzyme from Novikoff ascites hepatoma cells. It was activated by inorganic phosphate and AMP, in

agreement with the properties of the enzyme from other tissues, but not affected by fructose diphosphate at concentrations as high as 1.5 mM. This is in marked contrast to the liver enzyme (for example) which is activated by low concentrations of fructose diphosphate (Passonneau and Lowry, 1964; Underwood and Newsholme, 1965). In addition, the tumor enzyme is less sensitive to inhibition by ATP than is the liver enzyme. Phosphofructokinase from the latter tissue is inhibited by ATP concentrations as low as 0.1 mM, whereas the tumor enzyme was not inhibited until the level of ATP exceeded 1 mM. This difference in ATP sensitivity might be anticipated in view of the different metabolic activities of the two tissues. Thus, liver is essentially gluconeogenic in function, whereas the Novikoff cells demonstrate a high rate of glycolysis. In the former instance, phosphofructokinase activity would be expected to be in the inhibited state at most times.

Studies on the purified enzyme have led to the suggestion that regulatory mechanisms other than purely kinetic ones might also play a role in determining the enzyme activity in vivo. Evidence for interconversion of active and inactive forms has been found (Mansour, 1965; Mansour et al., 1965). Mansour and Ahlfers (1968) have proposed a model involving a reversible dissociation between an active dimer and inactive monomer form, ATP favoring dissociation to the monomer, and the activators fructose-6-phosphate and fructose diphosphate favoring formation of the active dimer form.

Glyceraldehyde-3-Phosphate Dehydrogenase and Pyruvic Kinase

The conversion of fructose diphosphate to 2 moles of pyruvate is accompanied by the generation of 4 moles of ATP. Aldolase catalyses the formation of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, the interconversion of these two compounds being mediated by triose phosphate isomerase. The oxidation of glyceraldehyde-3-phosphate to 3-phosphoglyceric acid involves the stoichiometric participation of inorganic phosphate (Warburg and Christian, 1939) and is accompanied by the phosphorylation of adenine nucleotides (Needham and Pillar, 1937). Negelein and Brömel (1939) identified 1, 3-diphosphoglyceric acid as an intermediate in this conversion, and Bücher (1947) demonstrated the involvement of a kinase which catalyzes the transfer of a phosphate group from 1, 3-diphosphoglyceric acid to ADP to form 3-phosphoglyceric acid and ATP. Phosphoenolpyruvic acid was identified as an intermediate in the conversion of 3-phosphoglyceric acid to pyruvic acid (Lohman and Meyerhof, 1934) and it was subsequently recognized that the formation of pyruvic acid from phosphoenolpyruvic acid is accompanied by the formation of ATP (Needham and Van Heyningen, 1935). The properties of glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, and pyruvic kinase have been reviewed by Velick and Furfine (1963), Malmström and Larssen-Raźnikiewicz (1962), and Boyer (1962) respectively. Racker (1965) has discussed the mechanism of the glyceraldehyde-3-phosphate dehydrogenase reaction in some detail.

An accumulation of hexose diphosphate and/or triose phosphates is frequently observed [e.g. during ischemia in brain (Lowry and Passonneau, 1964a), on addition of pyruvate to perfused rat heart (Williamson, 1965), or on addition of glucose to ascites tumor cells (Wu et al., 1965)] suggesting that glyceraldehyde-3-phosphate dehydrogenase is rate limiting under these conditions. As previously mentioned, studies on the Pasteur effect led Wu and Racker (1959a and b) to conclude that this reaction is limited by the available supply of inorganic phosphate. However, the Michaelis constant for inorganic phosphate [e.g. 1.5 mM for ascites cells (Wu and Racker, 1959b) and 2.3 mM for the enzyme from rabbit muscle (Cowey, 1967)] is considerably lower than the level of this compound which is generally found in the intact cell (see, for example Coe and Lee, 1969) and this has required the postulation of compartmentation as discussed above. In addition to regulation by phosphate availability, the activity of glyceraldehyde-3-phosphate dehydrogenase may be controlled through interaction with 1,3-diphosphoglyceric acid and NADH, both of which are potent inhibitors of the reaction (Furfine and Velick, 1965). Maitra and Chance (1965) failed to observe any evidence for phosphate control and suggested that the enzyme was strictly controlled by 1,3-diphosphoglyceric acid. Another possible regulatory mechanism is suggested by the observation of Uyeda and Racker (1965b) that the addition of mitochondria to a reconstructed glycolysing system in the presence of excess ADP and inorganic phosphate

resulted in a marked decrease in the amount of lactic acid produced. The resulting accumulation of hexose phosphates indicated that the block occurred below phosphofructokinase. Bálazs (1959) reported a similar observation and suggested that an inhibitor of glyceraldehyde-3-phosphate dehydrogenase was produced during mitochondrial respiration.

The possible role of competition between the transphosphorylating enzymes of glycolysis and the mitochondria for a common cofactor, such as ADP and/or inorganic phosphate, in regulating the rates of glycolysis and respiration has been discussed. The activity of pyruvic kinase may be regulated by a number of factors in addition to ADP availability. In 1949, for example, Meyerhof and Oesper reported inhibition of pyruvic kinase by ATP, an observation which has since been confirmed by others (McQuate and Utter, 1959; Reynard et al., 1961; Mildvan and Cohn, 1966). Inhibition by high levels of inorganic phosphate has been reported by Rose (1960), and Weber et al., (1965) described an inhibition produced by free fatty acids. Pyruvic kinase is inhibited by calcium, and it has been suggested that this is due, at least partially, to a competition with potassium ions, the latter being a required cofactor for the reaction (Kachman and Boyer, 1953).

The occurrence of two types of pyruvic kinase was reported in 1965 by Tanaka, Harano, Morimura and Mori. The muscle, or M type, is present in a variety of tissues, but the liver, or L type, is found only in liver and kidney. Some differences in the kinetic behavior of the two enzyme

types have been described. For example, the liver, but not the muscle type is activated by fructose diphosphate and inhibited by copper ions (Weber et al., 1965; Tanaka et al., 1967; Taylor and Bailey, 1967; Passeron et al., 1967; Carminatti et al., 1968).

1-2. ASPECTS OF THE METABOLISM OF PURINE NUCLEOTIDES

ATP Turnover and Purine Catabolism

The production and utilization of ATP in the intact cell occurs at a very rapid rate [e.g. in the Krebs-2 ascites cells ATP is produced at a velocity of 450 μmoles per hour per ml of packed cells (Yushok, 1964) and Coe (1966) has estimated that during the initial minutes of glucose metabolism by Ehrlich ascites cells the ATP can be turned over oxidatively in about 20 seconds and glycolytically in approximately 15 seconds]. The utilization of energy by the known cellular processes accounts for only a small fraction of the total energy which is available. Johnstone and Scholefield (1965) for example, have estimated that the synthesis of protein by Ehrlich ascites cells requires only some 3% of the total ATP which is produced. Calculations such as these suggest that most of the ATP turnover in the cell may be accounted for by the action of ATPases, the specific functions of which are as yet undefined.

Regardless of the pathways involved it is evident that the actual

level of ATP in the cell at any one moment is determined by the relative velocities of synthesis and utilization. Under most circumstances the cellular machinery for energy production is able to adapt to changes in the environment and maintain a relatively constant supply of available energy. In some instances, however, such as in the presence of metabolic inhibitors, the balance which is maintained between ATP production and utilization may be upset. When this occurs the acid soluble pool of high energy nucleotide phosphates is rapidly depleted and products of purine catabolism are produced (Wu and Racker, 1959b; McComb and Yushok, 1964a and b).

The catabolism of AMP may proceed either by 1) deamination to IMP (see below) followed by dephosphorylation to inosine or by 2) dephosphorylation to adenosine followed by deamination to inosine. Conway and Cooke (1939) suggested that the latter pathway is the predominant one in most tissues. More recently Burger and Lowenstein (1967) obtained similar results with extracts of heart, lung, brain, kidney, liver, and pancreas of rats, provided that ATP was not added to the extract. Deamination of AMP to IMP is stimulated by ATP (Muntz, 1953; Setlow and Lowenstein, 1967) and dephosphorylation to adenosine is inhibited by ATP (Baer et al., 1966; Burger and Lowenstein, 1967). Thus, while the rate of AMP dephosphorylation in extracts of rat heart was 16 times greater than the rate of deamination in the absence of ATP, in the presence of this compound the rate of deamination exceeded that of dephosphorylation by a factor of three (Burger and Lowenstein, 1967).

Burger and Lowenstein (1967) concluded that under most conditions the intracellular level of ATP is sufficiently high to inhibit the nucleotidase, allowing deamination of AMP to proceed largely via adenylic deaminase. Inhibition of 5'nucleotidase activity by nucleoside triphosphates has also been observed with the enzyme from sheep brain (Ipata, 1968) and Ehrlich ascites cells (Murray and Friedrichs, 1969).

Catabolism of guanine may proceed either through IMP, which is formed by the reductive deamination of GMP (see below), or through guanosine, the product of GMP dephosphorylation.

Cleavage of the nucleoside derivative to the free base and ribose-1-phosphate is catalyzed by nucleoside phosphorylase (Freidkin and Kalckar, 1961; Tar, 1967). Christman (1952) suggested that the most probable pathways of adenosine and guanosine catabolism are: adenosine $\longrightarrow$ inosine $\longrightarrow$ hypoxanthine and guanosine $\longrightarrow$ guanine $\longrightarrow$ xanthine respectively. Xanthine oxidase may then convert both hypoxanthine and xanthine to uric acid (Bray, 1963) which can be further oxidized to allantoin by the enzyme uricase (Mahler, 1963). In man the major end product of purine catabolism is uric acid whereas in most subprimates allantoin is the chief urinary end product of this metabolic pathway (Christman, 1952).

The catabolism of purines by tumor and normal tissues has been compared and it has been suggested that the loss of growth control in tumors might be a reflection of a decreased capacity of these cells to degrade

nucleotides (Bennet et al., 1960). As part of an investigation of the enzymes concerned with purine catabolism in tumors Read and Lewin (1957) measured the levels of adenosine deaminase, nucleoside phosphorylase and xanthine oxidase in azo-dye induced hepatomas. The concentrations of both nucleoside phosphorylase and xanthine oxidase were markedly decreased from the levels prevailing in normal liver, whereas no change in the level of adenosine deaminase was noted. Similar results were obtained in a study in which the levels of enzyme activity in Novikoff hepatoma and liver were compared (de Lamirande et al., 1958). The tumor in this case was found to lack both uricase and xanthine oxidase and to have decreased levels of nucleotidase, nucleoside phosphorylase, and guanase. In contrast, the level of adenosine deaminase was elevated above that present in liver. Increased activity of adenosine deaminase in hepatomas was also observed by Chan and co-workers (1959).

Metabolic studies have yielded results that are in accord with the measurements of enzyme activity in indicating a decrease in the catabolic activity of rapidly growing tumors. For example, the experiments of Bennet et al., (1960) demonstrated an almost total conservation of purines by tumor cells during periods of rapid growth. In a comparative study of the catabolism of purines by rapidly growing tumors and normal tissues, a marked difference in the pattern of product accumulation was noted between the neoplastic and normal cells (Wheeler and Alexander, 1961). When tumor cell extracts were incubated with purine mononucleotides labeled with C^{14} for a 60 minute

period no more than 6% of the total radioactivity recovered was found in allantoin and uric acid. In contrast, in extracts of normal liver these two products accounted for 41 to 98% of the original radioactivity, and in intestinal extracts 12 to 59% of the added radioactivity could be found in these two end products. Subsequent experiments with more slowly growing tumors however, have indicated that a deficiency in purine catabolism is not a requisite for neoplastic growth (Wheeler et al., 1962 and 1964).

The "Salvage" Pathway of Purine Metabolism

The ability of tissues to utilize preformed purine bases was indicated by the study of Lajtha and Vane (1958) in which it was demonstrated that hepatectomized rabbits incorporated less C^{14}-formate into DNA purines of the bone marrow than control rabbits, although C^{14}-formate incorporation into DNA thymine was unaltered. The results suggested that tissues with a low capacity for nucleotide formation utilized partially or totally preformed purines which were synthesized in the liver. The utilization of preformed purine bases for nucleotide formation has been found to be of widespread occurrence (Williams and LePage, 1958; Buchwald and Brittin, 1963; Sartorelli and Upchurch, 1963; Tar and Roy, 1966) and it has been proposed that the "salvage" pathway may be of special physiological significance in rapidly growing tissues (Murray, 1966a).

In 1951 Greenberg obtained evidence which indicated that IMP

was formed from hypoxanthine. Subsequent studies by Buchanan and co-workers (Williams and Buchanan, 1953; Korn and Buchanan, 1953) demonstrated that the formation of IMP from hypoxanthine required the presence of ATP and ribose-5-phosphate and involved the participation of at least two separate enzymes. Saffran and Scarano (1953) and Korn, Remy, Wasilejko, and Buchanan (1955) showed that the product of the first reaction is an activated derivative of ribose-5-phosphate, the condensation of which with the free base to yield the nucleotide derivative is mediated via the second enzyme. Phosphoribosylpyrophosphate (PRPP) was identified as the activated phosphoribosyl intermediate by Remy, Remy, and Buchanan (1955) and Kornberg, Lieberman, and Simms (1955a). The condensation of adenine and hypoxanthine with PRPP to yield AMP and IMP respectively was shown by Kornberg and co-workers to be catalyzed by two distinct enzymes. These workers also reported that IMP and GMP forming activities occurred in the same fraction (Kornberg et al., 1955b). Evidence for the occurrence of a single enzyme possessing both hypoxanthine and guanine phosphoribosyl transferase activity has come from studies of the enzyme from yeast and human sources (Kelley et al., 1967; Seegmiller et al., 1967; Miller and Bieber, 1968). Partial separation of these two enzyme activities from Ehrlich ascites cells was reported by Atkinson and Murray (1965).

Studies of the different phosphoribosyl transferase activities have revealed kinetic properties which may be of regulatory significance in the

intact cell. For example, the adenine, hypoxanthine, and guanine phosphoribosyl transferase activities of Ehrlich ascites cells are inhibited by the respective nucleoside monophosphates (Murray, 1966b; Hori and Henderson, 1966). Similar properties of the enzymes from human erythrocytes (Henderson et al., 1968) and bacteria (Berlin and Stadtman, 1966) have been demonstrated as has inhibition by pyrophosphate, the other reaction product (Henderson et al., 1968; Hori and Henderson, 1966). Low concentrations (10^{-5} to 10^{-6}M) of AMP, GMP, and dAMP cause an increase in the rate of the adenine phosphoribosyl transferase reaction of Ehrlich ascites cells (Hori et al., 1967). A decrease and increase in the activity of this enzyme is produced by the presence of high and low concentrations of ATP respectively (Murray, 1966b; Murray, 1967; Murray and Wong, 1967a). Murray (1967) has suggested that ". . . the ability of ATP both to stimulate and to inhibit the reaction . . . could be a mechanism controlling the activity of this enzyme in vivo." In contrast to the adenine enzyme, the rate of the hypoxanthine phosphoribosyl transferase reaction was decreased at all the concentrations of ATP tested (Murray, 1967).

Interconversion of Purine Mononucleotides

IMP is a common intermediate in the de novo biosynthesis of both AMP and GMP. In 1955 Abrams and Bentley described the formation of AMP from IMP by extracts of rabbit bone marrow and demonstrated that the

reaction required the presence of aspartic acid and a high energy phosphate compound, phosphoglyceric acid being most active in their preparations. In the same year the formation of adenylosuccinate (AMPS) from AMP and fumaric acid was shown to be catalyzed by a protein fraction from yeast autolysate and it was postulated that this compound (i.e. AMPS) might be an intermediate in the incorporation of the amino group into purine nucleotides (Carter and Cohen, 1955 and 1956). Lieberman (1956a and b) subsequently reported the enzymatic synthesis of AMPS from aspartic acid and IMP by extracts of E. coli , the reaction being accompanied by the stoichiometric cleavage of GTP to GDP and inorganic phosphate. Other nucleoside mono-, di-, and triphosphates could not replace GTP and of a number of possible amino donors tested only aspartic acid was active. A similar requirement for GTP was shown for the rabbit muscle enzyme (Davey, 1959). Inhibition of adenylosuccinate synthetase from E. coli by adenine and guanine nucleotides has been demonstrated (Wyngaarden and Greenland, 1963; Lieberman, 1956b; Nichol et al., 1967).

The formation of GMP from IMP is a two step process involving the initial oxidation ~~reduction~~ of IMP to XMP and the subsequent amination of XMP to give GMP. The latter reaction requires the presence of ATP and magnesium ions, the ATP being cleaved to AMP and pyrophosphate (Moyed and Magasanik, 1957; Abrams and Bentley, 1959). In extracts of rabbit bone marrow both glutamine and glutamate were found to serve as the amino group donor

(Abrams and Bentley, 1955) but studies with the purified enzyme from calf thymus demonstrated that glutamate cannot substitute for glutamine (Abrams and Bentley, 1959). In the latter preparation ammonium ions yielded approximately one-third as much activity as glutamine.

The deamination of AMP to IMP is catalyzed by the enzyme adenylic deaminase. Lowenstein's group have extensively studied the properties of this enzyme from calf brain and have made the following observations:

1. At low substrate concentrations the enzyme is activated by ATP, this compound raising the apparent affinity for AMP. ADP does not cause any activation.

2. GTP decreases the enzyme activity both in the presence and absence of ATP, although the inhibition may be overcome by increasing concentrations of ATP.

3. In the absence of ATP alkali metal ions activate the enzyme, the order of effectiveness being $Li^+ > Na^+ > K^+ > Rb^+ > NH_4^+ > Cs^+$. Lithium and to a lesser extent sodium reverse the inhibition produced by GTP.

4. In the presence of ATP, the activation by lithium and sodium ions is decreased and the other ions are inhibitory (Cunningham and Lowenstein, 1965; Setlow et al., 1966; Setlow and Lowenstein, 1967; 1968a and b).

Setlow et al., (1966) reported that the enzyme from brain, kidney, liver, lung, pancreas, spleen and testes all behaved similarly with respect to ATP

activation and GTP inhibition. In contrast to these findings Askari and Franklin (1965) observed that the activity of adenylic deaminase from human erythrocytes is not affected by ATP, and that only potassium and ammonium ions are able to activate the enzyme in the absence of ATP. In the presence of ATP Na^+, K^+, NH_4^+, Li^+, and Rb^+ are all effective activators of the erythrocyte enzyme. Adenylic deaminase from rabbit muscle behaves similarly to the calf brain enzyme in that it is activated by alkali-metal ions in the absence of ATP, however, in this case the order of effectiveness is $Na^+ = K^+ > Li^+ > NH_4^+ = Rb^+ > Cs^+$ (Smiley et al., 1967). In contrast to the enzyme from calf brain the rabbit muscle enzyme is activated by ADP as well as by ATP (Smiley and Suelter, 1967). Lee and Wang (1968) reported inhibition of adenylic deaminase from rabbit skeletal muscle by inorganic phosphate.

The reductive deamination of GMP to IMP has been described by Mager and Magasanik (1960). Using extracts of purine auxotrophs these authors demonstrated that ATP strongly inhibits this reaction. Results consistent with the operation of a similar pathway in extracts of rat liver were obtained by Guarino and Yüregir (1959).

1-3. POTASSIUM AND CELLULAR METABOLISM

Potassium is the major intracellular cation and it is not surprising that it affects the activity of a number of enzyme systems. Its effects

on the activities of phosphofructokinase, pyruvic kinase, and adenylic deaminase have already been mentioned. The discussion which follows will be concerned with some of the changes in the energy producing metabolic pathways which result from changes in the extracellular cationic environment, as well as with the effects of potassium on certain selected metabolic processes. Discussions of other aspects of the biochemistry and physiology of the alkali metals may be found in the reviews of Anderson and Ussing (1960), Brown and Stein (1960), Shaw (1960), Gerebtzoff and Schoffeniels (1960), Steinbach (1962), and Ussing (1960) as well as in the book Cell K by Kernan (1965).

Glycolysis and Respiration

The utilization of energy for the maintenance of ionic gradients across the plasma membrane has been referred to above. In view of the ability of the cell to regulate its energy producing processes very carefully, it is not surprising that alterations in the extracellular ionic environment, which result in changes in the activity of the cation pump, produce concomitant changes in the patterns of cellular metabolism. Keynes and Maizels (1954), in confirmation of an earlier observation by Solandt (1936) demonstrated that small increases in the extracellular potassium concentration stimulate the metabolic activity of frog muscle, without initiating contraction. It was suggested that the initial effect of the increase in the level of potassium ions was to increase the activity of the sodium pump, and that the

energy demands resulting from this stimulation were met by a greater rate of respiration. This proposed interplay between cation transport and energy metabolism has been confirmed by a number of studies on a variety of tissues. Erythrocyte glycolysis is stimulated by sodim and potassium ions when these are internal and external to the plasma membrane respectively. The stimulation has been shown to be counteracted by ouabain, the portion of ouabain sensitive glycolysis varying from 25 to 75% of the basal rate, which is independent of the transport process (Whittam and Ager, 1965). The respiratory activity of kidney cortex slices was reported by Whittam and Willis (1963) to be decreased approximately 40% by the addition of ouabain to the incubation medium. Similarly, inhibition of ion transport by Strophanthin-K or by the omission of sodium or potassium from the incubation medium produced a 30% to 40% decrease in the respiration of liver slices (Elshove and Van Rossum, 1963).

The initiation of potassium accumulation in ascites tumor cells is associated with increased respiratory and/or glycolytic activity. Gordon and de Hartog (1968) for example, demonstrated a two to threefold, ouabain sensitive, increase in the rate of lactic acid production upon potassium addition to potassium depleted Ehrlich ascites cells. Under similar conditions, but in the absence of glucose, a marked increase in the respiratory activity of these cells is observed (Levinson and Hempling, 1967; Levinson, 1967; Gordon et al., 1967). In accord with these observations Maizels et al.,

(1958a) had earlier found that a reduction in the concentration of extracellular potassium from 4.5 to 0.2 mM was accompanied by a 20% decrease in respiration, and a 70% reduction in the glycolytic activity of Ehrlich ascites cells.

In addition to the above examples cation transport affects the energy metabolism of a number of other tissues, including mammalian non-myelinated fibers (Rang and Ritchie, 1968), ciliary body (Riley, 1964), turtle and toad bladder (Klahr and Bricker, 1965; Handler et al., 1968), rat diaphragm (Clausen, 1965 and 1966), yeast (Peña et al., 1967 and 1969), and bacteria (Zarlengo and Schultz, 1966).

In contrast to the increases in respiration and/or glycolysis which accompany the addition of small amounts of potassium to the incubation medium, and which are related to changes in the activity of the cationic pump, high levels of extracellular potassium have been found, in some instances, to be inhibitory. For example, Mudge (1951) observed that the respiratory activity of kidney cortex slices was increased as potassium levels were elevated from 0 to 10 mM, but was inhibited in the presence of 80 mM external potassium. The respiration of heart (Merrick, 1957), liver (Boggio-Gilot and Demartiis, 1956), and erythrocytes (Ito, 1952) as well as the glycolytic activities of brain (Elliot and Bilodeau, 1962), liver and hepatomas (Ashmore et al., 1958) have all been found to be decreased in the presence of high extracellular concentrations of potassium.

Glycogen Metabolism

The level of extracellular potassium effects the metabolism of glycogen in a variety of tissues. Maximum glycogen deposition by liver slices occurs in an incubation medium in which all of the sodium ions have been replaced by potassium (Hastings, 1940; Hastings et al., 1952). It has been proposed that the lower rate which is observed in the presence of high levels of extracellular sodium is the consequence of greater phosphorylase activity under these conditions (Hastings, 1957). Similar to its effect on liver, a high level of extracellular potassium promotes the formation of glycogen by blood polynucleocytes (Bazin et al., 1956) and kidney slices (Marsh and Miller, 1953). In contrast, the deposition of glycogen by muscle (Stadie et al., 1947; Stadie and Zapp, 1947; Tuerkischer and Werth-eimer, 1948; Merrick, 1957) and brain (Kleinzeller and Rybova, 1957) is de-creased in the presence of elevated levels of extracellular potassium. Com-plete replacement of sodium with either choline or potassium produces an irreversible inhibition of glycogen synthesis in rat diaphragm (Clausen, 1968). Replacement of potassium ions by choline or the addition of ouabain to the incubation medium both cause an increase in the incorporation of glucose into glycogen by rat diaphragm and Clausen (1966) has suggested that the changes in the pattern of glucose metabolism are secondary to changes in the activity of the cationic pump.

Amino Acid Transport and Protein Synthesis

A relationship between the cationic composition of the extracellular medium and the carrier mediated uptake of amino acids has been well documented. In 1952 Christensen et al. noted that partial replacement of the sodium ions in the medium by either potassium or choline resulted in a decrease in the uptake of glycine. Subsequent work (Riggs et al., 1958) demonstrated a correlation between intracellular rather than extracellular potassium ions and glycine uptake and it was proposed that amino acid transport might be driven by the energy inherent in the assymetrical distribution of potassium ions. More recent reports have criticized this earlier proposal (Hempling, 1960; Hempling and Hare, 1961; Heinz, 1962) and attention has been switched from potassium to sodium ions. Kromphardt and co-workers (1963) for example, demonstrated a direct dependence of glycine influx on the external concentration of sodium. Heinz (1962) has shown that if the sodium level is held constant at 90 mM and the potassium concentration varied between 6 and 65 mM little change in the initial uptake of glycine is observed. If on the other hand potassium is increased at the expense of sodium a decrease in glycine influx occurs under conditions of high potassium low sodium concentrations. Heinz concluded that ". . . the inhibitory effect of potassium on the flux seems to be only apparent and rather due to the concomitant decrease in extracellular sodium." The studies of Vidaver (1964) on glycine uptake by pigeon red cells demonstrated that

glycine was concentrated only if a sodium gradient existed and that glycine moved in the direction of the gradient. In addition, if cells containing high sodium and low potassium concentrations were placed in a reverse medium they pumped glycine outward against its concentration gradient. These results support the hypothesis that the sodium gradient furnishes the energy for the transport of glycine and is in agreement with the observations of Crane (1964) with respect to sugar transport in intestine.

Kinetic analysis have demonstrated that at low sodium concentrations the K_m value for AIB transport in rabbit lymphnodes (Kipnis and Parrish, 1965) and amino acid uptake in the eye lens (Cotlier and Beaty, 1967) is increased. Changes in both the K_m and V_{max} values for amino acid uptake in Ehrlich ascites cells (Inui and Christensen, 1966), pigeon erythrocytes (Wheeler et al., 1965) and rabbit red cells (Wheeler and Christensen, 1967) accompany changes in the external sodium concentration.

In an investigation of the effects of dietary potassium levels on the anabolic capacity of rats, Frost and Sandy (1953) observed that animals suffering from a potassium deficiency were unable to utilize amino acids for the synthesis of new proteins. Similarly, it has been observed that chicks maintained on a potassium free diet incorporate significantly less amino acid into skeletal muscle protein than do control chicks (Rinehart et al., 1968). In vitro, potassium concentrations of between 60 and 100 mM were found to be optimum for protein synthesis in liver microsome preparations (Sachs,

1957) and similar findings were reported for extracts of E. coli (Lubin and Ennis, 1964; Schlessinger, 1964). A priming reaction for protein synthesis by E. coli extracts involving mRNA and ribosomes and requiring either ammonium or potassium ions was described by Lubin (1963). Nakamoto et al. (1963) made a similar observation and suggested that a high concentration of either ammonium or potassium ions is required for the formation of an aminoacyl-sRNA-ribosome complex. A role for potassium ions in the transfer of amino acid from sRNA to the polypeptide has been suggested (Lubin and Ennis, 1964) and more recently Sarker (1969) proposed that binding to the aminoacyl site, but not to the peptidyl site, is dependent on the presence of either ammonium or potassium ions.

1-4. OBJECTIVES OF THE PRESENT WORK

The present study was undertaken as part of a continuing investigation of the energy metabolism of tumor cells. Interest in the Novikoff ascites cells stemmed from the ability of these cells to convert glucose to glycogen and was heightened by the observation of Nigam (1966) that the utilization of glucose for glycogen synthesis resulted in a considerable alteration of the energy metabolism of this tissue. In the preliminary experiments it was established that the general pattern of metabolism in the presence of glucose could be markedly altered by either preincubating the cells prior to

the addition of exogenous substrates or by changing the ionic composition of the incubation medium. The experiments to be described were performed in an attempt to determine the nature of the factors involved in the regulation of the energy metabolism of these cells under the conditions discussed above.

CHAPTER II

MATERIALS AND METHODS

2-1. CHEMICALS

All common chemicals were of "reagent grade" and were used without further purification. All bases, nucleosides and nucleotides were obtained from Sigma Chemical Co., St. Louis, Missouri, USA, as were NADH, NADP, and all phosphorylated glycolytic intermediates used in the enzyme assays. Phosphoribosylpyrophosphate and cytochrome C were purchased from the Nutritional Biochemical Corporation, Cleveland, Ohio, USA, and hadacidin was obtained from Merck, Sharp and Dohme, Rahway, New Jersey, USA.

MN-cellulose powder 300 and poly(ethyleneimine) for the preparation of plates for thin layer chromatography were obtained from Brinkmann Instruments Inc., Westbury, New York, USA, and BASF Canada Ltd., Montreal, Quebec, Canada, respectively.

The following enzymes were purchased from Sigma Chemical Co.: hexokinase (type III), aldolase, α-glycerophosphate dehydrogenase, triose

phosphate isomerase, pyruvic kinase (type II), lactic dehydrogenase (type V), and 5'-adenylic acid deaminase. Phosphoglucose isomerase and glucose-6-phosphate dehydrogenase (A grade) were obtained from Calbiochem, Los Angeles, California, USA. Glucostat was obtained from Worthington Biochemical Corporation, Freehold, New Jersey, USA.

Glucose-2-C^{14}, adenine-8-C^{14}, and hypoxanthine-8-C^{14} were purchased from the Radiochemical Center, Amersham, England. Glycine-2-C^{14}, leucine-1-C^{14}, 1-aminocyclopentane-1-carboxylic acid-carboxyl-C^{14}, and inulin-carboxyl-C^{14} were obtained from New England Nuclear Corporation, Boston, Massachusetts, USA.

All solutions were made up with distilled water. Solutions of organic compounds were stored at -20°C. Labelled compounds were made up in stock solutions without the addition of carrier and diluted with the corresponding non-radioactive compound as desired.

2-2. MAINTENANCE OF TUMOR CELLS

The Novikoff ascites hepatoma was carried in male Sprague-Dawley rats weighing 200 to 250 g by weekly intraperitoneal injection of ascitic fluid.

The Ehrlich ascites carcinoma was grown in Swiss white mice weighing 20 to 25 g. The tumor was transplanted into the peritoneal cavity by injection of a suspension of cells usually derived from the solid form of

the tumor. The solid tumor material was finely minced and injected intraperitoneally to form the ascitic tumor. The first generation of ascites cells was frozen and stored on dry ice until used.

2-3. PREPARATION OF TUMOR CELLS

Rats which had been injected with tumor cells 7 days previously were sacrificed by decapitation. The ascitic fluid was removed from the peritoneal cavity and 6-8 ml placed in centrifuge tubes kept in an ice water mixture. After diluting the ascitic fluid to 12 ml with ice cold isotonic saline the tubes were centrifuged for 30 seconds at 800 xg on an International Equipment Company clinical model centrifuge. The cells were routinely washed 5 or 6 times with ice cold isotonic saline following which they were centrifuged for 90 seconds at 800 xg to obtain the packed cell volume. Finally the cells were diluted to 10 times their own volume with calcium-free Krebs-Ringer solution. For the experiments 1 ml of this suspension (equivalent to 12 mg dry weight of tissue) was used per 3 ml of incubation medium.

Ehrlich ascites cells were prepared in the same manner as the Novikoff ascites cells. The animals carrying the tumor were killed by cervical dislocation 6 or 7 days after transplantation, the ascitic fluid was removed, and the cells washed with ice cold isotonic saline as described above. The washed cells were packed for 90 seconds at 800 xg and diluted to 10

times their volume with calcium-free Krebs-Ringer solution. One ml of this suspension was used for the experiments.

2-4. PREPARATION OF HOMOGENATES

The washed Novikoff ascites cells were suspended in 10 times their own volume of ice cold one-quarter strength calcium-free Krebs-Ringer solution and allowed to stand in the cold for 10 minutes. The cells were then centrifuged at 800 xg for 2 minutes, resuspended in a volume of ice cold distilled water equal to the original volume of packed cells and homogenized for 2 minutes at $4^{o}C$. 1.5 ml of homogenate was then added to 4.5 ml of 4/3 strength calcium-free Krebs-Ringer solution and 1 ml of this suspension (equivalent to 0.125 ml of packed cells) per 3 ml incubation medium was used in the experiments. The above procedure is similar to that described by Nigam (1967a).

2-5. INCUBATION METHODS

All incubations were carried out under air and unless otherwise noted the basic medium used was a calcium-free Krebs-Ringer solution containing 145 mM NaCl, 5.8 mM KCl, 1.5 mM KH_2PO_4, and 1.5 mM $MgSO_4$ buffered at pH 7.4 with 10 mM sodium phosphate. Further additions were made as indicated in the individual experiments. The final volume was 3 ml. Incubations were performed at $37^{o}C$ and were generally carried out in 25 ml

Erlenmyer flasks in a shaker bath. Experiments in which the ionic environment was altered were performed in a medium similar to the above except that various amounts of sodium were replaced with other monovalent cations. The concentration of potassium and sodium ions in the K-103 medium were 103 mM and 59 mM respectively.

Unless otherwise noted additions following preincubation were made directly into the preincubation vessel.

The incubation medium for the homogenates was similar to the above but contained in addition 5.7 μmoles of NAD and 0.105 μmole of cytochrome C in 3 ml of solution. The potassium medium used for the homogenate experiments had all of the sodium replaced by potassium in the Krebs-Ringer solution and used 10 mM sodium phosphate as the buffer.

2-6. MEASUREMENT OF OXYGEN UPTAKE

The rate of respiration was measured by the conventional Warburg technique (Umbreit et al., 1964). Carbon dioxide was absorbed by 0.2 ml of 10% (w/v) KOH in the centre well with a Whatman No. 1 filter paper strip inserted in it. QO_2 values were calculated from the respiratory rate between 20 and 60 minutes after the initiation of the incubation or the addition of exogenous substrates.

2-7. DETERMINATION OF LACTIC ACID AND SEVEN MINUTE NUCLEOTIDE PHOSPHATE

Following the incubation period the contents of the incubation vessel were poured into tubes containing 3 ml of ice cold 5% perchloric acid (PCA) and the extraction allowed to proceed for 30 minutes at 2°C. Following centrifugation portions of the PCA supernatant were used for the determination of lactic acid and 7mnp.

Total lactic acid was determined colorimetrically following treatment with copper sulfate and calcium hydroxide, according to the method of Barker and Summerson (1941). The incorporation of radioactivity from glucose-2-C^{14} into lactic acid was measured using a modification of the method described by Wu (1964a). An aliquot of the PCA extract was added to an equal volume of 20% copper sulfate and approximately 0.5 g of calcium hydroxide was added. The mixture was allowed to stand for 30 to 40 minutes at room temperature with occasional shaking and then centrifuged. To a portion of the supernatant was added 50 µmoles of cold glucose and the solution was again treated with copper sulfate and calcium hydroxide. Following centrifugation an aliquot of the supernatant was counted for radioactivity.

For the determination of 7mnp approximately 30 mg of Norite were added to a portion of the PCA supernatant and the contents of the tube thoroughly agitated. The mixture was allowed to stand for 15 minutes at room temperature with frequent shaking. 0.2 ml of 95% ethanol was then

layered onto the suspension to reduce the amount of charcoal floating on the surface, and the tubes centrifuged for 10 minutes at 800 xg. The supernatant was decanted and the charcoal washed 3 times with 4 ml portions of distilled water. 3 ml of 1 N HCl were added to the washed charcoal and the mixture was heated at 100°C for 7 minutes, during which time the charcoal was maintained in suspension by continuous agitation with glass rods. At the end of the heating period the tubes were cooled by placing them in an ice water bath, centrifuged for 10 minutes at 800 xg and an aliquot of the supernatant analysed for inorganic phosphate by Bartlett's (1959) modification of the method of Fiske and SubbaRow (1925).

2-8. DETERMINATION OF INORGANIC PHOSPHATE

At the termination of the incubation period the contents of the vessel were poured into tubes containing 8 ml of calcium-free Krebs-Ringer solution and standing in an ice water mixture. Following centrifugation the supernatant was aspirated and the cells were extracted for 30 minutes at 2°C with 3 ml of 2.5% PCA. The PCA supernatant was added to approximately 30 mg of Norite, the adsorption procedure being similar to that used for 7mnp determinations. The tubes were centrifuged, the supernatant decanted into another tube and the charcoal washed 3 times with distilled water. The washings were added to the original supernatant and the volume adjusted to 20 ml with distilled water. An aliquot of this solution was analysed for inorganic

phosphate according to Bartlett's (1959) modification of the method of Fiske and SubbaRow (1925).

2-9. DETERMINATION OF GLYCOGEN

At the end of the incubation the contents of the vessel were poured into tubes standing in an ice water bath and the tubes centrifuged at 800 xg for 90 seconds. The supernatant was aspirated, 3 ml of 30% KOH added and the mixture was heated for 30 minutes at 100°C with occasional agitation with glassrods. For the determination of total glycogen 0.5 ml of a saturated solution of Na_2SO_4, followed by 4.5 ml of 95% ethanol was added at the end of the heating period and the resulting mixture brought to a boil. The tubes were centrifuged at 800 xg for 15 minutes, the supernatant decanted and the glycogen pellet washed with water and reprecipitated with 95% ethanol. The washed precipitate was resuspended in water and a portion used for the colorimetric determination of glycogen using the Anthrone reagent (Carroll et al., 1956). When the incorporation of radioactivity from glucose into glycogen was to be determined 5 mg of cold glycogen and 0.2 ml of saturated Na_2SO_4 were added to the KOH digest. The rest of the procedure was similar to that used for total glycogen except that the glycogen pellet was washed 3 times with distilled water. In the earlier experiments an aliquot of the washed glycogen was plated on aluminum planchets, dried under an infrared lamp and radioactivity determined as described below. In the latter

experiments the radioactivity was determined using the standard liquid scintillation counting technique.

2-10. MEASUREMENT OF AMINO ACID UPTAKE AND INCORPORATION INTO PROTEIN

In these experiments the incubation was terminated by adding the vessel contents to 8 ml of calcium-free Krebs-Ringer solution in tubes standing in an ice water bath. Following centrifugation, the supernatant was aspirated and the cells washed once with ice cold calcium-free Krebs-Ringer solution. The washed cells were extracted for 30 minutes at 2°C with 3 ml of 5% trichloroacetic acid (TCA) and an aliquot of the TCA supernatant counted for the determination of amino acid uptake. For the determination of radioactivity incorporated into protein the TCA precipitate was washed twice with 4 ml portions of 5% TCA and subsequently extracted with 6 ml of 95% ethanol at 50 to 60°C for 15 minutes. The ethanol extract was aspirated and the residue washed once with a 5 ml volume of ethanol:ether (1:1 by volume). The lipid-free residue was then heated with 3 ml of 5% TCA at 90°C for 20 minutes to hydrolyse the nucleic acids. Following centrifugation, the precipitate was washed once with 5% TCA and then dissolved in 0.2 ml of hyamine by heating at approximately 50°C for 10 to 15 minutes. This solution was transferred quantitatively to counting vials by rinsing the tubes with scintillation fluid and radioactivity determined as described

below.

2-11. ENZYMATIC ANALYSIS

At the desired time the reaction was stopped by pouring the contents of the incubation vessel into a centrifuge tube standing in an ice water mixture and containing 0.16 ml of 50% PCA. After standing with occasional stirring for approximately 30 minutes at 2°C the suspension was centrifuged for 3 minutes at 800 xg. The supernatant was poured off into a new tube and neutralized with KOH and the $KClO_4$ allowed to precipitate. The mixture was centrifuged for 10 minutes at 800 xg and the supernatant used for the analysis of ATP, ADP, AMP, hexose monophosphates, fructose diphosphate and triose phosphates, and glucose. In the following procedures the optical densities at 340 mμ were measured in 3 ml quartz cuvettes in a Beckman DU spectrophotometer.

Hexose Monophosphates and ATP

These compounds were determined in the same incubation mixture according to the method of Wu (1964b). The supernatant obtained by the above procedure was adjusted to pH 7.4 and the volume made up to 4 ml with distilled water. 1.5 ml of this solution was used for the assay which was performed in an incubation mixture containing (in 3 ml): triethanolamine, pH 7.4, 150 μmoles; $MgCl_2$, 15 μmoles; glucose, 30 μmoles; NADP, 0.3 μmole; glucose-6-phosphate dehydrogenase, 0.4 μg; phosphoglucose

isomerase, 0.5 µg. The increase in optical density at 340 mµ resulting from the reduction of NADP by the conversion of glucose-6-phosphate to 6-phosphoglucono- δ -lactone was a measure of the amount of hexose monophosphates present in the assay mixture. 10 µg hexokinase was added when the readings were constant, and the additional increase in optical density gave the amount of ATP present. A change in optical density of 0.14 units was equivalent to the reduction of 0.1 µmole of NADP.

ADP

ADP was determined according to the method of Nigam (1966). 2 ml of the same solution which was used for the assay of ATP were added to an incubation mixture containing in a final volume of 3 ml: Tris, pH 7.4, 100 µmoles; triethanolamine, pH 7.5, 50 µmoles; $MgSO_4$, 10 µmoles; KCl, 100 µmoles; NADH, 0.43 µmole; phosphoenolpyruvate, 0.5 µmole; lactate dehydrogenase, 0.46 units; pyruvic kinase, 0.85 units. Trace amounts of lactic acid were removed by incubation with lactate dehydrogenase prior to the addition of pyruvic kinase. ADP is utilized in the conversion of phosphoenolpyruvic acid to pyruvic acid. The decrease in optical density at 340 mµ resulting from the subsequent oxidation of NADH by the conversion of pyruvate to lactate was a measure of the amount of ADP present. The oxidation of 0.1 µmole of NADH yielded a change in optical density of 0.13 units.

AMP

The neutralized perchloric acid supernatant was adjusted to pH 6.1 and the volume made up to 4 ml with distilled water. AMP was measured in an incubation mixture containing (in 3 ml): sodium succinate, pH 6.1, 900 µmoles; $MgCl_2$, 20 µmoles; adenylic deaminase, 0.02 µMolar units. The decrease in optical density at 265 mµ resulting from the deamination of AMP was a measure of the amount of AMP present. The deamination of 0.1 µmole of AMP resulted in an optical density change of 0.20 units. The procedure used was similar to that described by Munch-Peterson and Kalckar (1957).

Fructose Diphosphate and Triose Phosphates

The neutralized PCA extract was adjusted to pH 7.4, the volume made up to 4 ml with distilled water, and an aliquot removed from the assay. The assay mixture contained (in 3 ml): triethanolamine, pH 7.4, 150 µmoles; NADH, 5 µmoles; aldolase, 20 µg; triose phosphate isomerase, 6 µg; α-glycerophosphate dehydrogenase, 6 µg. Aldolase converts fructose diphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. The latter two compounds are interconvertible through the action of triose phosphate isomerase. The conversion of dihydroxyacetone phosphate to α-glycerophosphate by α-glycerophosphate dehydrogenase results in the oxidation of NADH. The resulting decrease in optical density at 340 mµ was a measure of the

amounts of fructose diphosphate and triose phosphates present. The oxidation of 0.1 μmole of NADH gave an optical density change of 0.13 units. The method used was similar to that described by Wu (1964b).

Glucose

An aliquot of the PCA supernatant was adjusted to pH 7.4 and glucose was determined using the commercially available Worthington Glucostat Kit. Glucostat is a coupled enzyme system based on the following simplified scheme of reactions:

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

$$H_2O_2 + \text{reduced chromogen} \xrightarrow{\text{peroxidase}} \text{oxidized chromogen}$$

The resulting optical density in the region of 400 mμ is a measure of the amount of glucose present in the assay mixture.

Phosphoribosylpyrophosphate

Phosphoribosylpyrophosphate was determined according to the method of Henderson and Khoo (1965a). The reaction was terminated by pouring the contents of the incubation vessel into tubes standing in a boiling water bath. The cells were heated in this manner for 30 seconds and then cooled by placing the tubes in a bath of ice water. The cells were spun

down at 800 xg for 90 seconds and an aliquot of the supernatant used for the assay. The assay mixture contained, in a final volume of 1.13 ml, 0.18 μmole of adenine-8-C^{14} and 0.1 ml of a 10000 xg supernatant from Ehrlich ascites cells containing the phosphoribosyl transferase enzyme (Henderson and Khoo, 1965a). 0.1 ml of 67.5% TCA was added at the termination of the assay and the TCA supernatant was extracted with water saturated butanol to remove any unreacted adenine (Ellis and Scholefield, 1962). After the butanol extraction the radioactivity in an aliquot of the remaining fraction was determined.

2-12. THIN LAYER CHROMATOGRAPHY

The incubation was terminated in the same manner as that described above for the enzymatic analysis of adenine nucleotides. After a 30 minute extraction period, 30 mg of Norite were added to the PCA supernatant, the adsorption procedure being similar to that described above. After washing the charcoal with water, 8 ml of a solution of 95% ethanol:pyridine: water (50:10:40 by volume) was added and the mixture transferred to 25 ml Erlenmyer flasks and shaken at 37°C for 3 hours to elute the purine derivatives. The mixture was subsequently decanted into tubes, centrifuged, and the supernatant taken to dryness using a Buchler Instruments rotary evapomix. The residue was dissolved in 0.5 ml of distilled water and extracted 6 times with 2 ml of water saturated n-butanol to extract the free bases

and nucleosides (Ellis and Scholefield, 1962). Both the butanol extract and the remaining aqueous fraction were brought to dryness as described above and the residues were redissolved in 0.2 ml of 50% ethanol. 0.1 ml of these solutions was used for thin layer chromatography. The solution obtained from the aqueous fraction was run, with appropriate standards, on PEI-cellulose layers in 0.1N sodium formate buffer, pH 2 (Randerath and Randerath, 1964). The solution derived from the butanol extract was run on cellulose plates in a solvent system consisting of n-butanol:methanol: water:ammonia (S.Gr. 0.90), (60:20:20:1 by volume) (Randerath and Randerath, 1967). The developed chromatograms were analysed under short wave ultraviolet light. The major catabolic product was identified as hypoxanthine by comparing its Rf with standards run on cellulose layers using solvent systems of 1) water, 2) water saturated butanol, and 3) n-butanol: methanol:water:ammonia (S. Gr. 0.90), (60:20:20:1 by volume) (Randerath and Randerath, 1967).

2-13. INCORPORATION OF PURINES INTO THE ACID SOLUBLE FRACTION

Following the desired incubation time the contents of the incubation vessels were poured into centrifuge tubes containing 8 ml of ice cold, calcium-free Krebs-Ringer solution and centrifuged at 800 xg for 90 seconds. The supernatant was discarded and the cells were extracted at 2°C with 3 ml of 2.5% PCA for 30 minutes. The radioactivity in 0.2 ml aliquots of the

PCA extract was determined.

2-14. INCORPORATION OF HYPOXANTHINE-8-C^{14} INTO THE ADENINE NUCLEOTIDE POOL

The initial procedure was similar to that used for determining the incorporation of purines into the acid soluble fraction. 30 mg of Norite was added to the PCA extract and adsorption allowed to proceed for 15 minutes. The charcoal was washed once with distilled water and eluted with 8 ml of ethanol:pyridine:water (50:10:40 by volume) as previously described. The eluant was brought to dryness, redissolved in 0.3 ml of 70% PCA and heated for 60 minutes at 100°C to hydrolyze the nucleotides to the free bases (Marshak and Vogel, 1951). Following hydrolysis the solution was made up to 3 ml with distilled water, and adsorbed onto, and eluted from Norite. The eluant was brought to dryness, redissolved in 50% ethanol and run with cold adenine as carrier on thin layer cellulose plates in n-butanol:methanol: water:ammonia (S. Gr. 0.90), (60:20:20:1 by volume) (Randerath and Randerath, 1967). The adenine spot was located under short wave ultraviolet light, scraped off the plate, and the radioactivity determined as described below.

2-15. DETERMINATION OF INTRACELLULAR WATER AND ION LEVELS

The intracellular water content was determined using inulin-carboxyl-C^{14}. At the end of the incubation period the contents of the incubation

vessels were poured into tubes and centrifuged for 90 seconds at 800 xg. The supernatant was discarded and the cells were suspended in 3 ml of ice cold iso-osmotic choline chloride containing approximately 1 μcurie of inulin-carboxyl-C^{14}. The cells were centrifuged, the supernatant withdrawn, the sides of the tube carefully dried and the cells resuspended in choline chloride. Following recentrifugation an aliquot of the supernatant was taken for the determination of radioactivity present. Duplicate vessels were used for the determination of the wet weight at the various time intervals tested. The dry weight was measured by heating an aliquot of the cell suspension obtained prior to the start of the incubation at approximately 120°C until a constant weight was obtained. The intracellular water was calculated by subtracting the dry weight from the wet weight and then subtracting the inulin space from this value.

The procedure used for the determination of the intracellular sodium and potassium levels was similar to the method described by Pappius and Elliot (1956). Following the incubation period the cells were washed twice with 4 ml of ice cold iso-osmotic choline chloride buffered at pH 7.4 with 10 mM Tris (Hempling, 1962). 0.4 ml of concentrated nitric acid was added and the tissue digested at approximately 60°C until the solution became clear. The digest was made up to 10 ml with distilled water. To 4 ml of this solution was added 1 ml of 1M NaCl and this fraction was used for the determination of potassium ions. The remaining portion of the digest was used for

the sodium determinations. Sodium and potassium were measured using a Beckman Model B spectrophotometer with flame attachment. The values which were obtained in this manner represented the total of both intracellular and extracellular ions. Correction for extracellular ions was made by calculating the amount of these ions which were present using the values for extracellular water which were determined as described above.

2-16. MEASUREMENT OF RADIOACTIVITY

In most experiments radioactivity was determined using a series 3000 Packard Instrument Company liquid scintillation counter. Aliquots of the material to be counted (maximum volume of 300λ) were placed in glass or plastic vials containing 10 ml of a scintillation mixture consisting of 5.0 g of 2, 5-diphenyloxazole (PPO), 50 mg of 1, 4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl-POPOP), and 80 g of naphthalene in 1 litre of toluene:dioxane:ethanol (1:1:1 by volume). Correction was routinely made for background and, when necessary, for error due to quenching.

In some of the earlier experiments a portion of the radioactive glycogen suspension was plated on aluminum planchets, dried under an infrared lamp, and the radioactivity determined using a Nuclear-Chicago Model 470 gas flow detector.

In some experiments radioactive spots were scraped from thin layer chromatographic plates and counted in vials containing 15 ml of

scintillation fluid consisting of 4 g PPO, and 100 mg dimethyl-POPOP in 1 litre of toluene.

CHAPTER III

PRELIMINARY STUDIES ON THE ENERGY METABOLISM OF THE NOVIKOFF ASCITES HEPATOMA

The present investigation was undertaken in an attempt to define more clearly the existing relationships between the utilization and production of cellular energy. Because of the major contribution made by glycolysis to the production of energy in tumor cells glucose was the substrate of choice. In addition to serving as a major energy source glucose, as a result of its conversion to glycogen, is also a relatively large energy consumer in the Novikoff ascites cells. Interest in the relationship between the metabolism of glucose and other cellular processes in this tissue are of particular interest in view of the changes which glycogen synthesis introduces into the normal pattern of tumor energy metabolism (Nigam, 1966). Variations in the level of ATP upon the addition of glucose to aerobically metabolizing tumor cells have been observed (e.g. Chance and Hess, 1959a) and changes in the steady state level of ATP in the presence of glucose, as compared to the endogenous value, have been reported (e.g. Nigam, 1966). It was therefore of interest to know what effect glucose was having on the intracellular high energy phosphate levels under the present experimental condi-

tions. This was initially determined through measurement of the readily hydrolyzable nucleotide phosphate. In addition to glycogen synthesis, glycolysis, respiration, and the levels of high energy nucleotide phosphate, the active uptake of amino acids and their incorporation into protein were determined as two other processes which are dependent on the intracellular supply of energy.

3-1. THE EFFECTS OF GLUCOSE ON THE METABOLISM OF NOVIKOFF ASCITES HEPATOMA CELLS

The results of Tables I and II show the effects of the addition of 10 mM glucose on the metabolism of Novikoff ascites cells. In confirmation of previous findings (Nigam, 1966), the level of high energy nucleotide phosphate was decreased in the presence of glucose. However, the reduced rate of respiration in the presence of glucose and the relatively low incorporation of glucose into glycogen, compared to that converted to lactic acid, are in contrast to the results reported by Nigam (1966) in which a Crabtree effect was not observed and the ratio of glucose utilized for glycogen formation to that incorporated into lactic acid was approximately two.

In the presence of glucose the rates of glycine and leucine incorporation into protein were reduced by 35% and 30% respectively, although glucose addition did not alter amino acid uptake. These results are in

TABLE I

RESPIRATION, GLYCOLYSIS, GLYCOGEN SYNTHESIS, AND 7 MINUTE NUCLEOTIDE PHOSPHATE LEVELS IN NOVIKOFF ASCITES HEPATOMA CELLS

Additions	QO_2	μatoms glucose-2-C^{14}* incorporated/ ml packed cells		μmoles 7mnp/ ml packed cells
		Lactic acid	Glycogen	
Nil	9.4	-	-	4.1
10 mM glucose	7.7	25	22	2.7

For the determinations of glucose-2-C^{14} incorporation into lactic acid and glycogen, and for the 7mnp determinations the incubation time was 25 minutes. The QO_2 values were calculated from the respiratory rates between 20 and 60 minutes after the start of the incubation and represent the average of 4 separate experiments.

*μatoms glucose-2-C^{14} refer to μatoms of radioactive carbon from glucose-2-C^{14}.

TABLE II

THE EFFECTS OF GLUCOSE ON AMINO ACID UPTAKE AND INCORPORATION INTO PROTEIN

Additions	μmoles/ml packed cells			
	Glycine-2-C^{14} uptake	Glycine-2-C^{14} incorporation x 10^2	ACPC-C^{14} uptake	Leucine-1-C^{14} incorporation x 10^2
Nil	2.5	34	5.3	40
10 mM glucose	2.5	22	5.7	28

The incubation time was 60 minutes. The extracellular concentrations of the amino acids were 2 mM. The values presented represent the average of from 2 to 5 separate experiments.

contrast to previous studies which demonstrated that protein synthesis by tumor cells was similar in the presence and absence of glucose (Quastel and Bickis, 1959).

3-2. THE EFFECTS OF PREINCUBATION

Because of the relatively low rates of glycogen synthesis which were observed (Table I) several attempts were made to increase the activity of this pathway relative to the glycolytic utilization of glucose. The results presented in Table II are from a typical experiment in which the cells were preincubated for 10 minutes prior to the addition of exogenous substrate. This treatment produces several changes in the metabolic pattern. For example, in the experiment presented preincubation resulted in a 55% increase in the level of high energy phosphate and a 70% increase in the amount of glucose incorporated into glycogen during the 25 minute incubation period. The amount of lactic acid produced was slightly decreased following preincubation, and the ratio of glycogen to lactate formation was increased from 0.65 to 1.3. In spite of these alterations in the pattern of glucose metabolism preincubation did not produce a significant difference in respiratory activity, the magnitude of the Crabtree effect being similar in both control and preincubated cells.

The effects of preincubation on glycine uptake and incorporation into protein are presented in Table IV. A small but consistent increase in

TABLE III

THE EFFECTS OF PREINCUBATION ON RESPIRATION, GLYCOLYSIS, GLYCOGEN SYNTHESIS, AND 7 MINUTE NUCLEOTIDE PHOSPHATE LEVELS

Time of preincubation (minutes)	QO_2		μatoms glucose-2-C^{14} incorporated/ ml packed cells		Glycogen / Lactic acid	μmoles 7mnp/ ml packed cells
	No additions	Plus 10 mM glucose	Lactic acid	Glycogen		
0	7.4	6.6	29	19	0.65	2.2
10	7.4	6.3	24	32	1.3	3.4

Glucose was added to a final concentration of 10 mM at zero time or following a 10 minute preincubation and the incubation continued for an additional 25 minutes. The QO_2 values represent the average of four separate experiments and were determined as described in Table I for the non-preincubated cells and between 20 and 60 minutes following the addition of glucose to the preincubated cells.

TABLE IV

THE EFFECTS OF PREINCUBATION ON GLYCINE UPTAKE AND INCORPORATION INTO PROTEIN

Additions	Time of preincubation (minutes)	μmoles glycine-2-C^{14}/ml packed cells	
		Uptake	Incorporation x 10^2
2 mM glycine	0	4.2	32.1
	10	4.3	33.0
2 mM glycine plus 10 mM glucose	0	4.2	24.6
	10	4.0	27.7

Glycine, or glycine plus glucose, was added at zero time or following a preincubation of 10 minutes and the incubation continued for an additional 60 minutes.

protein synthesis in the presence of glucose was observed following preincubation. Glycine uptake in the presence or absence of glucose and glycine incorporation in the absence of glucose were unaffected by this treatment.

3-3. THE EFFECTS OF INCREASED EXTRACELLULAR POTASSIUM CONCENTRATIONS

It has been well documented that maximum glycogen deposition in liver slice preparations is obtained in an incubation medium in which all of the sodium ions have been replaced by potassium ions (Hastings et al., 1952). Experiments were therefore performed to determine the effects of increased extracellular potassium levels on the glucose metabolism of Novikoff ascites cells. Preliminary results indicated that the conversion of glucose to glycogen by the tumor cells was inhibited, rather than increased, in the presence of elevated levels of extracellular potassium. The results presented in Tables V and VI are typical of the changes in the metabolic pattern which were produced when the concentration of potassium in the incubation medium was increased. In these experiments two-thirds of the sodium was replaced by potassium (K-103 medium). This change in the extracellular ionic environment results in a decrease in the rate of respiration in the absence of glucose, but has little, if any, effect on the respiratory activity in the presence of glucose. Increasing the concentration of extracellular potassium produced little change in the energy state of the cell if glucose was omitted

TABLE V

THE EFFECTS OF INCREASED EXTRACELLULAR POTASSIUM LEVELS ON RESPIRATION, GLYCOLYSIS, GLYCOGEN SYNTHESIS, AND 7 MINUTE NUCLEOTIDE PHOSPHATE LEVELS

Additions	Incubation medium	QO_2	μatoms glucose-2-C^{14} incorporated/ ml packed cells		μmoles 7mnp/ ml packed cells
			Lactic acid	Glycogen	
Nil	Control	9.4	-	-	4.1
	K-103	7.4 (79)	-	-	4.6 (112)
10 mM glucose	Control	7.7	29	24	2.7
	K-103	7.7 (100)	14 (48)	11 (47)	1.2 (44)

The incubation time was 25 minutes. The K-103 incubation medium is a normal Krebs-Ringer phosphate buffer in which some of the sodium ions have been replaced by potassium ions so that the final concentration of sodium ions is 59 mM and that of potassium ions is 103 mM. The QO_2 values represent the average of 4 separate experiments and were determined as described in Table I. The numbers in parenthesis refer to percentages of the control values.

from the incubation medium, but the level of high energy nucleotide phosphate in the presence of glucose was reduced by more than 50% under these conditions. Both glycolysis and glycogen synthesis were also decreased by approximately one-half when the extracellular potassium concentration was increased to 103 mM.

The effects of increased levels of extracellular potassium on glycine uptake and incorporation into protein are presented in Table VI. In confirmation of previous studies (Christensen et al., 1952) replacement of sodium by potassium resulted in a considerable reduction in glycine uptake. Protein synthesis was also decreased in the K-103 medium, the inhibitory effects of potassium on protein synthesis being much greater in the presence of exogenous glucose than in its absence.

The effects of varying concentrations of extracellular potassium on the metabolism of Novikoff ascites and Ehrlich ascites cells are shown in Table VII. In contrast to the marked inhibitory effects which potassium exerts on the metabolism of the Novikoff tumor the Ehrlich ascites cells were found to be relatively insensitive to changes in the sodium and potassium content of the external medium. Replacement of sodium chloride by choline chloride produced little change in the metabolic activity or the energy levels of either tumor, indicating that the inhibitions observed in the presence of increased levels of potassium are not solely a consequence of the concomitant decreases in the concentration of sodium.

TABLE VI

THE EFFECTS OF INCREASED EXTRACELLULAR POTASSIUM LEVELS ON GLYCINE UPTAKE AND INCORPORATION INTO PROTEIN

Additions	Incubation medium	μmoles glycine-2-C^{14}/ml packed cells	
		Uptake	Incorporation x 10^2
2 mM glycine	Control	3.0	34
	K-103	1.6 (53)	26 (76)
2 mM glycine plus 10 mM glucose	Control	2.9	26
	K-103	1.6 (55)	3 (12)

The experimental conditions were as described in Table V except that the incubation time was 60 minutes. The numbers in parenthesis refer to the percentages of the control values.

TABLE VII

THE EFFECTS OF INCREASING EXTRACELLULAR POTASSIUM CONCENTRATIONS ON GLUCOSE METABOLISM AND 7 MINUTE NUCLEOTIDE PHOSPHATE LEVELS IN NOVIKOFF ASCITES AND EHRLICH ASCITES CELLS

Concentrations of Na^+ and K^+ in the incubation medium (mM)		Novikoff Ascites			Ehrlich Ascites	
		µmoles 7mnp/ml packed cells	µmoles glucose equivalents/ml packed cells		µmoles 7mnp/ml packed cells	µmoles glucose equivalents of lactic acid formed/ml packed cells
Na^+	K^+		Lactic acid	Glycogen**		
155	7.3	3.5	71	34	4.4	62
106	56	3.2 (92)	56 (79)	20 (59)	4.4 (100)	58 (94)
59	103	2.4 (68)	39 (55)	9 (26)	4.3 (98)	54 (87)
10	152	1.0 (29)	23 (32)	1 (3)	3.8 (86)	45 (73)
10*	7.3	3.3 (94)	58 (82)	39 (115)	4.5 (102)	62 (100)

The incubation time was 60 minutes and the concentration of glucose was 10 mM. The numbers in parenthesis refer to percentages of the control values (i.e. in the presence of 155 mM sodium).

*Choline chloride was added to a final concentration of 145 mM.

**The values for glycogen represent the increase in total glycogen over the content at the start of the incubation, which was 22 µmoles glucose equivalents/ml packed cells.

3-4. THE METABOLIC EFFECTS OF SODIUM REPLACEMENT BY RUBIDIUM OR LITHIUM

It has been frequently observed that many of the biological effects of potassium are also produced by rubidium, whereas lithium tends to exhibit biological properties in common with sodium. The effects of partial replacement of sodium in the Krebs-Ringer buffer solution by these two ions are presented in Table VIII. Rubidium mimics the metabolic effects of potassium in every respect. On the other hand, the effects of lithium substitution are somewhat more complex. Unlike the effects of sodium substitution by rubidium or potassium, replacement of sodium by lithium had little effect on the level of 7mnp. Although the uptake of glycine by cells incubated in the choline or lithium substituted medium was similar, protein synthesis in the latter instance was greatly decreased. Incubation in the presence of lithium resulted in a decrease in the conversion of glucose to both lactic acid and glycogen, although as with protein synthesis, these inhibitions were not as great as those observed when either potassium or rubidium was the substituting cation.

TABLE VIII

THE EFFECTS OF SUBSTITUTING OTHER MONOVALENT CATIONS FOR SODIUM ON THE METABOLISM OF NOVIKOFF ASCITES HEPATOMA CELLS

Major cation in the incubation medium	μmoles 7mnp/ml packed cells	μmoles glycine-2-C^{14}/ml packed cells		μmoles lactic acid/ml packed cells	μatoms glucose-2-C^{14} incorporated into glycogen/ml packed cells
		Uptake	Incorporation x 10^2		
Sodium	2.8	4.5	27	128	15
Choline	2.7 (96)	3.3 (73)	27 (100)	121 (94)	14 (93)
Lithium	2.5 (89)	3.0 (66)	13 (48)	86 (67)	5 (33)
Potassium	1.2 (43)	2.0 (44)	4 (15)	55 (43)	3 (20)
Rubidium	1.2 (43)	2.0 (44)	4 (15)	60 (47)	3 (20)

The incubation time was 60 minutes. The concentration of the substituting cations was 96 mM, except potassium which was 103 mM. The concentrations of glycine and glucose were 2 mM and 10 mM respectively. Glycogen synthesis was determined in separate experiments. The numbers in parenthesis refer to the percentages of the control values.

SUMMARY

1. Incubation of Novikoff ascites hepatoma cells in the presence of glucose results in a decrease in protein synthesis and in the 7mnp level from the values obtained in the absence of glucose. A Crabtree effect is observed.

2. Preincubation prior to the addition of glucose results in an increase in the level of 7mnp and in glycogen synthesis as compared to non-preincubated cells. A small increase in protein synthesis and decrease in glycolysis is produced by preincubation. A Crabtree effect is exhibited by the preincubated cells.

3. Replacement of sodium by potassium in the incubation medium results in inhibitions of glycolysis, glycogen and protein synthesis, and amino acid transport. The level of 7mnp is decreased in the presence of glucose and high levels of extracellular potassium but the energy level in the absence of glucose is unaffected by increased potassium concentrations.

4. The inhibitions produced by potassium are mimicked in every respect by rubidium. Lithium has a somewhat lesser inhibitory action and, with the exception of amino acid transport, choline has no effect on the measured parameters of cellular metabolism.

CHAPTER IV

STUDIES ON THE REGULATION OF GLUCOSE METABOLISM AND THE EFFECTS OF GLUCOSE ON OTHER ENERGY REQUIRING PROCESSES

The observations described in Chapter III raise a number of questions concerning the relationship between the metabolism of glucose and the general energy metabolism of the cell. What, for example, is the relationship between the different levels of energy in the preincubated and non-preincubated cells and the different patterns of glucose metabolism under these two conditions, what changes in the metabolic state of the cell occur during the preincubation period that result in the different metabolic pattern following this treatment, how does potassium alter the energy state of the cell and what is the relationship between the reduced energy levels and the other metabolic inhibitions observed in the presence of increased levels of this ion? The experiments described in this chapter were performed in an attempt to answer these and similar questions.

4-1. TIME COURSE EXPERIMENTS

In order to investigate more carefully the metabolism of glucose

and the effects of glucose on the level of cellular high energy nucleotide phosphate time course experiments were performed, the results of which are presented in Figures 2, 3, and 4.

Under both experimental conditions employed (i.e. in the control and K-103 media) the pattern of glycogen synthesis was observed to be similar; an initial lag period followed by a slowly increasing rate of glycogen deposition. Whereas in the control cells the rate of glycogen deposition eventually exceeded that of lactic acid formation glycolysis in cells incubated in the K-103 medium was at all times the predominant of the two pathways. The major difference in glycolytic activity between the control and K-103 cells occurs 10 minutes after the addition of glucose, at which time the rate of lactic acid formation is increased in the former and decreased in the latter medium. Although the glycolytic rates during the latter half of the incubation period are similar under both experimental conditions, the maximum rate attained in the K-103 medium is less than one-half of the maximum rate exhibited by the control cells (0.7 μmoles glucose equivalents/ml packed cells/minute in the K-103 medium as compared to a corresponding value of 1.5 for the control cells).

The effects of glucose and of extracellular potassium concentration on the levels of high energy nucleotide phosphate in Novikoff ascites cells are presented in Figure 3A. In both the control and K-103 media the high energy nucleotide phosphate content is decreased by approximately 66%

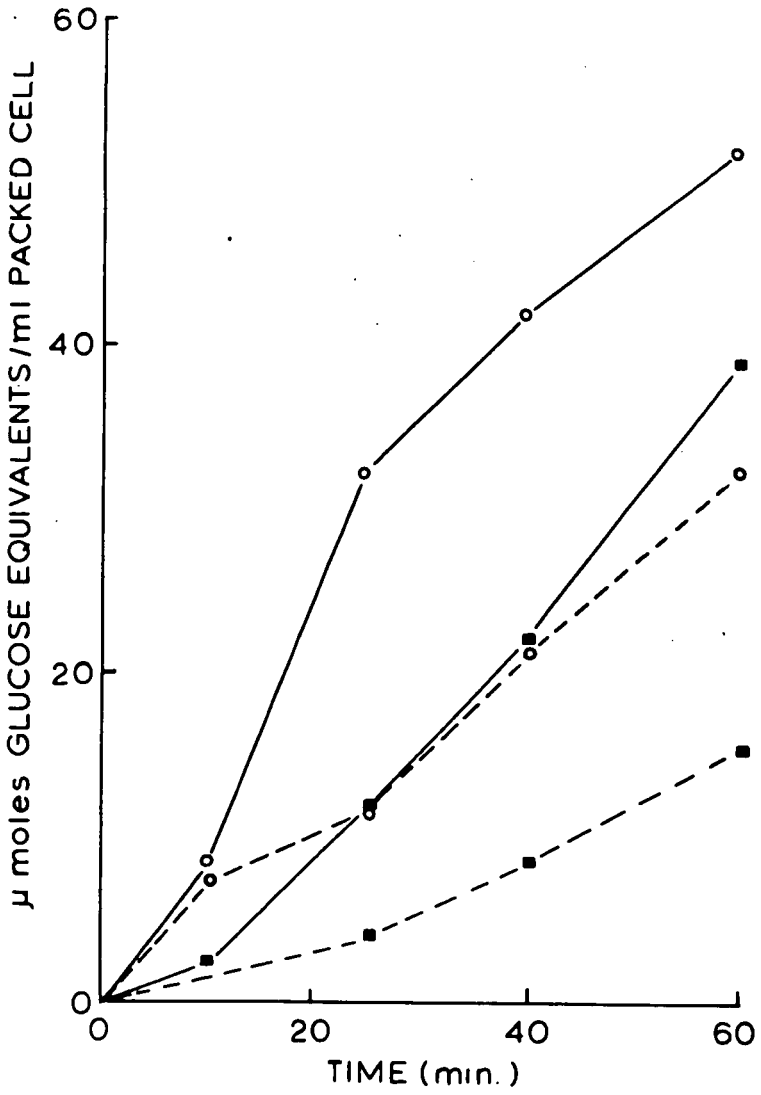

FIGURE 2. Glycolysis and glycogen synthesis in the control and K-103 media. The concentration of glucose was 10 mM. ○, lactic acid; ■, glycogen (increase in glucose equivalents); ———, control; — — — —, K-103.

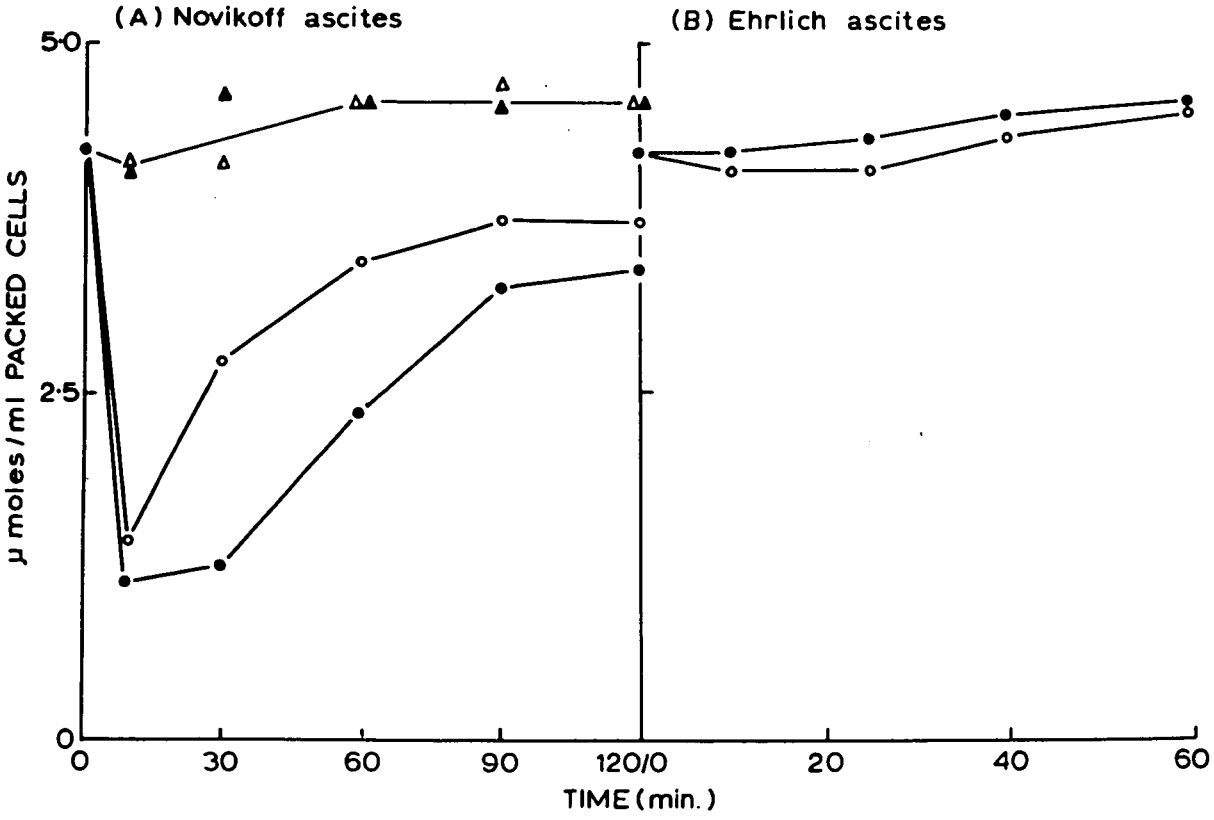

FIGURE 3. The effects of glucose on the levels of 7mnp in Novikoff ascites cells and Ehrlich ascites cells. A) open figures, control medium; closed figures, K-103 medium; △ and ▲ no additions; ○ and ● , plus 10 mM glucose. B) ● , control medium no additions; ○ , control medium plus 10 mM glucose.

within 10 minutes after the addition of glucose. As with glycolysis the greatest difference between the control and K-103 cells with respect to the regeneration of high energy nucleotide phosphate occurs between 10 and 30 minutes following the addition of glucose. The maximum rate of 7mnp formation in the K-103 medium is considerably less than the maximum rate observed in the control cells, the values for the experiment presented being 37 mμmoles/ml packed cells/minute for the K-103 cells as compared to 65 mμmoles/ml packed cells/minute for the control cells.

In contrast to its effects on the Novikoff tumor, and in confirmation of previous findings (Quastel and Bickis, 1959; Ibsen et al., 1959) glucose addition did not significantly alter the energy state of the Ehrlich ascites cell (Figure 3B).

Figure 4 presents the results of shorter time courses designed to investigate in more detail the metabolic activity during the initial minutes of the incubation period. The events of the first few minutes in both the control and K-103 media are similar and are characterized by:

1) an extremely rapid decrease in the level of 7mnp during the first minute, the minimum level being reached after one to three minutes of incubation;

2) an initial period of rapid lactic acid production lasting approximately one minute, following which glycolysis enters an inhibited phase; and

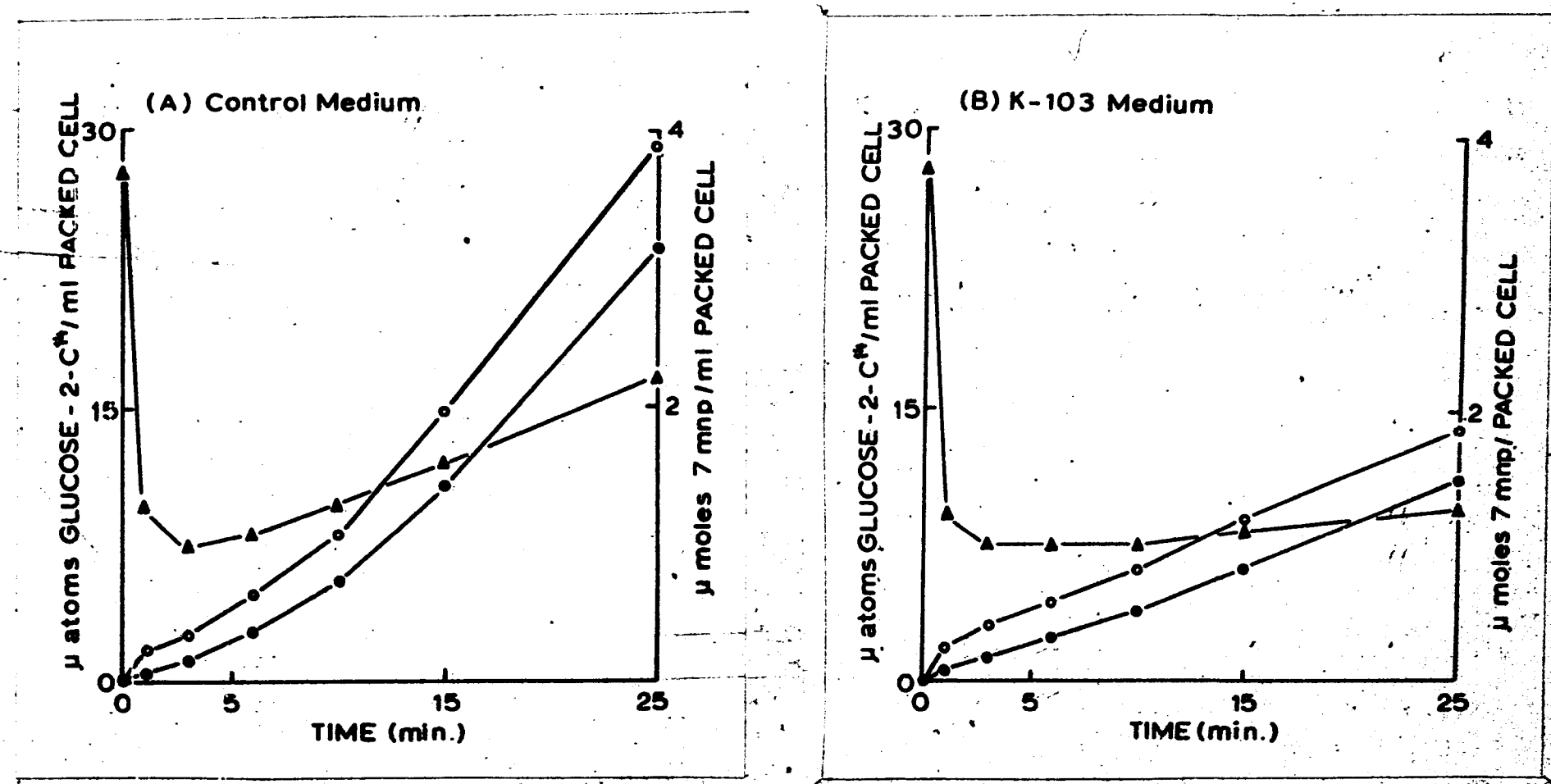

FIGURE 4. 7mnp levels and glucose-2-C^{14} incorporation into lactic acid and glycogen. The concentration of glucose was 10 mM. ▲ , 7mnp; ○ , lactic acid; ● , glycogen.

3) Low rates of glucose conversion to glycogen.

This is as far as the similarity between the two experimental conditions goes, however. In the control cells the rates of glycolysis and glycogen synthesis as well as the level of 7mnp all increase from three minutes onwards, but cells incubated in the K-103 medium demonstrate little change in any of these parameters during the remainder of the 25 minute incubation period. The results presented in Figures 2 and 3A indicate that with longer incubation times glucose metabolism in the K-103 cells eventually increases, the onset of greater activity following upon the onset of 7mnp reaccumulation.

4-2. THE EFFECTS OF VARIOUS SUGARS ON THE LEVEL OF SEVEN MINUTE NUCLEOTIDE PHOSPHATE

Is the depletion of the cell's content of high energy nucleotide phosphate unique to glucose or will other sugars produce a similar effect? The results of a typical experiment in which the level of 7mnp was determined after 10 minutes incubation in the presence of glucose, fructose, mannose, galactose, or ribose are shown in Table IX. Of the five sugars tested only glucose and mannose cause a significant decrease in the energy content of the cell.

The regeneration of 7mnp in the absence of glucose is shown in Table X. In these experiments the cells were preincubated for one

TABLE IX

THE EFFECTS OF VARIOUS SUGARS ON THE 7 MINUTE NUCLEOTIDE PHOSPHATE LEVEL OF NOVIKOFF ASCITES HEPATOMA CELLS

Additions	µmoles 7mnp/ml packed cells
Nil	3.4
Glucose	1.5
Fructose	3.6
Mannose	1.0
Galactose	3.0
Ribose	3.7

The sugars were added to a final concentration of 5 mM at zero time and the incubation allowed to proceed for 10 minutes.

TABLE X

THE REGENERATION OF 7 MINUTE NUCLEOTIDE PHOSPHATE IN THE ABSENCE OF GLUCOSE

Time (minutes)	μmoles 7mnp/ml packed cells	
	Control	K-103
0*	0.5, 0.7	0.6, 0.6
10	3.1, 3.0	2.6, 2.5
30	3.1, 3.3	2.8, 3.1
60	3.2, 3.7	3.0, -
90	3.6, 3.6	3.1, 3.3

The cells were preincubated for 1 minute in the presence of 10 mM glucose in either the control or K-103 medium and the preincubation stopped by placing the vessels on ice. The cells were then centrifuged and washed once with ice cold Krebs-Ringer phosphate buffer. The subsequent incubation was performed in the same medium as the preincubation except that glucose was not present. The results of two separate experiments are presented.

*Following the preincubation and washing procedures.

minute in the presence of glucose in either the control or K-103 medium. Following the preincubation period the cells were washed once with ice cold Krebs-Ringer solution and subsequently incubated in the absence of exogenous substrate. The incubation was performed in the same medium as the preincubation except that glucose was not present. Under these conditions the regeneration of 7mnp is virtually complete within 10 minutes and is similar in both the control and K-103 media.

4-3. INTRACELLULAR LEVELS OF WATER, SODIUM IONS, AND POTASSIUM IONS

Tissues which are maintained in the cold tend to lose potassium to, and to take up sodium from, the surrounding fluid. In order to remove extraneous blood elements from the tumor cells following their removal from the peritoneal cavity, the cells are washed several times with ice cold saline (see Materials and Methods). As a consequence of this process the tumor is maintained at a temperature of approximately $4^{o}C$ for 20 to 30 minutes and it seemed likely that some potassium loss and sodium gain would have occurred during this time. Experiments were performed in order to determine 1) the levels of intracellular sodium and potassium ions following the washing process and 2) the effect of incubation in the presence and absence of glucose on the tissue content of these two ions. In view of the large inhibitions of cellular metabolism produced by increases in extracellular

potassium the effects of incubation in the K-103 medium on the intracellular ion levels were determined.

In the experiments which are described no attempt was made to differentiate between soluble and bound ionic forms and only total sodium and potassium contents are presented.

The Effects of Washing

The results presented in Table XI show that following the washing procedure the sodium content of the cells is relatively high, the respective levels of sodium and potassium ions being 0.33 and 0.29 μequivalents/mg dry weight.

The Effects of Incubation in the Absence of Glucose

Cells incubated in either the control or K-103 medium in the absence of glucose exhibit a pronounced decrease in the level of intracellular sodium ions, the most rapid loss in both cases occurring within the first 10 minutes of the incubation period (Table XI). In the presence of increased levels of potassium ions a rapid initial uptake of potassium is observed, after which the intracellular level of this ion remains relatively constant. Little change in the absolute content of potassium ions in the control cells is observed, although an increase in concentration in terms of intracellular water does occur (Figure 5A).

TABLE XI

THE EFFECTS OF GLUCOSE ON THE INTRACELLULAR LEVELS OF SODIUM IONS, POTASSIUM IONS, AND WATER

Additions	Time (minutes)	Control				K-103			
		ml H_2O/mg dry weight x 10^3	μequivalents/mg dry weight x 10			ml H_2O/mg dry weight x 10^3	μequivalents/mg dry weight x 10		
			Na^+	K^+	$Na^+ + K^+$		Na^+	K^+	$Na^+ + K^+$
a) Nil	0	3.0	3.3	2.9	6.2	3.0	3.3	2.9	6.2
	2	2.8	3.5	2.9	6.4	3.0	2.4	5.7	8.1
	10	2.2	2.4	2.8	5.2	3.0	1.7	6.5	8.2
	25	2.2	1.9	2.9	4.8	2.7	1.3	6.2	7.5
	40	1.9	1.4	3.0	4.4	2.6	1.2	6.3	7.5
	60	1.8	1.3	3.0	4.3	2.9	1.1	6.9	8.0
b) 10 mM glucose	2	2.6	3.4	2.1	5.5	2.8	2.4	5.5	7.9
	10	2.2	3.4	1.3	4.7	3.1	2.4	6.1	8.5
	25	2.1	2.5	2.7	5.2	3.4	2.4	6.6	9.0
	40	2.1	2.1	3.5	5.6	3.7	2.4	7.2	9.6
	60	2.2	1.7	3.9	5.6	4.0	2.4	8.1	10.5

Each value represents the average of three (Na^+/K^+) or five (H_2O) separate experiments.

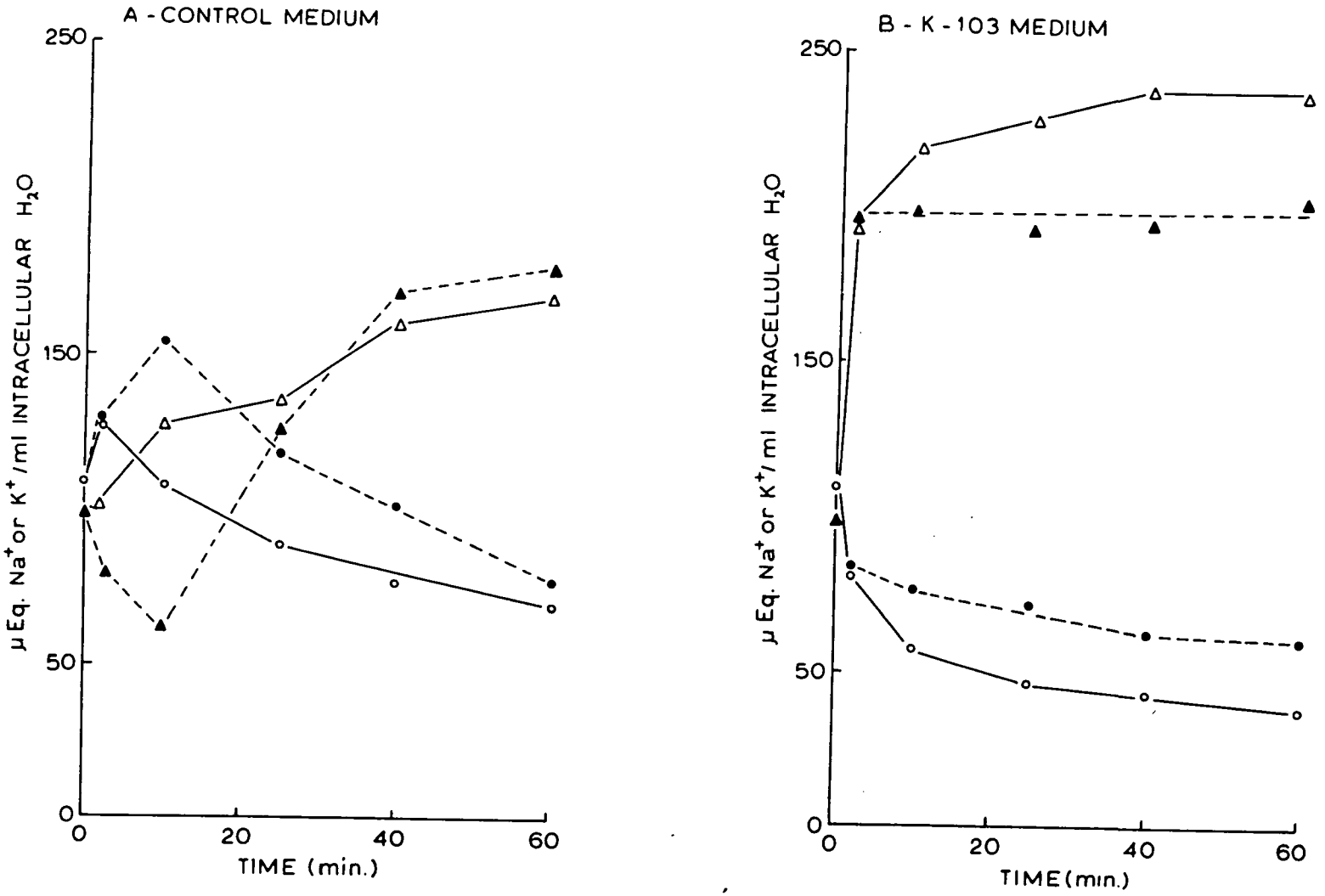

FIGURE 5. The effects of glucose on the intracellular concentration of sodium and potassium ions. The data is from Table XI. open figures, control; solid figures, plus 10 mM glucose; ○ and ● , Na^+ ; △ and ▲ , K^+ .

The Effects of Incubation in the Presence of Glucose

As observed in the absence of glucose, cells incubated in the control medium in the presence of exogenous glucose undergo a loss of intracellular water. However, the addition of glucose causes a considerable change in the pattern of net ion flow. Under these conditions the movements of sodium and potassium ions are consistent with a dependence of potassium accumulation, and sodium extrusion, on the functioning of an energy dependent cationic pump. Thus, during the initial minutes following the addition of glucose, when the level of intracellular energy is minimal, a net decrease in the content of potassium ions and little change in the sodium ion content occurs. It is not until the level of intracellular high energy nucleotide phosphate has begun to increase that the flow of ions in a direction compatible with the reestablishment of the normal ionic gradients is initiated.

In the presence of glucose, as in its absence, a large increase in the level of potassium ions occurs when the incubation is performed in the K-103 medium. However, when glucose is present in the incubation medium the sodium level remains constant and a considerable increase in the intracellular water occurs. In spite of the differences in the absolute levels of sodium and potassium ions following incubation in the K-103 medium in the presence and absence of glucose, the values are quite similar when calculated in terms of intracellular water (Figure 5B).

4-4. THE MEMBRANE AND POTASSIUM INHIBITIONS

It has previously been observed that many of the effects which potassium produces on the metabolism of intact cells do not occur in tissue extracts, suggesting that the action of potassium is mediated via the plasma membrane. The experiments described in this section were performed in order to determine what role, if any, the membrane plays in the potassium induced decreases in metabolic activity which occur under the present experimental conditions.

Homogenates

The results of a typical experiment in which the effects of increased potassium concentration on the glycolytic activity of homogenates of Novikoff ascites cells were determined are presented in Table XII. In contrast to the intact cell, and in agreement with earlier findings (Wenner et al., 1953), maximum glycolysis in the homogenate occurred in the high potassium medium. This result does not necessarily indicate that all of the inhibitory influences of potassium with respect to glycolysis are directed at the cellular plasma membrane but demonstrates only a difference in response between the integrated enzyme systems of the intact cell and the more diffuse conditions which are present after homogenization. The failure of exogenous ATP to stimulate lactic acid production in the presence of a high concentration of potassium ions indicates that under these

TABLE XII

THE EFFECTS OF SODIUM AND POTASSIUM IONS ON GLYCOLYSIS IN HOMOGENATES OF NOVIKOFF ASCITES HEPATOMA CELLS

Cell preparation	Additions	μatoms glucose-2-C^{14} incorporated into lactic acid/ml packed cells or/ 10 ml of homogenate	
		Control	Potassium
Intact cell	10 mM glucose	57	31
Homogenate	10 mM glucose	3	42
Homogenate	10 mM glucose and 2 mM ATP	18	44
Homogenate	10 mM glucose and 2 mM ADP	11	37
Homogenate	10 mM glucose and 2 mM AMP	4	40

In the intact cell preparation the K-103 medium was used. For the homogenate the potassium medium had all of the sodium replaced by potassium, with the exception of the 10 mM sodium phosphate buffer. The incubation medium for the homogenate contained 5.7 μmoles of NAD and 0.105 μmoles of Cytochrome C in a final volume of 3 ml. 10 ml of homogenate contained the equivalent of 1.25 ml of packed cells.

conditions glycolysis was not limited by the availability of this compound. The addition of 2 mM ATP to the control medium, on the other hand, resulted in a sixfold increase in the glycolytic rate, suggesting that in this case at least one of the factors limiting the velocity of lactate production was the availability of utilizable energy. ADP was able to support a somewhat lower rate of glycolysis than was ATP and the addition of AMP had virtually no effect on the production of lactate in the control medium.

The Effects of Ouabain

The results described above demonstrate that in the absence of the intact cell potassium is not inhibitory and suggest a possible role for the membrane in the potassium mediated inhibitions. One of the functions performed by the membrane is the active transport of cations, a process which has been shown to be intimately associated with cellular energy metabolism. The functioning of an active cation pump under the present experimental conditions was indicated by the results discussed in section 4-3, and this in turn suggested that at least some of the difference between the glycolytic activity in the control and K-103 media might be the result of a more "active" transport mechanism in the former case. In order to test this possibility the effects of ouabain, an inhibitor of active transport (Glynn, 1964), on various aspects of the cells energy metabolism were determined. Ouabain at 0.3 mM or greater decreased the total lactic acid

produced in the control medium by approximately 15%, and the difference between the control and K-103 values by approximately 25% (Table XIII). Glycogen synthesis in both media was unaltered by ouabain addition as was glycolysis in the K-103 cells. The results of other experiments demonstrated that neither the respiratory activity nor the level of 7mnp was altered by the addition of ouabain to either the control or K-103 medium.

4-5. THE EFFECTS OF PREINCUBATION

Glycolysis, Glycogen Synthesis, and 7mnp Levels

A considerable difference in the metabolism of glucose between cells subjected to a 10 minute preincubation prior to the addition of glucose and cells not receiving this treatment was noticed (Table III). This difference was now investigated more thoroughly by the performance of time course experiments in which glucose-2-C^{14} conversion to lactic acid and glycogen, as well as the 7mnp levels were determined. The results of a typical experiment are presented in Figure 6A and B. A number of changes upon preincubation are evident, perhaps the most striking of which is the failure of the addition of glucose to produce an energy depletion in the preincubated cells. Just as the level of high energy nucleotide phosphate shows only minor fluctuations when glucose is added following preincubation, so the rates of both glycolysis and glycogen synthesis are relatively constant under these conditions.

TABLE XIII

THE EFFECTS OF OUABAIN ON GLYCOLYSIS AND GLYCOGEN SYNTHESIS

Incubation medium	Concentration of ouabain (mM)	Lactic acid		Increase in total glycogen (μmoles glucose equivalents/ml packed cells)
		μmoles/ml packed cells	Control - (K-103)*	
a) Control	0.0	122	66	24
	0.1	119	63 (96)	24
	0.3	103	47 (71)	24
	0.5	107	51 (77)	21
	0.7	106	50 (76)	21
b) K-103	0.0	56	-	2
	0.7	59	-	2

The incubation time was 60 minutes and the concentration of glucose was 10 mM. The glycogen content at zero time was 36 μmoles of glucose equivalents/ml packed cells.

*The difference between the μmoles of lactate produced under the indicated conditions in the control medium and that produced in the K-103 medium in the absence of ouabain. The figures in parenthesis refer to the percentages of the difference when ouabain was not present in the control medium.

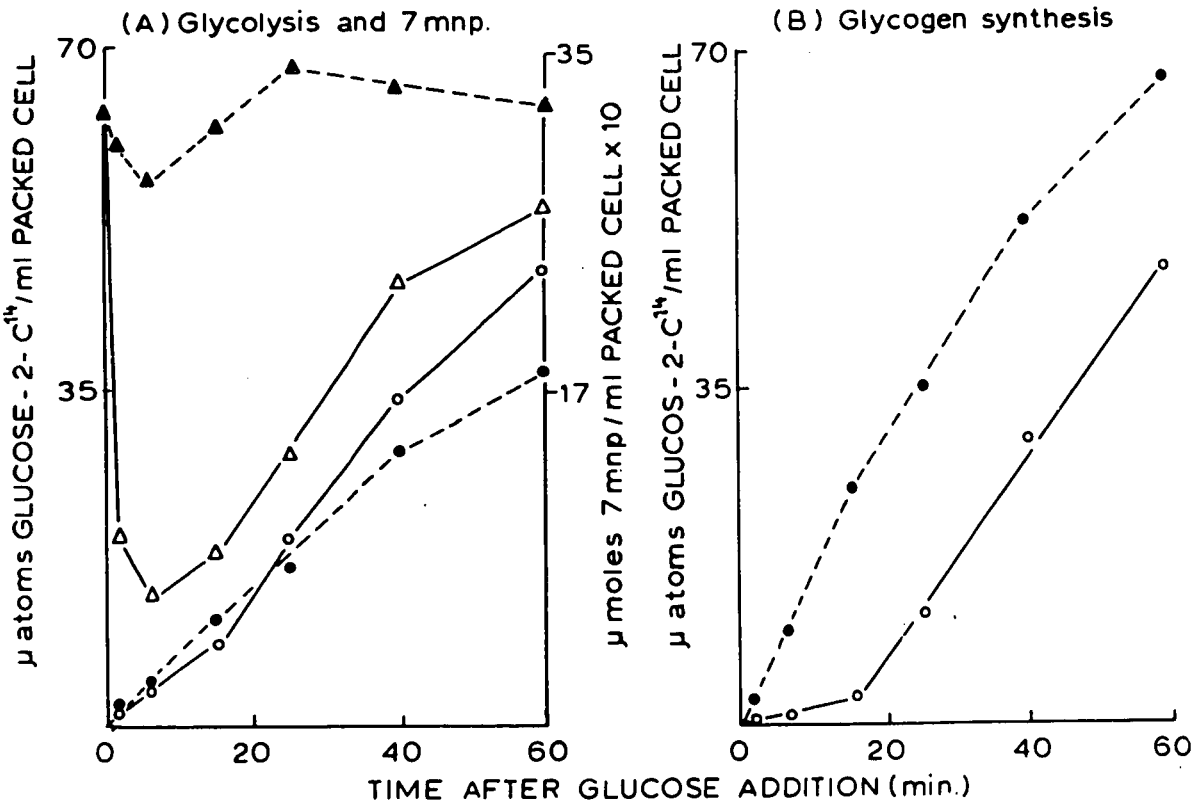

FIGURE 6. The effects of preincubation on 7mnp levels and glucose-2-C^{14} incorporation into glycogen and lactic acid. Experimental conditions were as described in Table III. (A) and (B) open figures, control; solid figures, 10 minute preincubation. (A) △ and ▲ , 7mnp; ○ and ● , lactic acid.

The greater incorporation of glucose into glycogen following preincubation is seen to be the consequence of the lag period in the control cells, the rates of glycogen synthesis under both experimental conditions being similar once this period has elapsed. In contrast to the effect of preincubation on glycogen synthesis this treatment results in a decrease in the formation of lactic acid. The results of Figure 6A show that the major differences between the control and preincubated conditions with respect to glycolytic activity occur during the first 30 minutes following glucose addition. During this time the preincubated cells exhibit a linear rate of lactic acid formation which is considerably less than the maximum velocity attained by the control cells.

These results also afford an explanation for the failure of preincubation to alter the respiratory activity (Table III). They demonstrate that during the latter half of the incubation period, when the QO_2 values were determined, the ratios of glycogen to lactic acid produced are similar under both conditions. For the experiment presented the ratios obtained 25 minutes after the addition of glucose were 0.56 and 2.11 for the control and preincubated cells respectively, whereas the corresponding values for the rates between 25 and 60 minutes were 1.31 and 1.55.

Glycine Uptake and Incorporation into Protein

The results of Table XIV demonstrate that the stimulatory effects of preincubation on glycine incorporation into protein are not a consequence

TABLE XIV

THE EFFECTS OF PREINCUBATION ON GLYCINE UPTAKE AND INCORPORATION INTO PROTEIN

	Time of preincubation (minutes)	Time after glucose and glycine addition (minutes)	μmoles glycine-2-C^{14}/ml packed cells	
			Uptake	Incorporation x 10^2
a)	0	10	2.0	0.4
		25	3.4	6.0
		40	3.6	13.9
		60	4.2	24.6
b)	10	10	2.0	4.3
		25	3.0	12.0
		40	3.4	20.8
		60	4.0	27.8

The concentrations of glucose and glycine were 10 mM and 2 mM respectively.

of increased rates of glycine uptake. The 10 minute values for glycine uptake are similar under both experimental conditions but a tenfold difference in glycine incorporation into protein is observed at this time. With the exception of the first 10 minutes protein synthesis in both the control and preincubated cells exhibit similar rates throughout the incubation period.

The Effects of the Time of Preincubation on 7mnp Levels

In the experiments described thus far, the time of preincubation had arbitrarily been chosen as 10 minutes. The results presented in Table XV show the effects of different preincubation times on the level of 7mnp 10 minutes after the addition of glucose. After 5 to 7.5 minutes of preincubation the addition of glucose no longer results in a depletion of cellular high energy nucleotide phosphate. The same pattern was obtained with cells incubated in the K-103 medium. As a consequence of these results the use of 10 minutes as a standard preincubation time was continued.

Preincubation and Extracellular Potassium

The absence of a decrease in the levels of high energy nucleotide phosphate following glucose addition to preincubated cells provided an experimental situation in which the effects of increased concentrations of extracellular potassium ions on cellular metabolism in the absence of energy changes could be investigated. The results presented in Table XVI show that under these conditions both glycolysis and glycogen synthesis are still

TABLE XV

THE EFFECTS OF THE TIME OF PREINCUBATION ON THE GLUCOSE INDUCED DEPLETION OF 7 MINUTE NUCLEOTIDE PHOSPHATE

Time of preincubation (minutes)	µmoles 7mnp/ml packed cells	
	Control	K-103
0	1.2	1.0
2.5	1.5	1.2
5	3.8	3.6
7.5	4.0	4.4
10	4.0	4.5
15	4.0	4.6
25	4.1	4.6

Following the indicated time of preincubation glucose was added to a final concentration of 10 mM and the incubation continued for an additional 10 minutes. Both the preincubation and incubation were performed in the same medium.

TABLE XVI

THE EFFECTS OF POTASSIUM IONS ON GLYCOLYSIS, GLYCOGEN SYNTHESIS AND 7 MINUTE NUCLEOTIDE PHOSPHATE LEVELS FOLLOWING PREINCUBATION

Time after glucose addition (minutes)	Control			K-103		
	μatoms glucose-2-C^{14} incorporated/ ml packed cells		μmoles 7mnp/ ml packed cells	μatoms glucose-2-C^{14} incorporated/ ml packed cells		μmoles 7mnp/ ml packed cells
	Lactic acid	Glycogen		Lactic acid	Glycogen	
1	2.2	2.9	2.9	1.3 (59)	3.3 (113)	3.0 (103)
10	13.4	19.2	2.9	11.0 (82)	19.4 (101)	3.2 (110)
25	31.2	45.3	3.2	26.4 (84)	40.5 (90)	3.5 (109)
40	42.1	66.5	3.1	34.9 (83)	55.9 (84)	3.4 (109)
60	51.3	82.9	3.4	40.5 (79)	64.7 (78)	3.3 (97)

The cells were preincubated for 10 minutes prior to the addition of glucose to a final concentration of 10 mM. The numbers in parenthesis refer to the percentages of the corresponding control values. Both the preincubation and incubation were performed in the same medium.

decreased in the K-103 medium. With the exception of the first minute, the glycolytic rate was consistently 15 to 20% below the control values but a progressive decrease in the velocity of glycogen synthesis was observed in the K-103 medium. For example, the rate of glucose conversion to glycogen was 20% lower than the control value between 10 and 25 minutes but was 54% lower between 40 and 60 minutes following the addition of glucose.

Preincubation has no effect on glycine uptake by cells incubated in either the control or K-103 medium (Table XVII) but does result in a marked increase in protein synthesis under the latter conditions. In spite of the large stimulatory effect of preincubation, however, the incorporation of glycine into protein is still decreased in the presence of increased levels of extracellular potassium.

4-6. THE EFFECTS OF POTASSIUM IONS ON GLYCOGENOLYSIS

The decreased deposition of glycogen which is observed in the presence of increased levels of extracellular potassium might reflect an increase in the rate of glycogen breakdown rather than a decrease in the activity of the synthesizing pathway. In order to test this possibility the experiment presented in Table XVIII was performed. Cells were preincubated in the control medium for 10 minutes prior to the addition of glucose-2-C^{14} and the incubation allowed to proceed for an additional 15 minutes. At the end of this time the cells were spun down and washed with Krebs-Ringer solution.

TABLE XVII

THE EFFECTS OF POTASSIUM IONS ON GLYCINE UPTAKE AND INCORPORATION INTO PROTEIN FOLLOWING PREINCUBATION

	Time of preincubation (minutes)	Additions	Control		K-103	
			μmoles glycine-2-C^{14}/ml packed cells		μmoles glycine-2-C^{14}/ml packed cells	
			Uptake	Incorporation x 10^2	Uptake	Incorporation x 10^2
a)	0	2 mM glycine	3.7	40	2.0	25
		2 mM glycine and 10 mM glucose	3.7	24	2.1	6
b)	15	2 mM glycine	3.7	38	1.9	21
		2 mM glycine and 10 mM glucose	3.7	27	2.0	13

The incubation time following the addition of substrates was 60 minutes. The experimental procedure was as described in Table XVI.

TABLE XVIII

THE EFFECTS OF POTASSIUM IONS ON GLYCOGENOLYSIS

Incubation medium	Additions	Time (minutes)	μatoms glucose-2-C^{14} in glycogen/ml packed cells
-	-	0	23.6
Control	Nil	60	19.4
	10 mM glucose	60	20.0
K-103	Nil	60	20.0
	10 mM glucose	60	21.4

Following a 10 minute preincubation the cells were incubated for 15 minutes in the control medium in the presence of 5 mM glucose-2-C^{14} to prelabel the glycogen. The cells were then washed with Krebs-Ringer phosphate buffer at room temperature and reincubated according to the indicated conditions.

Following resuspension an aliquot was removed for the determination of glycogen-C^{14} content at zero time and the remainder of the cells were incubated according to the conditions indicated in Table XVIII. At the end of 60 minutes the total radioactivity remaining in the glycogen was determined. In agreement with the results of Nigam (1967a) the loss of label from the glycogen was very slow. Moreover, the decrease in glucose-2-C^{14} content was the same whether the cells were incubated in the control or in the K-103 medium.

4-7. THE REVERSIBILITY OF THE POTASSIUM INHIBITIONS

Does incubation in the K-103 medium produce irreparable cellular damage or are the metabolic inhibitions observed under these conditions of a reversible nature? In order to answer this question cells were preincubated for 60 minutes in either the control or K-103 medium in the presence of glucose, then washed, resuspended, and reincubated in the control medium. If potassium produces irreversible tissue damage the metabolic inhibitions should persist on subsequent incubation in the control medium. If, on the other hand, the metabolic effects are reversible the activities of both the control and K-103 preincubated cells should be similar upon reincubation in the control medium.

Glycolysis and Glycogen Synthesis

The results of this treatment on glycolysis and glycogen synthesis

are presented in Table XIXa. Following an initial lag period of approximately 10 minutes lactic acid formation by cells preincubated in the K-103 medium is similar to that demonstrated by the control cells. Although the lag period with respect to glycogen synthesis is longer this activity in the K-103 pretreated cells also approaches the control value during the latter half of the incubation period. The decreased levels of high energy nucleotide phosphate in cells which were preincubated in the K-103 medium are most probably the result of the loss of purine bases during the washing which followed the preincubation (see Chapter V).

A comparison of the results presented in Tables XIXa and b shows that decreasing the extracellular potassium ion concentration does in fact result in an increase in metabolic activity. Furthermore, in confirmation of the results presented in Table XVI, glucose metabolism is decreased in the K-103 medium in the absence of a change in the cellular energy content. The metabolic activity of both the control and K-103 preincubated cells is similar during the latter half of the incubation in the presence of elevated potassium levels.

Glycine Uptake and Incorporation into Protein

Table XX presents the results of a typical experiment in which the effects of preincubation on glycine uptake and incorporation into protein were determined. For both control and K-103 pretreated cells the uptake

TABLE XIX

GLYCOLYSIS AND GLYCOGEN SYNTHESIS FOLLOWING A 60 MINUTE PREINCUBATION IN THE CONTROL OR K-103 MEDIUM

Incubation medium	Time (minutes)	Control*			K-103*		
		µatoms glucose-2-C^{14} incorporated/ ml packed cells		µmoles 7mnp/ml packed cells	µatoms glucose-2-C^{14} incorporated/ ml packed cells		µmoles 7mnp/ml packed cells
		Lactic acid	Glycogen		Lactic acid	Glycogen	
a) Control	10	10.5	16.5	2.5	8.1	9.3	1.6
	30	27.9	42.3	2.6	23.6	27.7	1.6
	60	42.9	68.7	2.7	39.6	50.3	1.8
	Δ 30-60	15.0	26.4	-	16.0	22.6	-
b) K-103	10	10.1	15.6	2.6	5.7	7.9	1.7
	30	24.1	36.4	2.7	17.2	17.7	2.0
	60	36.6	50.2	3.0	29.4	29.7	2.0
	Δ 30-60	12.5	13.8	-	12.2	12.0	-

*Preincubation medium: The cells were preincubated for 60 minutes in the presence of 10 mM glucose in either the control or K-103 medium. They were then washed once with ice cold Krebs-Ringer phosphate buffer and reincubated in the indicated medium in the presence of 10 mM glucose.

TABLE XX

GLYCINE UPTAKE AND INCORPORATION INTO PROTEIN FOLLOWING A 60 MINUTE PREINCUBATION IN THE CONTROL OR K-103 MEDIUM

Preincubation medium	Incubation medium	µmoles 7mnp/ml packed cells	µmoles glycine-2-C^{14}/ml packed cells	
			Uptake	Incorporation x 10^2
Control	Control	3.3	6.0	17
K-103	Control	2.3	5.9	16

The experimental procedure was similar to that described in Table XIX. The incubation medium contained 10 mM glucose and 2 mM glycine. The incubation time was 60 minutes.

and incorporation of glycine is similar upon a second incubation in the control medium. As with the results obtained with respect to glucose metabolism this similarity of metabolic activity occurred despite a lower level of 7mnp in the cells which were preincubated in the presence of increased extracellular potassium.

Intracellular Water, Sodium, and Potassium Content

The effects of a second incubation in a normal Krebs-Ringer solution on the water, sodium ion, and potassium ion content of cells which have experienced a 60 minute incubation in either the control or K-103 medium are shown in Table XXI. During the washing which precedes the second incubation there is a loss of water and potassium ions from both the control and K-103 pretreated cells, the latter also taking up a large amount of sodium ions during this period. During the subsequent incubation the K-103 pretreated cells lose water, sodium and potassium and by the end of 60 minutes incubation the levels of these materials are relatively similar in cells which were preincubated in either the control or K-103 medium.

The results presented in Tables XIX, XX, and XXI all demonstrate that the inhibitions which occur in the presence of increased extracellular potassium concentrations are dependent on the maintenance of elevated potassium levels. Once this condition is removed the metabolic activity returns to the same level as that exhibited by the control cells.

TABLE XXI

THE INTRACELLULAR LEVELS OF SODIUM IONS, POTASSIUM IONS, AND WATER FOLLOWING A 60 MINUTE PREINCUBATION IN THE CONTROL OR K-103 MEDIUM

Time (minutes)	Control*			K-103*		
	ml H_2O/mg dry weight x 10^3	µequivalents/mg dry weight x 10		ml H_2O/mg dry weight x 10^3	µequivalents/mg dry weight x 10	
		Na^+	K^+		Na^+	K^+
60**	2.7	2.6	4.5	4.7	2.3	10.4
0	2.5	2.8	4.0	4.1	4.8	4.4
2	1.9	2.5	4.1	3.3	5.0	3.3
10	1.9	2.0	3.7	2.6	3.6	2.8
25	1.9	1.3	3.8	2.2	2.5	2.8
40	2.1	0.9	3.8	2.0	2.0	3.0
60	1.9	1.0	3.9	1.9	1.3	2.8

*Preincubation medium: The experimental procedure was similar to that described in Table XIX. The incubation was performed in the presence of 10 mM glucose in the control medium only.

**Values determined at the conclusion of the preincubation period.

The values represent the average of three (Na^+/K^+) or five (H_2O) separate experiments.

4-8. THE CONTROL OF GLYCOLYSIS

Glucose Utilization and Inorganic Phosphate Levels

In an attempt to more thoroughly define the factors which are involved in the regulation of glucose and energy metabolism during the initial minutes of incubation in the non-preincubated cells the experiments presented in Tables XXII and XXIII were performed.

Table XXII shows the intracellular levels of inorganic phosphate and the amounts of glucose utilized at various times following the initiation of the incubation. Glycolysis, glycogen synthesis, and 7mnp levels are also presented, and follow the typical pattern (see, for example, Figure 6). The picture presented by glucose utilization is similar to that observed with respect to glycolysis: an initial burst of activity, an inhibited phase, and a subsequent increase in rate. Longer time courses demonstrated that the velocity of glucose utilization was linear between 15 and 40 minutes. Although the patterns of glucose utilization and lactic acid production are similar, the absolute values are very different and a large difference exists between the ATP utilized for phosphorylation and that produced by glycolysis. The consequences of this discrepancy will be discussed in more detail in the discussion which follows.

A second factor which emerges from Table XXII is the large difference between the amount of glucose which is phosphorylated and the

TABLE XXII

GLYCOLYSIS, GLYCOGEN SYNTHESIS, GLUCOSE UTILIZATION, INORGANIC PHOSPHATE AND 7 MINUTE NUCLEOTIDE PHOSPHATE LEVELS IN NOVIKOFF ASCITES HEPATOMA CELLS

Time (minutes)	µmoles/ml packed cells			µatoms glucose-2-C^{14} incorporated/ml packed cells	
	Glucose utilized	Pi	7mnp	Lactic acid	Glycogen
0	-	6.5	3.5	-	-
1	13	3.6	1.2	1.6	-
3	12	4.2	0.9	2.6	1.1
6	19	4.4	0.9	4.2	-
10	20	5.2	1.2	7.3	4.8
15	29	6.9	1.5	13.0	9.3
25	46	7.9	2.1	25.4	22.0

The concentration of glucose was 5 mM. Each value represents the average of three or four separate experiments.

amount which is recovered as either lactic acid or glycogen. For example, after 3 minutes these two products account for somewhat less than one-third of the glucose which has been utilized by this time. This low recovery of glucose suggests that an accumulation of phosphorylated intermediates must occur. Furthermore, the rapid depletion of inorganic phosphate which takes place in the first minute of the incubation suggests that inorganic phosphate may become limiting for triose phosphate dehydrogenase during this time.

Phosphorylated Intermediates

Table XXIII shows the results of experiments in which hexose monophosphate and fructose diphosphate (including triose phosphates) levels were determined. For comparative purposes the levels of these intermediates in preincubated cells were measured in the same experiments. Included also are the levels of inorganic phosphate and ATP.

In agreement with the results obtained from 7mnp determinations the regeneration of ATP is largely complete within 40 minutes after the addition of glucose to the non-preincubated cells. As is the case with respect to ATP, the changes in the level of inorganic phosphate are considerably smaller in the preincubated than in the control cells.

The large accumulation of fructose diphosphate in the control cells is indicative of extensive phosphofructokinase activity and limited further metabolism of this compound. In contrast to the control cells, no

TABLE XXIII

INTRACELLULAR LEVELS OF HEXOSE MONOPHOSPHATES, FRUCTOSE DIPHOSPHATE, INORGANIC PHOSPHATE, AND ATP IN NOVIKOFF ASCITES HEPATOMA CELLS

	Time of preincubation (minutes)	Time after glucose addition (minutes)	μmoles/ml packed cells			
			HMP	FDP	Pi	ATP
a)	0	0	0.0	0.0	6.2	2.6
		1	0.18	7.1	3.4	0.5
		6	0.22	9.6	3.3	0.2
		15	0.29	8.7	4.3	0.8
		25	0.39	3.3	6.6	1.3
		40	0.51	1.4	6.9	2.1
b)	10	0	0.0	0.0	6.1	2.8
		1	0.75	1.6	5.2	2.3
		6	0.61	1.5	6.1	2.5
		15	0.74	1.1	7.0	2.5
		25	0.80	0.9	6.5	2.4
		40	1.00	0.9	7.3	2.5

Each value represents the average of two separate experiments. The glucose concentration was 10 mM.

HMP: hexose monophosphates.

FDP: fructose diphosphate, includes triose phosphates.

excessive accumulation of intermediates occurs when glucose is added following preincubation.

In both the control and preincubated cells a slow, linear increase in the level of hexose monophosphates occurs between 6 and 40 minutes. The decrease in glucose phosphorylation in the control cells after one minute, in spite of the relatively low levels of hexose monophosphates, indicates that the available supply of utilizable energy may become limiting for the hexokinase reaction at this time.

SUMMARY

1. Incubation of Novikoff ascites hepatoma cells in the presence of exogenous glucose results in a rapid depletion of the cellular energy content. In the absence of glucose the cell's energy store is replenished within 10 minutes, but in the presence of glucose a steady state level is not attained until approximately 60 minutes have elapsed. A similar loss of cellular energy is produced by mannose but not by fructose, galactose, or ribose.

2. Glucose utilization and lactic acid production both undergo a phase of decreased activity between one and 10 minutes after the addition of glucose. Fructose diphosphate shows a large accumulation at this time. The incorporation of glucose into glycogen is very slow during this period.

3. Following a period of rapid glycolytic activity, which corresponds to the period of maximum energy regeneration, the velocity of lactate production decreases. As a consequence of this decrease, and the increase in the rate of glycogen synthesis following the initial lag period, glucose conversion to glycogen exceeds the glycolytic utilization of glucose during the later portions of the incubation.

4. Measurements of the intracellular sodium and potassium levels indicate that in the presence of glucose the cation pump is limited by the availability of ATP during the first 5 to 10 minutes of the incubation period.

5. Preincubation prior to the addition of glucose eliminates the drop in cellular energy levels and results in linear rates of glycolysis and glycogen synthesis between one and 40 minutes after the addition of glucose. Under these conditions the rate of glucose conversion to glycogen exceeds at all times the velocity of glucose utilization by the glycolytic pathway.

6. Incubation in the presence of glucose and increased levels of extracellular potassium ions results in a similar decrease in the cellular energy level as is observed in the control cells. The rate of high energy nucleotide phosphate regeneration in the absence of glucose is similar in both the control and K-103 cells but a much slower rate of regeneration in the presence of glucose is exhibited by cells incubated in the high potassium medium.

7. Cells incubated in the K-103 medium demonstrate both a longer period of inhibited glycolysis, and a longer lag with respect to glycogen synthesis than do the control cells. Moreover, the maximum rate of both of these pathways is slower in the presence of elevated potassium than in the control medium.

8. Incubation in the K-103 medium results in a large increase in the intracellular content of potassium ions. In the presence of both glucose and increased extracellular potassium an elevation in the intracellular water level is observed.

9. In the absence of cellular energy changes potassium induced inhibitions of glycolysis, glycogen synthesis, amino acid uptake and protein synthesis are still observed.

10. Incubation in the presence of increased extracellular potassium does not produce irreversible cellular damage, as evidenced by the decrease in intracellular water and the absence or reduction of metabolic inhibitions during a subsequent reincubation in the control medium.

11. Maximum glycolysis in homogenates is obtained in the presence of a high concentration of potassium. Incubation in the presence of ouabain decreases the difference in glycolytic activity between the control and K-103 cells, although no effect of ouabain on respiration, glycogen synthesis or energy levels is observed.

CHAPTER V

THE EFFECTS OF GLUCOSE ON THE ADENINE NUCLEOTIDE POOL

The results described in Chapter IV have served to indicate an intimate association between the intracellular concentration of ATP and the various metabolic activities which have been investigated. The experiments which are described in this chapter were performed first in an attempt to elucidate some of the factors which are important in regulating the rate at which the level of ATP is restored and second to determine what the effects of different rates of ATP regeneration on the cells metabolic activity would be.

5-1. THE ADENINE NUCLEOTIDE POOL

The investigation of the regulation of ATP regeneration was initiated by determining the levels of adenosine mono-, di-, and triphosphates following glucose addition to freshly prepared cells (Table XXIV and Figure 7). As previously demonstrated a rapid drop in ATP occurred within the first minute of the incubation, the minimum level being reached at approximately 3 minutes. Following this time ATP regeneration proceeded

TABLE XXIV

THE EFFECTS OF GLUCOSE ON THE ADENINE NUCLEOTIDE POOL OF NOVIKOFF ASCITES HEPATOMA CELLS

Time (minutes)	μmoles/ml packed cells				IMP**	Hypoxanthine**
	ATP	ADP	AMP	Total		
0	2.4, 2.4	0.5, 0.4	0.1, 0.2	3.0, 3.0	*	*
1	0.7, 0.6	0.9, 1.1	1.0, 0.9	2.6, 2.6	+	+
3	0.5, 0.3	0.7, 1.0	1.2, 0.9	2.4, 2.2	+	+
6	0.7, 0.4	0.6, 0.7	0.6, 0.7	1.9, 1.8	+	+
10	0.8, 0.6	0.5, 0.6	0.4, 0.4	1.7, 1.6	*	+
15	1.2, 1.0	0.4, 0.5	0.4, 0.3	2.0, 1.8	*	+
25	1.7, 1.5	0.2, 0.5	0.1, 0.2	2.0, 2.2	*	+
40	2.1, 2.1	0.3, 0.4	0.1, 0.1	2.5, 2.6	*	+
60	2.2, 2.2	0.3, 0.4	0.1, 0.1	2.6, 2.7	*	*

The incubation medium contained 10 mM glucose. The results of two separate experiments are presented.

*Not detectable.

**Detected by visual inspection of thin layer chromatograms under short wave ultraviolet light.

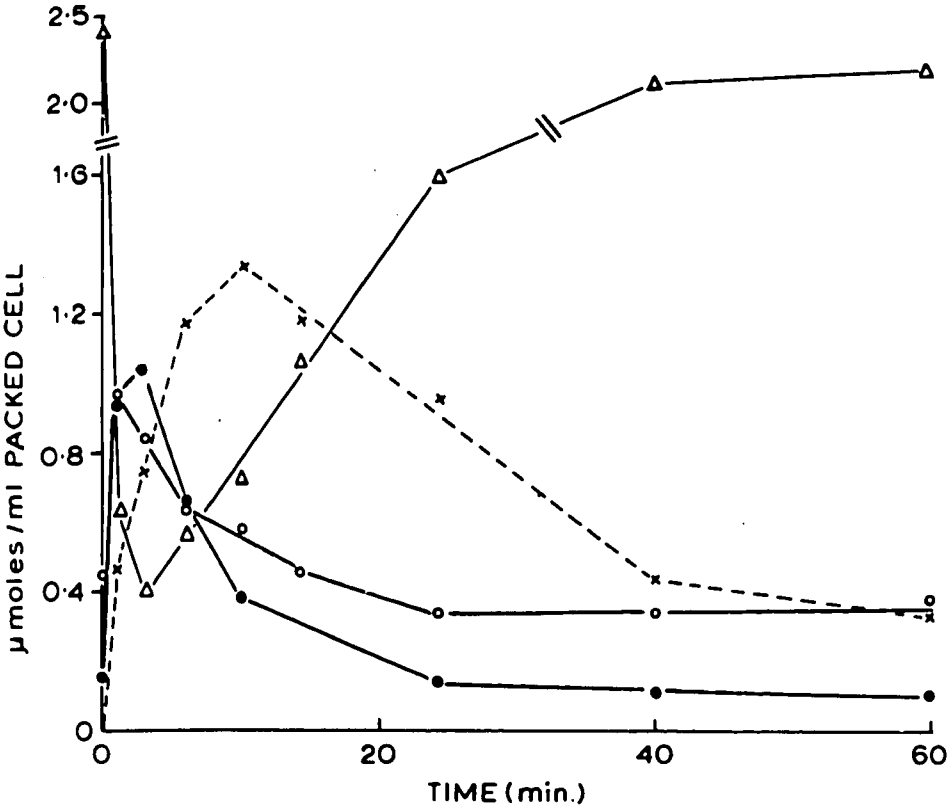

FIGURE 7. The effects of glucose on the levels of adenine nucleotides. The data is from Table XXIV. △ , ATP; ○ , ADP; ● , AMP; X , the difference between the total of (AMP + ADP + ATP) at the indicated times and the total present at 0 time.

linearily for 20 to 30 minutes, a steady state level being attained within one hour. The levels of both AMP and ADP increased during the first 3 minutes, AMP reaching a maximum value slightly later than ADP. However, the decrease in the concentration of ATP was not equally balanced by the increases in the levels of adenosine mono- and diphosphates and the total adenine nucleotide pool decreased by approximately 20% within 3 minutes. Following their initial accumulation the levels of both AMP and ADP decreased rapidly, the drop in the concentration of these compounds between 3 and 10 minutes being accompanied by a further loss of adenine nucleotides, indicating that some of the AMP is further catabolized during this time. As indicated in Figure 7, the loss of adenine nucleotides was maximum at approximately 10 minutes, slightly less than 50% of the original adenine nucleotide pool having been catabolized by this time. Chromatographic analysis of the acid soluble fraction indicated that hypoxanthine accumulated as the major product of purine catabolism. Visual inspection of the developed chromatograms under short wave ultraviolet light revealed the presence of hypoxanthine at all times tested between 1 and 40 minutes after the addition of glucose, the largest and darkest spots being present at 6, 10, and 15 minutes. Small amounts of IMP were detectable between 1 and 6 minutes of the incubation period, but neither adenosine nor inosine could be detected at any of the times which were tested.

Changes in the levels of the various adenine nucleotides following

the addition of glucose to the K-103 medium are presented in Table XXV. In the same experiments the nucleotide levels in the control cells were also determined for comparative purposes. As with the control cells a rapid decrease in the level of ATP is observed in cells incubated in the K-103 medium. In the latter case, however, a slightly greater decrease in the total pool of adenine nucleotides is evident and the difference curve does not reach a maximum until approximately 30 to 40 minutes have elapsed (Figure 8). In the presence of increased extracellular potassium, hypoxanthine again accumulated as the major product of purine catabolism and was still detectable after 90 minutes of incubation. In the same experiment hypoxanthine could not be detected after 40 minutes in the control medium. Although the initial increase in the levels of AMP and ADP are similar under both experimental conditions, a slightly slower return to the steady state is exhibited by the cells incubated in the K-103 medium.

5-2. PURINE PHOSPHORIBOSYL TRANSFERASE

The failure of IMP, AMP, or ADP to accumulate after 25 minutes suggests that the formation of IMP from hypoxanthine and PRPP might play a limiting role with respect to ATP regeneration during the latter half of the incubation period. The experiments to be described were designed to study the activity of this reaction under the various experimental conditions which have been employed in the present investigation with the hopes

TABLE XXV

THE EFFECTS OF POTASSIUM ON THE ADENINE NUCLEOTIDE POOL OF NOVIKOFF ASCITES HEPATOMA CELLS FOLLOWING THE ADDITION OF 10 mM GLUCOSE

Time (minutes)	Control				K-103			
	μmoles/ml packed cells				μmoles/ml packed cells			
	ATP	ADP	AMP	Total	ATP	ADP	AMP	Total
0	2.7	0.6	0.1	3.4	2.7	0.6	0.1	3.4
5	0.3	0.8	1.0	2.1	0.2	0.7	1.1	2.0
10	0.5	0.6	0.4	1.5	0.3	0.6	1.0	1.9
20	1.1	0.4	0.2	1.7	0.3	0.6	0.6	1.5
40	1.6	0.3	0.3	2.2	0.6	0.5	0.2	1.3
60	1.9	-	0.2	-	0.9	0.5	0.1	1.5
90	1.8	0.4	0.2	2.4	1.7	0.3	*	2.0

*Not detectable

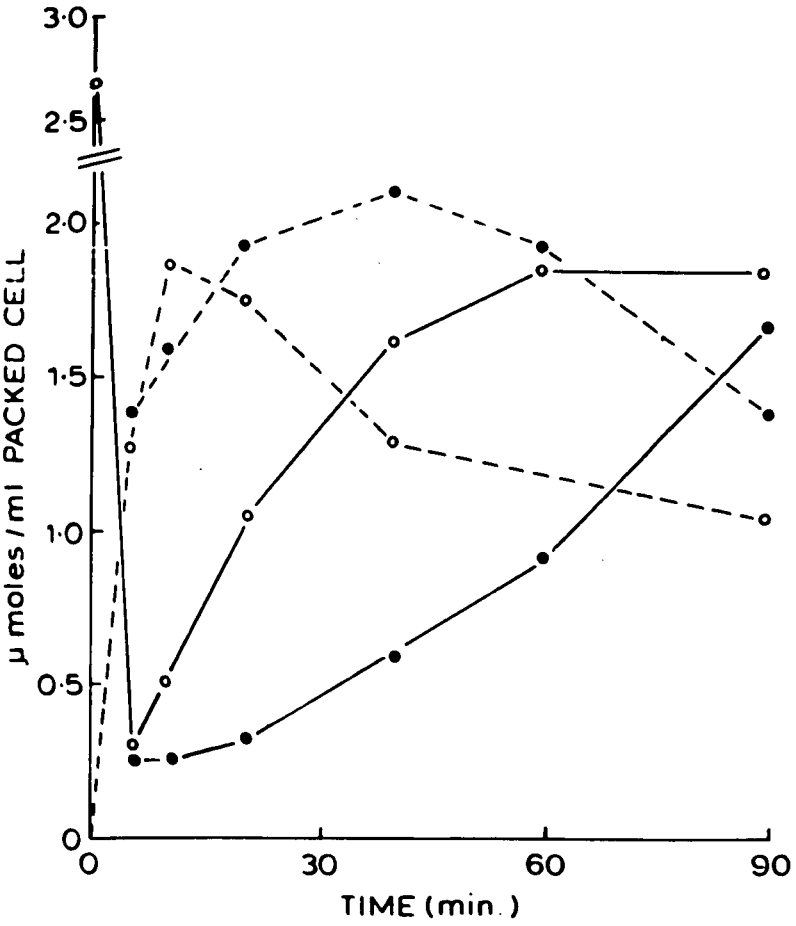

FIGURE 8. The effects of potassium ions on the level of ATP and on the total adenine nucleotide pool. The data is from Table XXV. ———, ATP; — — — —, difference curve (calculated as described in Figure 7); ○, control; ●, K-103.

of elucidating possible regulatory mechanisms.

The Effects of Glucose and Preincubation

In agreement with the results of other investigators (LePage, 1953; Harrington, 1958; Thomson et al., 1958) the incorporation of adenine into the acid soluble fraction was markedly stimulated by the addition of glucose (Table XXVI). However, as demonstrated by the ratio of incorporation in the presence and absence of glucose a lag period of 20 to 30 minutes occurred before the maximum stimulatory effects of glucose were evident.

In order to determine if this period resulted from an intrinsic property of the reaction per se or if it was in some manner related to the variations in ATP levels which occur during the same time period (Figure 7), the effect of preincubation in the absence of glucose on the incorporation of adenine into the acid soluble fraction was determined. The results presented in Figure 9 show that the lag period was eliminated by this treatment and suggest a connection between the reduced concentrations of ATP and the failure of glucose to initially stimulate adenine incorporation in the non-preincubated cells. The actual relationship between the concentration of ATP and the velocity of adenine incorporation is obscured however, by the additional observation that the maximum velocity of adenine incorporation in the control cells is 30% greater than that observed in the preincubated cells (48 mμmoles/

TABLE XXVI

THE EFFECTS OF GLUCOSE ON THE TIME COURSE OF INCORPORATION OF ADENINE INTO THE ACID SOLUBLE FRACTION OF NOVIKOFF ASCITES HEPATOMA CELLS

Additions	mμmoles adenine-8-C^{14} incorporated/ml packed cells				
	10 mins.	20 mins.	30 mins.	45 mins.	60 mins.
Nil (A)	80	90	120	160	190
5 mM glucose (B)	110	340	670	950	1080
Ratio B/A	1.4	3.8	5.6	5.9	5.7

The concentration of adenine was 0.1 mM.

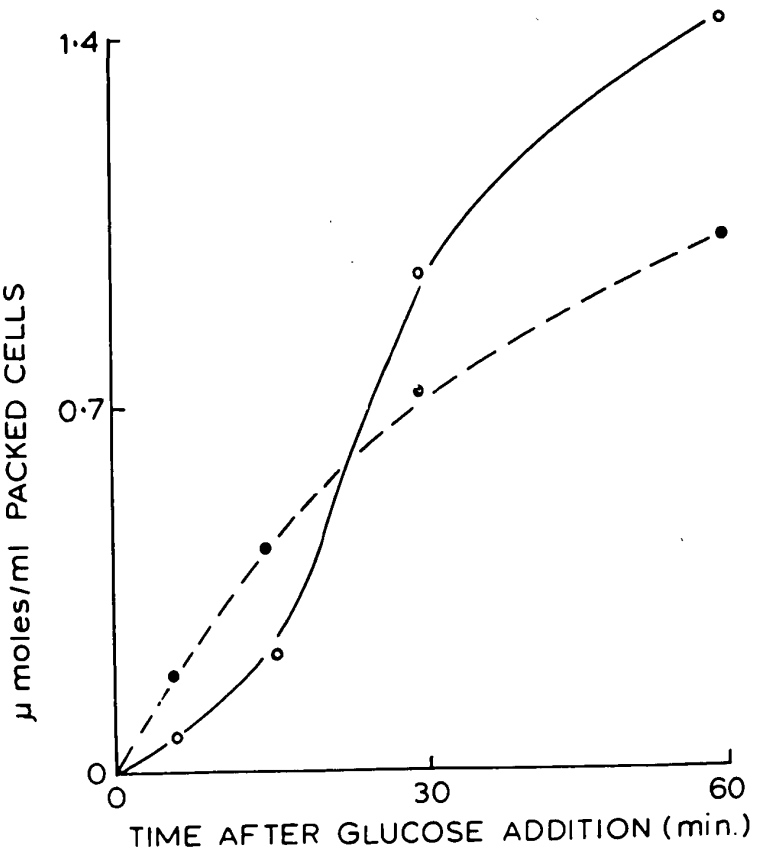

FIGURE 9. The effects of preincubation on the incorporation of adenine-8-C^{14} into the acid soluble fraction. The concentrations of glucose and adenine were 10 mM and 0.1 mM respectively. ○ , control cells; ● , 10 minute preincubation.

minute/ml packed cells as compared to 36 mμmoles/minute/ml packed cells) in spite of the higher concentration of ATP in the latter case.

The effects of glucose and preincubation on the incorporation of adenine into the acid soluble fraction of cells incubated in the K-103 medium are presented in Table XXVII. The results obtained with the control cells are similar to those previously described. Incubation in the presence of increased extracellular potassium does not alter the low rate of adenine incorporation in the absence of glucose, but in the presence of glucose a marked inhibitory effect of potassium is apparent. Under these conditions no stimulatory effect of glucose is observed within the first 30 minutes. However, following preincubation adenine incorporation is similar in both the control and K-103 media. This latter result indicates that increased concentrations of potassium ions do not directly inhibit the adenine phosphoribosyl transferase reaction and that in the absence of an effect on the level of ATP the availability of substrate for this reaction does not become limiting in the K-103, as compared to the control medium.

Phosphoribosylpyrophosphate and Purine Phosphoribosyl Transferase Activity

The intracellular environment immediately following the addition of glucose to non-preincubated cells is not favorable to the synthesis of PRPP. Thus, not only is the level of ATP reduced, but the concentration of inorganic phosphate, a required cofactor for PRPP synthetase (Preiss and Handler, 1957; Murray and Wong, 1967b) is also decreased. In

TABLE XXVII

THE EFFECTS OF INCREASED EXTRACELLULAR POTASSIUM LEVELS ON THE INCORPORATION OF ADENINE INTO THE ACID SOLUBLE FRACTION OF NOVIKOFF ASCITES HEPATOMA CELLS

	Time of preincubation (minutes)	Additions	Time after substrate addition (minutes)	mμmoles adenine-8-C^{14} incorporated/ ml packed cells	
				Control	K-103
a)	0	0.1 mM adenine	30	174, 131	160, 110
			60	268, 199	260, 162
b)	0	0.1 mM adenine and 10 mM glucose	30	1030, 650	180, 92
			60	1580, 1320	910, 306
c)	10	0.1 mM adenine and 10 mM glucose	30	690, 570	686, 587
			60	950, 866	1060, 1070

The results of two separate experiments are presented. In "c" glucose and adenine were added following the preincubation. Both the preincubation and incubation were performed in the same medium.

addition, the levels of both AMP and ADP, inhibitors of this reaction (Wong and Murray, 1969) are elevated at this time. The experiments presented in Figure 10A and B were performed in order to determine what relationship, if any, exists between the lag period prior to the maximum stimulatory effect of glucose on adenine incorporation and the ability to synthesis PRPP. Although the rate of PRPP synthesis was found to be maximal 10 to 12 minutes after the addition of glucose the initial velocity of adenine uptake did not reach a maximum value until the preincubation time in the presence of glucose approached 20 minutes. This result indicates that factors other than, or in addition to PRPP availability are involved in regulating the activity of the phosphoribosyl transferase reaction during this period.

The results of two typical experiments in which the effects of various preincubation conditions on both adenine and hypoxanthine incorporation were studied are presented in Table XXVIII. The typical stimulation of adenine uptake by preincubation either in the presence or absence of glucose is observed. Following preincubation with glucose the maximum velocity of incorporation was approximately twice that observed when the preincubation was performed in the absence of exogenous substrate. The failure of combined endogenous and glucose preincubations to significantly increase the rate of adenine incorporation over that observed following an endogenous preincubation alone (Table XXVIIIa) indicates that the greater stimulatory effect

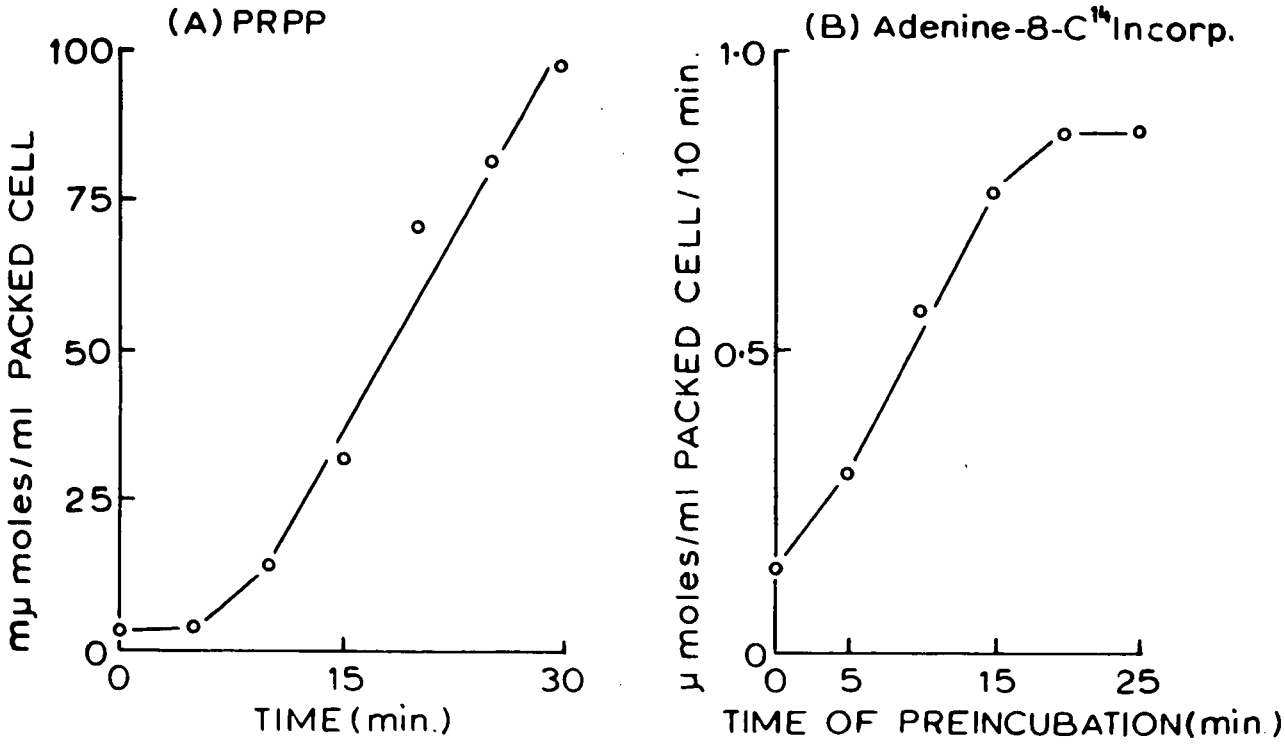

FIGURE 10. (A) The effects of time on the intracellular levels of PRPP. The concentration of glucose was 10 mM. (B) The effects of preincubation in the presence of glucose on the initial rates of adenine-8-C^{14} incorporation into the acid soluble fraction. The cells were preincubated in the presence of 10 mM glucose for the indicated period of time prior to the addition of 0.1 mM adenine-8-C^{14}.

TABLE XXVIII

THE EFFECTS OF PREINCUBATION ON THE INCORPORATION OF ADENINE AND HYPOXANTHINE INTO THE ACID SOLUBLE FRACTION OF NOVIKOFF ASCITES HEPATOMA CELLS

Experiment number	Time of preincubation (minutes)	Conditions of preincubation	mμmoles adenine-8-C^{14} or hypoxanthine-8-C^{14} incorporated/ml packed cells	
			Adenine	Hypoxanthine
1	0	-	90	65
	10	5 mM glucose	503	206
	10	no additions*	284	145
	25	5 mM glucose	600	275
	25	10 mins. with no additions plus 15 mins. with 5 mM glucose	324	157
2	0	-	93	72
	10	no additions**	264	135
	10	10 mM glucose	392	190
	15	10 mM glucose	540	243
	20	10 mM glucose	590	278
	30	10 mM glucose	515	260
	45	10 mM glucose	377	188
	60	10 mM glucose	262	128
	75	10 mM glucose	263	108

Following the indicated preincubation adenine or hypoxanthine was added to a final concentration of 0.1 mM.

*Glucose was added to a final concentration of 5 mM following the preincubation.

**Glucose was added to a final concentration of 10 mM following the preincubation.

The incubation time following the addition of substrates was 10 minutes.

resulting from pretreatment with glucose is not simply a reflection of increased concentrations of PRPP under these conditions. The results presented in Table XXVIIIb show that the initial velocity of adenine incorporation into the acid soluble fraction was progressively decreased once the preincubation time in the presence of glucose exceeded 30 minutes.

Although the velocity of incorporation was lower the results obtained with hypoxanthine as the added base were in all other respects essentially the same as those obtained with adenine.

The Effects of Preincubation on PRPP Formation

The initial velocity of either adenine or hypoxanthine incorporation following preincubation in the absence of glucose is less than the maximum rate attained when glucose is added to the preincubation medium. The effects of preincubation in the absence of glucose on the rate of PRPP accumulation are presented in Figure 11. Similar to its effects on adenine uptake this treatment eliminates the lag period of PRPP formation. However, the velocity of PRPP accumulation following preincubation is less than the maximum velocity demonstrated by the control cells, the respective values for the results presented being 6.3 and 10.3 mμmoles/minute/ml packed cells.

The pattern of PRPP accumulation in the control cells is complicated by the utilization of this compound for the resynthesis of nucleotides

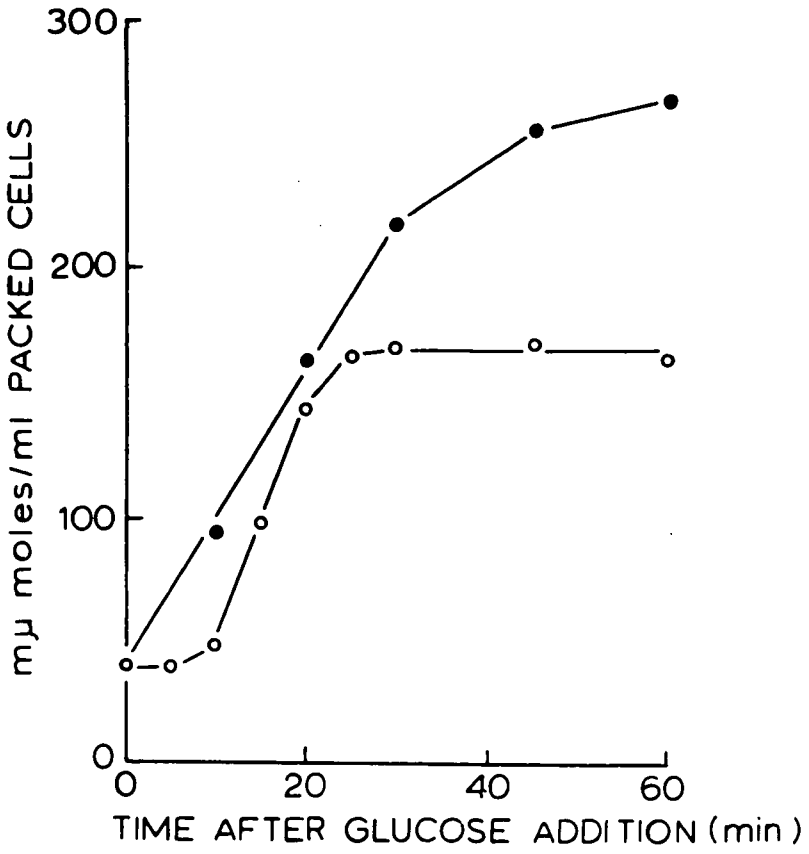

FIGURE 11. The effects of preincubation on the synthesis of PRPP. 10 mM glucose was added at 0 time (○) or following a 10 minute preincubation (●).

from hypoxanthine under these conditions. The difference in the velocities of PRPP synthesis between the control and preincubated cells is therefore probably even greater than that which is observed when the rates of accumulation of "free" cellular PRPP are determined.

5-3. ALTERATIONS IN THE RATE OF ATP REGENERATION

The Effects of Hadacidin on the Conversion of Hypoxanthine to Adenine Nucleotides

The formation of AMP from IMP involves two reactions. The first reaction, the combination of aspartic acid with IMP to form adenylosuccinate, has been shown by Shigeura and Gordon (1962a and b) to be inhibited by hadacidin (N-hydroxy-N-formyl glycine) which is a competitive inhibitor with respect to aspartic acid. Table XXIX presents the results of experiments in which the effects of increasing concentrations of hadacidin on the incorporation of hypoxanthine-8-C^{14} into adenine nucleotides were studied. The incorporation of adenine into the acid soluble fraction exhibited little change in the presence of the highest concentration of hadacidin tested, this being the case in both the control and K-103 media. In the two experiments presented the uptake of hypoxanthine in the control cells was decreased by 22% and 35% in the presence of 0.5 mM hadacidin. In the presence of increased extracellular potassium levels the effect of hadacidin on hypoxanthine uptake was not as great, the maximum inhibitions being somewhat less

TABLE XXIX

THE EFFECTS OF HADACIDIN ON THE INCORPORATION OF ADENINE AND HYPOXANTHINE INTO THE ACID SOLUBLE FRACTION AND ON THE INCORPORATION OF HYPOXANTHINE INTO THE ADENINE NUCLEOTIDE POOL

Incubation medium	Concentration of hadacidin (mM)	mμmoles incorporated/ml packed cells		mμmoles hypoxanthine-8-C^{14} converted to adenine nucleotides/ml packed cells
		Adenine-8-C^{14}	Hypoxanthine-8-C^{14}	
a) Control	0.0	868, 765	452, 322	279, 189
	0.1	- -	308, 268	79, 51
	0.5	948, 777	295, 252	25, 20
	1.0	- -	281, 249	14, 12
	2.0	937, 815	295, 252	9, 8
b) K-103	0.0	233, 144	126, 112	- 19
	0.1	- -	115, 98	- 10
	0.5	228, 153	109, 96	- 4
	1.0	- -	109, 97	- 3
	2.0	276, 168	124, 101	- 3

The concentration of purine base was 0.1 mM and that of glucose was 10 mM. The incubation time was 30 minutes. The results of two separate experiments are given.

than 50% of those observed in the control cells. Approximately 60% of the hypoxanthine which was taken up from the control medium was converted into adenine nucleotides. Hadacidin concentrations of 0.5 mM or greater inhibited this conversion by 90% or more and 0.5 mM was chosen as the concentration at which subsequent experiments were performed.

In comparison to the control cells a relatively small percentage of the hypoxanthine which was taken up from the K-103 medium was incorporated into the adenine portion of the nucleotide pool. In view of the previous finding that glucose has no stimulatory effect on the uptake of adenine for at least 30 minutes (Table XXVII) in the presence of increased potassium levels, it seems probable that the low conversion of hypoxanthine to adenine nucleotides in the K-103 medium is a reflection of decreased IMP formation, rather than a direct effect of potassium on either the hypoxanthine phosphoribosyl transferase reaction or on one of the enzymes involved in the conversion of IMP to AMP.

The Effects of Hadacidin on ATP Regeneration

The results of a time course experiment in which ATP levels in the presence and absence of 0.5 mM hadacidin were measured are presented in Table XXX. The initial depletion of ATP was unaffected by the presence of hadacidin. 30 minutes after the addition of glucose the level of ATP in the inhibited cells was only 75% of that in the control cells indicating that

TABLE XXX

THE EFFECTS OF HADACIDIN ON THE REGENERATION OF ATP

Time (minutes)	μmoles ATP/ml packed cells		Ratio B/A
	No additions (A)	0.5 mM hadacidin (B)	
0	2.4	2.4	1.0
3	0.3	0.3	1.0
10	0.4	0.3	0.75
30	1.2	0.9	0.75
60	1.8	1.1	0.61

Each value represents the average of four separate experiments. 10 mM glucose was present under both experimental conditions.

in the latter case one-quarter of the ATP which was resynthesized between 3 and 30 minutes arose from the conversion of hypoxanthine and IMP back to adenine nucleotides. The difference between the ATP levels of the control and inhibited cells increased during the latter half of the incubation period when a greater portion of the regenerated ATP arises from hypoxanthine. The concentration of ATP after 60 minutes was 40% lower in the presence of hadacidin than in its absence.

The Effects of Adenine, Hypoxanthine, and Hadacidin on ATP Regeneration

The inhibition of ATP regeneration in the presence of hadacidin should be reversed by the addition of adenine to the incubation medium. The results presented in Figure 12 show that not only was this the case, but also that the addition of adenine, either in the presence or absence of hadacidin, produced a marked increase in the rate of ATP accumulation over the control value. In contrast to adenine, hypoxanthine did not reverse the inhibition produced by hadacidin and had little, if any, effect on the restoration of ATP levels in the absence of hadacidin.

5-4. ATP REGENERATION AND ENERGY METABOLISM

The experiments to be described were performed in order to determine the effects of different rates of ATP regeneration on the various metabolic activities which were discussed in Chapters III and IV.

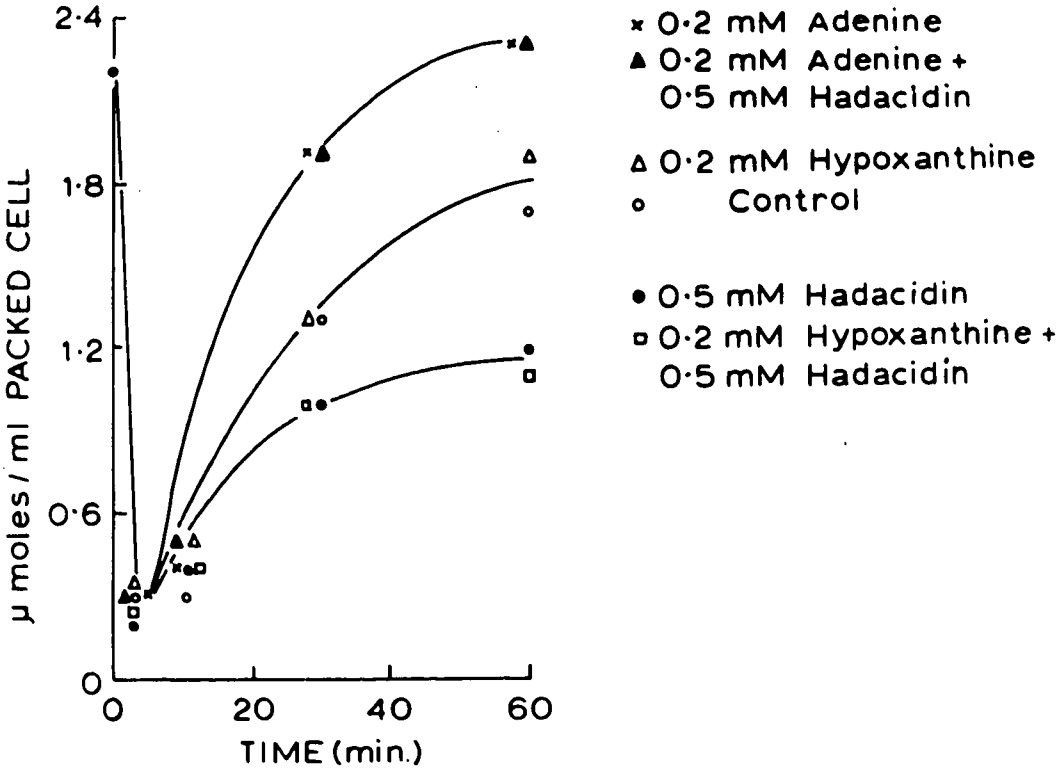

FIGURE 12. The effects of adenine, hypoxanthine, and hadacidin on the regeneration of ATP. The concentration of glucose was 10 mM.

Figure 13A demonstrates that the formation of lactic acid was unaffected by the addition of either adenine or hadacidin. On the other hand, the lag period with respect to glycogen synthesis was decreased in the presence of adenine, although the activity of this pathway once the lag period had elapsed was not affected (Figure 13B). The incorporation of glucose into glycogen was decreased by approximately 25% in the presence of hadacidin.

The effects of adenine and hadacidin on respiratory activity are presented in Table XXXI. Unlike the lack of an effect on glycolysis, the addition of these compounds resulted in small, but significant, changes in the rate of oxygen consumption.

Incubation in the presence of hadacidin did not alter the uptake of glycine-2-C^{14} and produced little change in the amount of glycine incorporated into protein (Table XXXII). Although a small increase in protein synthesis in the presence of hadacidin was evident between 35 and 90 minutes in the experiments which are presented in Table XXXII, this effect was not observed in all the experiments performed and its significance is unclear. A small increase in glycine uptake was apparent in the presence of adenine. The effect of adenine on protein synthesis was similar to that observed with respect to glucose incorporation into glycogen; i.e. adenine decreased the duration of the lag but did not alter the rate of glycine incorporation once the lag period had elapsed.

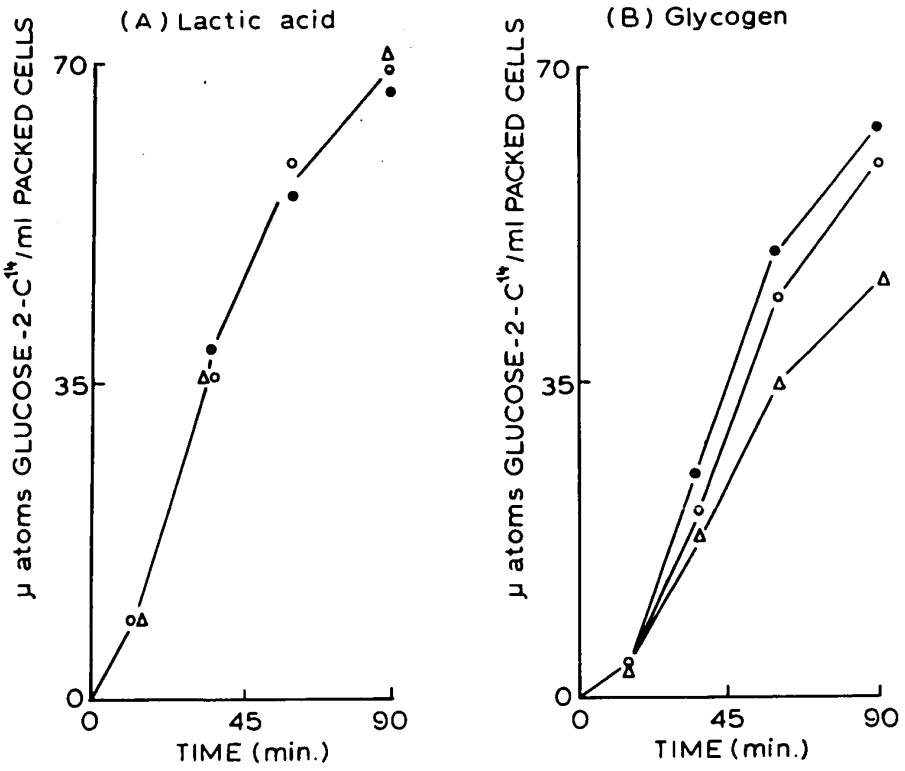

FIGURE 13. The effects of adenine and hadacidin on glucose-2-C^{14} incorporation into lactic acid and glycogen. The concentration of glucose was 10 mM. ○ , control; ● , 0.1 mM adenine; △ , 0.5 mM hadacidin.

TABLE XXXI

THE EFFECTS OF ADENINE AND HADACIDIN ON THE RESPIRATORY ACTIVITY OF NOVIKOFF ASCITES HEPATOMA CELLS

Experiment number	QO_2		
	No additions	0.1 mM adenine	0.5 mM hadacidin
1	7.2	7.8	6.9
2	7.6	8.1	6.5
3	6.7	7.6	5.6
4	6.5	7.4	6.3
5	7.2	7.6	5.4
Average ± S.D.	7.0 ± 0.4	7.7 ± 0.3	6.1 ± 0.6
p		0.02	0.05

p is calculated with respect to the control value. 10 mM glucose was present under all experimental conditions. The QO_2 values were determined as described in Table I.

TABLE XXXII

THE EFFECTS OF ADENINE AND HADACIDIN ON GLYCINE UPTAKE AND INCORPORATION INTO PROTEIN

Time (minutes)	μmoles glycine-2-C^{14}/ml packed cells					
	Control		Plus 0.1 mM adenine		Plus 0.5 mM hadacidin	
	Uptake	Incorporation x 10^2	Uptake	Incorporation x 10^2	Uptake	Incorporation x 10^2
15	3.1, 3.2	3.1, 1.1	3.0, 3.0	3.7, 1.3	3.1, 3.1	3.1, 1.1
35	4.2, 4.6	16.2, 8.5	4.5, 4.8	20.1, 11.7	4.2, 4.6	17.6, 8.6
60	4.9, 5.9	26.6, 17.5	5.4, 5.6	31.2, 20.8	4.5, 5.0	30.6, 19.7
90	5.3, 5.8	35.6, 25.0	6.0, 6.3	38.6, 28.8	5.3, 5.6	38.5, 28.9

The results of two separate experiments are presented. 2 mM glycine and 10 mM glucose were present under all experimental conditions.

5-5. THE EFFECTS OF ADENINE AND HADACIDIN FOLLOWING PREINCUBATION

In order to verify that the effects of adenine and hadacidin which have been described were in fact due to the differences in ATP regeneration which are produced by these two compounds, their action on the metabolism of cells receiving a 10 minute preincubation prior to the addition of substrates was determined (Table XXXIII). Under these conditions the presence of hadacidin did not alter any of the metabolic activities where were measured. With the exception of respiration adenine also had little effect on the cell's metabolic activity following preincubation. The stimulation of respiration by adenine under these conditions is most probably a consequence of its conversion to ATP. It is interesting that, as with the non-preincubated cells, it was respiration and not glycolysis that responded to adenine addition.

The apparent sensitivity of respiration to small changes in the concentration of adenine nucleotides implies that the electron transport system is very tightly coupled to phosphorylation in these cells. Experiments in which the effects of DNP on oxygen uptake were determined indicated that this is in fact the case. Thus the addition of 0.05 mM DNP to the control medium, in the presence of 10 mM glucose produced a four to fivefold stimulation of respiratory activity. Under similar conditions the addition of DNP resulted in only a 2.5 fold increase in the respiration of Ehrlich ascites cells (Ellis and Scholefield, 1961).

TABLE XXXIII

THE EFFECTS OF HADACIDIN AND ADENINE FOLLOWING PREINCUBATION IN THE ABSENCE OF GLUCOSE

Additions*	QO_2	µatoms glucose-2-C^{14} incorporated/ ml packed cells		µmoles glycine-2-C^{14}/ml packed cells	
		Lactic acid	Glycogen	Uptake	Incorporation x 10^2
Nil	7.6	53	71	3.8	33.6
0.5 mM hadacidin	7.8	48	68	3.9	34.6
0.1 mM adenine	8.5	47	74	4.1	35.4

The cells were preincubated for 10 minutes prior to the additions and the incubation continued for an additional 60 minutes. The QO_2 values were determined from the respiratory rate between 20 and 60 minutes after the additions were made.

*In addition to hadacidin or adenine, 10 mM glucose, or 10 mM glucose plus 2 mM glycine were added following the preincubation period.

SUMMARY

1. Incubation of freshly prepared Novikoff ascites hepatoma cells in the presence of glucose results in a rapid depletion of the cellular ATP. Small increases in the levels of both AMP and ADP occur, but do not compensate for the total loss of ATP, and a net loss of adenine nucleotides results. Hypoxanthine accumulates as the major catabolic product of ATP breakdown.

2. In the presence of increased extracellular potassium the pattern of adenine nucleotides is similar to that observed in the control cells. However, a somewhat greater loss of adenine nucleotides occurs under these conditions.

3. Studies on the incorporation of adenine-8-C^{14} into the acid soluble fraction indicate that during the first 10 to 12 minutes in the control cells the purine phosphoribosyl transferase reaction may be limited by the availability of PRPP. However, with longer times of incubation, or following preincubation in the absence of glucose, other factors appear to be involved in the regulation of this reaction.

4. In the presence of glucose, and in the absence of a preincubation the uptake of adenine is markedly decreased by increased extra-

cellular potassium levels, although this ion does not alter the rate of adenine uptake following an initial endogenous preincubation.

5. The regeneration of ATP is stimulated by adenine and decreased in the presence of hadacidin.

6. Incubation in the presence of hadacidin results in a decrease in respiration and glycogen synthesis, but this compound has little effect on glycolysis, protein synthesis or glycine transport.

7. Incubation in the presence of adenine results in an increase in both respiration and glycine uptake. The lag periods of both glycogen and protein synthesis are reduced in the presence of adenine although this compound does not alter the rates of these pathways once the lag period has elapsed. No effect of adenine on glycolysis is observed.

CHAPTER VI

DISCUSSION

The addition of glucose to tumor cells, in the presence of oxygen, generally has little, if any effect on either the energy level or anabolic capability of the cell. Time course experiments have revealed a transient decrease in the level of ATP following the addition of glucose but steady state conditions are normally reestablished within minutes (see above, Chapter I). In view of these previous findings the extremely deleterious effect of glucose on the energy state of the Novikoff ascites tumor which has been described in the present work was unexpected and considered to be of sufficient interest to merit further investigation.

6-1. THE EFFECTS OF THE ADDITION OF GLUCOSE ON THE INTRACELLULAR ENERGY LEVEL

Rapid catabolism of adenine nucleotides may occur in the presence of metabolic inhibitors (Wu and Racker, 1959b; McComb and Yushok, 1964a and b). However, these experimental conditions are anticipated to be inimical to the normal functioning of the cell and are therefore not

comparable to the environment in which the addition of glucose produces its effects in the present study.

The present observation is not unique, and an adverse effect of hexose addition on the energy metabolism of other tissues has been observed. For example, Harris and co-workers (1954) found that addition of high concentrations of glucose to lens tissue resulted in a loss of ATP and an inability to accumulate potassium ions. Fructose infusion into rats results in a rapid breakdown and loss of hepatic ATP and a consequent inhibition of glycogen and protein synthesis (Mäenpää et al., 1968; Burch et al., 1969). The addition of glucose to dilute suspensions of Ehrlich ascites cells produces a rapid depletion of the cellular ATP content (Lassen, 1965; Overgäard-Hansen, 1965).

Are the present findings related in any way to these previously described effects of glucose? The effect of glucose on the energy levels of Ehrlich ascites cells depends upon the concentration of the cell suspension and Overgäard-Hansen (1965) has suggested that the drop in ATP and the subsequent deamination of AMP are dependent on strict aerobic conditions. In the present work no significant change in the level of high energy nucleotide phosphate was caused by the addition of glucose to a 3.3% (by volume) suspension of Ehrlich ascites cells (Figure 3). This is the same concentration of cells used with the Novikoff tumor and it is therefore reasonable to assume that the "aerobic state" of both tumors was similar at the time of

glucose addition. This result indicates that the response of the Novikoff cells to the addition of glucose is determined by different, or additional factors than those responsible for the previously described effects of glucose in the Ehrlich cells. This conclusion is further supported by the failure of glucose to cause a loss of adenine nucleotides if it is added to the Novikoff tumor following a preincubation period (Figure 6). In contrast, catabolism of adenine nucleotides was observed when glucose was added to Ehrlich cells after a 15 minute preincubation (Lassen, 1965; Overgäard-Hansen, 1965).

The effect of fructose on the ATP content of rat liver has been related to the rapid phosphorylation of that compound by the enzyme fructokinase (Mäenpää et al., 1968). In the present study a relationship between the affinity of the various sugars tested for hexokinase (Sols and Crane, 1954a) and their ability to cause a depletion of high energy nucleotide phosphate is indicated by the results of Table IX. It is important to note that in these experiments fructose did not have an effect on the level of 7mnp. In view of the report by Nigam (1967a) that the Novikoff cells incorporate fructose and glucose into glycogen at similar rates it was anticipated that these two sugars, at the concentration used, would have similar effects on the energy state of the cell. An explanation for the apparent discrepancy between the results of Nigam (1967a) and those of the present work may be provided by the study of Letnansky (1968) in which strain variations

in the ability of Ehrlich ascites cells to metabolize fructose were demonstrated. Although the results presented in Table IX suggest that the rate of sugar phosphorylation plays a role in the production of the ATP drop, the failure of glucose to elicit a similar effect following preincubation indicates that additional factors are involved.

Cells depleted of potassium begin to reaccumulate this ion as soon as favorable conditions are reestablished. The reaccumulation of potassium ions requires more energy than the maintenance of the normal ionic gradients, a fact which is reflected in the increased rates of respiration and/or glycolysis that accompany this process (Levinson, 1967; Gordon and de Hartog, 1968). In the present study it was found that following the washing procedure the intracellular levels of sodium ions were high and of potassium ions were low (Table XI). It is therefore proposed that the effects of glucose on the energy levels are consequences of the combined utilization of ATP by hexokinase for the phosphorylation of glucose and by the cation pump for the reestablishment of the normal ionic gradients. Once the initial drop in ATP has been produced, the internal environment of the cell favors further catabolism of ATP and the other adenine nucleotides (see below). During the preincubation period there is a decrease in the velocity of intracellular water and ionic changes, indicating a reduction in the utilization of ATP for ion transport. When glucose is added at this time the energy balance is no longer upset by the resulting increase

in ATP utilization and the catabolic sequence is not initiated.

6-2. ANALYSIS OF THE ENERGY RELATIONSHIPS FOLLOWING GLUCOSE ADDITION

In the above paragraphs it has been suggested that the addition of glucose to freshly prepared Novikoff ascites cells results in a shift in the balance between the production and utilization of ATP and that this in turn results in the observed decrease in the level of high energy nucleotide phosphate. The nature of the energy relationships involved in producing this effect may be obtained from the following calculations.

In the first instance it is assumed that the 1 minute values for glucose utilization and lactic acid production presented in Table XXII represent initial velocities. It is also assumed that glucose phosphorylation proceeds largely to the monophosphate level during the first 30 seconds so that the accumulation of fructose diphosphate is minimal during this period. From the data of Levinson and Hempling (1967) it can be calculated that the active transport of cations could account for the utilization of 1 μmole of ATP within the first 30 seconds. Under these circumstances the difference between the amount of ATP utilized for glucose phosphorylation and cation transport at the end of 30 seconds and that produced by glycolysis during the same time interval would be -5.9 μmoles/ml packed cells. The results of the experiments presented in Table XXIV demonstrate that the

actual decrease in the level of ATP after 1 minute is only 1.7 μmoles/ml packed cells. Assuming that other factors are constant the difference between the actual and the calculated decreases in ATP must be accounted for in terms of extra oxidative ATP production. If 50% of the observed drop in ATP occurs within the first 30 seconds then oxidative phosphorylation should account for the production of 5 μmoles of ATP/ml packed cells during this period. To produce this amount of ATP the rate of respiration must be increased following the addition of glucose. [i.e. If the QO_2 in the absence of glucose is taken as 9.4 (Table I) and the P/O ratio is then 3, then a doubling of the respiratory rate would result in the production of 10 μmoles of ATP/ml packed cells/minute or 5 μmoles in 30 seconds. If the QO_2 in the absence of glucose is lower (e.g. 7.4 in Table III) then a greater stimulation in respiration would be required to produce the same increase in the rate of ATP production.] The postulated increase in oxygen consumption is in agreement with the findings of Chance and Hess (1959a) that the respiratory activity of tumor cells may be increased by as much as tenfold upon the addition of glucose. In the present study the exact extent of the shift in the energy balance towards catabolism would depend on the relative contribution to ATP utilization made by other cellular activities (e.g. protein synthesis, ATPases, etc.) as well as on the actual change in respiration elicited by the addition of glucose.

6-3. THE CATABOLISM OF ADENINE NUCLEOTIDES

The initial decrease in ATP levels which follows the addition of glucose is accompanied by a temporary increase in both AMP and ADP concentrations, and is followed by a decrease in the total adenine nucleotide pool (Table XXIV and Figure 7). The brief accumulation of IMP during the initial minutes suggests that there is deamination of AMP by adenylic acid deaminase and is consistent with the findings of others (McComb and Yushok, 1964b; Overgäard-Hansen, 1965). Unlike earlier reports in which inosine was found to be the major catabolite produced following ATP depletion (McComb and Yushok, 1964b; Overgäard-Hansen, 1965), it is now found that hypoxanthine accumulates as the chief product of purine catabolism. A comparison of the relative activities of IMP nucleotidase and inosine phosphorylase in extracts of Novikoff hepatoma shows that the latter enzyme is 3 to 4 times more active than the former (de Lamirande et al., 1958). A similar ratio of activities in the intact cell would be consistent with an accumulation of hypoxanthine rather than inosine. Although inhibition by hypoxanthine might tend to decrease the activity of the phosphorylase reaction (Friedkin and Kalckar, 1961) this effect would be minimized by the dilution of hypoxanthine in the incubation medium.

The initial accumulations of AMP and IMP indicate that catabolism of the adenine nucleotides during the first minutes following the addition

of glucose is limited by the relative activities of AMP deaminase and 5'IMP nucleotidase. However, the changes in the intracellular environment are such that conditions soon favor the further catabolism of AMP. Thus the decrease in the level of inorganic phosphate and the increase in ADP concentrations would favour an increase in AMP deaminase activity (Lee and Wang, 1968; Smiley and Seulter, 1967). Similarly, an increase in nucleotidase activity would be expected to accompany the decrease in the concentration of ATP (Murray and Friedrichs, 1969). The initial effects of glucose on the ATP levels are therefore magnified because of the kinetic properties of the various enzymes involved in the catabolism of adenine nucleotides.

6-4. THE CONTROL OF ATP REGENERATION

The addition of glucose to tumor cells in which the ATP level has been reduced by anaerobiosis or by the addition of metabolic inhibitors results, in many cases, in a rapid restoration of the ATP concentration. In contrast, Laws and Strickland (1967) reported that following an anaerobic preincubation the regeneration of ATP in Ehrlich ascites cells in the presence of glucose was very slow. The long period required for the restoration of the steady state in these studies was attributed only to the balance between the synthesis and breakdown of ATP. Overgäard-Hansen (1965) demonstrated that following catabolism to inosine the time required for the restoration of the

steady state level of ATP was 5 times longer than that required if catabolism had proceeded only as far as AMP, indicating that factors in addition to the simple production/utilization balance are involved in regulating the regeneration process in the former case.

In the present study the maximum rate of ATP regeneration occurred between 10 and 25 minutes after the addition of glucose and 65 to 75% of the ATP formed during this period could be accounted for in terms of the decreased levels of AMP and ADP (Table XXIV and Figure 7). Subsequently, most, if not all of the regenerated ATP appeared to be derived from hypoxanthine. The conversion of hypoxanthine to AMP is implicated as the rate limiting sequence in the regeneration of ATP during the latter half of the incubation period on the basis of the following observations. First, in spite of the continuous decrease in the concentration of hypoxanthine, the levels of IMP, AMP, or ADP did not increase during this period. Second, the addition of adenine, but not of hypoxanthine, resulted in a marked increase in the rate of ATP regeneration. The former observation suggests that ATP production is limited by the rate of IMP formation from hypoxanthine and PRPP or by the cleavage of AMPS to yield AMP and fumaric acid. Evidence for the latter is found in the report of Balis and Salser (1964) in which it is demonstrated that some tumors have a low level of adenylosuccinate lysase and that with these tumors AMPS accumulates in the incubation medium. Incubation in the presence of adenine not only eliminates the necessity for the

conversion of IMP to AMP but also results in a more rapid formation of ribonucleotide than occurs if hypoxanthine is the substrate (Table XXVIII). This latter finding is in agreement with the observation of Henderson and Khoo (1965b) that Ehrlich ascites cells incorporate adenine into the acid soluble nucleotide fraction at a considerably greater rate than hypoxanthine.

6-5. THE REGULATION OF PHOSPHORIBOSYL TRANSFERASE

The suggestion that the regeneration of ATP might be partially controlled by the rate of ribonucleotide formation from hypoxanthine prompted a closer investigation of the phosphoribosyl transferase reaction. It was felt that the low levels of ATP which are present shortly after the addition of glucose might limit the synthesis of PRPP and that this in turn might limit the velocity of the phosphoribosyl transferase reaction. The lag period prior to the establishment of the maximum rate of PRPP synthesis, and the elimination of this period by preincubation, are consistent with this proposal (Figure 11). However, the continuing increases in the velocities of adenine and hypoxanthine incorporation into the acid soluble fraction after the rate of PRPP synthesis was maximal, (Table XXVII and Figure 10) indicated that during the first 30 minutes of the incubation the phosphoribosyl transferase reaction is regulated by factors other than, or in addition to, the availability of PRPP. Adenine and hypoxanthine phosphoribosyl transferase activities from Ehrlich ascites cells are inhibited by AMP and IMP respectively (Hori

and Henderson, 1966; Murray, 1966a). The initial accumulation of these two compounds and the increase in the velocity of the phosphoribosyl transferase reaction which accompanies the decrease in the mononucleotide levels between 10 and 25 minutes (Table XXVIII) are consistent with a regulatory role for AMP and IMP during this period. It is important to note, however, that even at its lowest level the concentration of AMP is many times greater than the K_i reported by Murray (1966a) for the enzyme from Ehrlich ascites cells. Similar observations led Murray to suggest that all, or part, of the adenine nucleotide pool is unable to act on the phosphoribosyl transferase enzyme in the intact cell. If, as postulated, the purine mononucleotides play a regulatory role under the present conditions the same conclusion must be drawn.

The lower rates of PRPP synthesis and of adenine incorporation into the acid soluble fraction which occur following preincubation in the absence of glucose are suggestive of regulation of these processes by nucleoside triphosphates (Figures 9 and 11). Control of phosphoribosyl transferase activity by these compounds is also indicated by the decreases in the initial velocity of this reaction which occur if the preincubation time in the presence of glucose exceeds 20 to 30 minutes (Table XXVIII). In accord with this suggestion are the findings that both PRPP synthetase (Kornberg et al., 1955a; Switzer, 1967; Wong and Murray, 1969) and purine phosphoribosyl transferase (Murray, 1966a; Berlin and Stadtman, 1966) are inhibited by nucleoside triphos-

phates. An alternate possibility is that only the rate of PRPP synthesis is affected by these compounds and that as the concentration of the nucleoside triphosphates increases the supply of PRPP becomes limiting for the phosphoribosyl transferase reaction. Control by the availability of PRPP would, however, be in contrast to experiments with Ehrlich ascites cells in which it was found that PRPP does not normally limit the formation of ribonucleotides from a single purine base (Henderson and Khoo, 1965b).

The postulated controls indicate that the period of rapid PRPP formation and phosphoribosyl transferase activity which occurs between 10 and 25 minutes after the addition of glucose is the consequence of a permissive environment of purine nucleotides. Thus the level of ATP is high enough to support high rates of PRPP formation but the concentrations of the nucleoside triphosphates are still relatively low, minimizing inhibition by these compounds. In addition the activity of the phosphoribosyl transferase enzyme would be further enhanced by the decreasing levels of AMP and IMP during this period.

These experiments demonstrate that when the contribution of hypoxanthine to the regeneration of ATP is minimal (between 10 and 25 minutes) the activities of the enzymes involved in PRPP synthesis and the formation of ribonucleotides from the free base are maximal. On the other hand, when most of the ATP being formed is derived from hypoxanthine, the enzymes involved in this process are in a progressively inhibited state. The results indicate

that the rate of ATP regeneration during the latter half of the incubation period is strictly regulated by its own concentration, and by the levels of the other high energy nucleoside triphosphates.

6-6. ATP LEVELS AND ENERGY METABOLISM

Glycolysis

ATP serves a dual role as substrate and regulator of the glycolytic sequence. In the present study the accumulation of phosphorylated intermediates which occurs following the addition of glucose to freshly prepared tumor cells (Table XXIII) is suggestive of a regulatory pattern focused around the control of phosphofructokinase by adenine nucleotides. The initial drop in the level of ATP, in conjunction with the increased levels of AMP and ADP, is consistent with an activation of phosphofructokinase shortly after the addition of glucose, and the large accumulation of fructose diphosphate indicates an imbalance between the formation and subsequent metabolism of this compound. The decrease in the level of inorganic phosphate, most probably the result of trapping in fructose diphosphate, suggests that phosphate may become limiting for glyceraldehyde phosphate dehydrogenase, a situation which would favor the further accumulation of fructose diphosphate. The availability of inorganic phosphate, rather than of ADP, is implicated as limiting the conversion of fructose diphosphate to lactic acid by the observation that both ADP and fructose diphosphate accumulate during the same

period. In addition, the inverse relationship which exists between the levels of inorganic phosphate and fructose diphosphate after 6 minutes is consistent with control by the former compound. The decrease in the rate of glucose utilization which occurs 1 minute after the start of the incubation is most probably the consequence of limited ATP availability. However, the decrease in the concentration of inorganic phosphate would render hexokinase more sensitive to product inhibition (Rose et al., 1964) and, therefore, control by glucose-6-phosphate may also limit glucose utilization at this time.

6 to 10 minutes after the addition of glucose the levels of ATP and inorganic phosphate begin to increase and the above sequence is reversed. The rates of both glucose utilization and lactic acid production are accelerated at this time, the latter activity further enhancing the regeneration of ATP. The changes in the levels of the adenine nucleotides after 10 minutes would cause a progressive inhibition of phosphofructokinase. Inhibition of this enzyme by ATP at a relatively high FDP:ATP ratio is in accord with the findings of Wu (1966) that phosphofructokinase from Novikoff ascites cells is insensitive to activation by fructose diphosphate. The lack of an effect of either adenine or hadacidin on the formation of lactic acid (Figure 13) suggests that ADP plays a more significant role in the regulation of phosphofructokinase than ATP does. Thus, in the presence of these compounds (Figure 12) the rate of ATP regeneration showed considerable variation but the levels of ADP were similar under the different experimental conditions.[1]

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[1] J.W. Gurd and P.G. Scholefield, unpublished observations.

It is significant that glycolysis undergoes a phase of very rapid activity between 10 and 30 minutes after the initial depletion of ATP (Figures 2 and 6). The above discussion indicates that this period occurs when the level of ATP is high enough to support a high rate of hexose phosphorylation but the relative amounts of adenosine mono-, di-, and triphosphates are such that phosphofructokinase is still in a relatively activated state.

Glycogen Synthesis and Protein Synthesis

The low activities associated with the glycogen and protein synthesizing pathways during the period of minimum ATP levels are consistent with the known energy dependence of these processes. The dependence of the cell's anabolic activities on the maintenance of a minimum concentration of intracellular ATP is further emphasized by 1) the reduction in the lag periods of these two activities which accompanies the greater rate of ATP regeneration in the presence of adenine (Table XXXII and Figure 13), and 2) the initial stimulatory effects of preincubation on both glycogen and protein synthesis (Table XIV and Figure 6). The increase in the initial rates of protein synthesis but not in the uptake of glycine following preincubation (Table XIV), demonstrates a dichotomy between these two processes and a much greater sensitivity of the former activity to changes in the energy state of the cell. These findings are similar to those reported by Riggs and Walker (1963) in which transport and incorporation of amino acids in Ehrlich ascites cells were shown to be independent of one another under a

variety of experimental conditions.

Decreases in the level of ATP are accompanied by simultaneous decreases in the concentrations of the other nucleoside triphosphates (Letnansky, 1964; Burch et al., 1969). The participation of UTP in glycogen synthesis and the observations that aerobic energy may be required for the formation of glycogen and of uridine nucleotides may afford an explanation for the inhibitory effects of hadacidin on glucose conversion to glycogen in the non-preincubated cells (Figure 13). Thus less efficient formation of UTP in the presence of hadacidin, resulting from the decrease in respiration under these conditions (Table XXXI), may render UTP limiting for glycogen synthesis. This may be the case even though, as in the present study, the concentration of ATP exceeds that at which maximum glycogen formation occurs in the control cells. In contrast to its effect on the incorporation of glucose into glycogen, hadacidin did not have any effect on protein synthesis. This is consistent with the above suggestion because tumor cells utilize both glycolytic and oxidative energy with equal efficiency for the formation of protein (Quastel and Bickis, 1959).

Ion Transport and Amino Acid Transport

The changes in the concentrations of sodium and potassium ions which follow the addition of glucose to the control cells (Table XI, Figure 5) are indicative of a failure of the cation pump to function during the period of minimum ATP levels. A similar effect of ATP depletion on the ability of

Ehrlich ascites cells to accumulate potassium ions has been observed (Weinstein and Hempling, 1964; Levinson, 1967). In contrast to the transport of ions the uptake of amino acids was unaffected by the variations in the levels of ATP following glucose addition. The ability of cells incubated in either the control or K-103 medium to accumulate glycine was similar in the presence and absence of glucose, with or without preincubation (Tables IV, VI, XIV, and XVII). The failure of amino acid uptake to be affected by the various incubation conditions employed suggests that either the lowest levels of ATP reached were still great enough to support maximum rates of uptake, or that a high energy intermediate other than ATP was capable of supporting the active accumulation of amino acids during the periods of minimum ATP concentrations. The latter suggestion is supported by the previous observation of Johnstone and Scholefield (1959) that in spite of the immediate decrease in the level of ATP which follows the addition of DNP to Ehrlich ascites cells, a 10 minute lag period occurs before amino acid uptake is inhibited. A similar lag in the present study would permit uptake, in the control medium at least, to continue unaltered until the levels of ATP had begun to increase.

The results which have been presented demonstrate that both glycogen (Figure 2) and protein synthesis (Table XIV) can proceed at maximum rates when the concentration of ATP is only 40 to 60% of the steady state level, that cation transport is initiated during the early stages of ATP

recovery (Table XI and Figure 5) and that relatively low levels of ATP can support maximum rates of glucose phosphorylation (Table XXII) and PRPP formation (Figure 11). Similar observations with respect to metabolic activity in the presence of low concentrations of ATP have been made by others. For example, Wu and Racker (1959b) found that glucose phosphorylation in Ehrlich ascites cells proceeds at a high rate when the level of intracellular ATP is quite low, and Lassen (1965) demonstrated that following glucose induced depletion of ATP the accumulation of Rb^{86} by Ehrlich ascites cells begins when the concentration of ATP is only 20 to 50% of the control value. These results, in conjunction with the lack of an effect of hadacidin on protein synthesis (Table XXXII), and the similar metabolic behavior of cells with ATP levels differing by as much as 40% (Tables XIX and XX) demonstrate that the intact cell is able to perform numerous energy requiring metabolic activities at levels of ATP considerably lower than those which are normally present.

6-7. THE METABOLIC EFFECTS OF POTASSIUM IONS

During the course of the present investigation it was observed that replacement of sodium by potassium in the incubation medium resulted in an inhibition of the various metabolic activities studied. The experiments on the effects of increased concentrations of extracellular potassium were initially stimulated by the observation of Hastings and co-workers (1952)

that maximum glycogen formation by liver slices occurs in a medium in which all of the sodium has been replaced by potassium and by a desire to increase the rate of glycogen deposition by the Novikoff ascites cells in the present study. Unexpectedly this treatment (i.e. increasing the level of extracellular potassium) resulted in a severe inhibition of glucose incorporation into glycogen and this observation prompted the additional experiments on the metabolic effects of potassium which have been described.

In the present study it is necessary to differentiate between those effects of potassium which are due to changes in the ionic environment per se and those which are a consequence of the decreased rate of ATP regeneration which occurs in the K-103 medium. For example, in the absence of an affect on the concentration of ATP, glycolysis, glycogen synthesis, and protein synthesis are all decreased in the presence of elevated extracellular potassium levels (Tables XVI and XVII). In contrast, the severe inhibition of adenine incorporation into the acid soluble fraction which follows glucose addition to non-preincubated cells does not occur if the cells are preincubated prior to the addition of exogenous substrates (Table XXVII). The decrease in the latter activity thus appears to be solely the consequence of the effect of potassium on the cell's energy supply. The inhibitions of the former activities, in the absence of preincubation, would seem to be the expression of the combined effects of a specific inhibitory action of potassium ions and the decreased rate of ATP restoration under these conditions.

Protein Synthesis

The dual effects of potassium in the non-preincubated cells are perhaps best exemplified by the experiments on protein synthesis (Tables VI and XVII). Following the glucose induced depletion of ATP, glycine incorporation into protein of cells incubated in the K-103 medium was reduced by 75 to 90% but in the absence of a decrease in the ATP level the inhibition was only 25 to 50% (Tables VI and XVII). The greater effects of potassium in the former case are most readily explained on the basis of the prolonged period of low ATP concentrations under these conditions.

The amino acid incorporating ability of the microsomal fraction from rat liver is decreased in the presence of excess potassium ions (Sachs, 1957) suggesting that the present inhibitions may be due to a direct action of potassium on the mechanisms involved in protein synthesis. Similar effects of increased levels of extracellular potassium on the protein synthesizing activities of LM strain mouse fibroblasts (Kuchler, 1967) and Ehrlich ascites cells (Riggs and Walker, 1963) have been reported.

Glucose Metabolism

As mentioned above, it had been anticipated that an increase in the concentration of extracellular potassium would stimulate glycogen synthesis in the Novikoff ascites cells as it does in normal liver. Earlier

studies had demonstrated that slices of Novikoff hepatoma behave similarly to liver in exhibiting less glycogen breakdown in a high potassium than in a high sodium medium (Ashmore et al., 1958). However, the inhibition of glycogen deposition which is observed in the present study resembles more closely the reaction of muscle (Stadie et al., 1947) than of liver to environmental ionic changes. In this connection it is interesting to note that hepatomas have been found to resemble muscle more closely than liver in other respects as well. For example, the enzymes pyruvic kinase (Tanaka et al., 1965) and aldolase (Adelman et al., 1967) of hepatomas are of the muscle rather than the liver type and the overall pattern of fructose metabolism by rapidly growing hepatomas is similar to that of muscle (Ashmore et al., 1963).

Hastings (1957) has suggested that the response of liver glycogen metabolism to changes in the ionic environment reflects changes in the activity of phosphorylase. The results presented in Table XVIII demonstrate that glycogenolysis is similar in both the K-103 and control media, indicating that in the present study the effects of potassium are exerted on the anabolic rather than the catabolic pathway of glycogen metabolism.

Experiments on the effects of cations on the carbohydrate metabolism of rat diaphragm led Clausen (1966) to propose that the changes in glycolysis and glycogen synthesis which accompany variations in the concentrations of extracellular ions are secondary to changes in the activity of the cation pump. In agreement with the results reported by Clausen, the

addition of ouabain in the present study caused a decrease in the glycolytic activity of cells incubated in the control medium. Ouabain did not, however, alter the rate of lactate formation in the presence of increased levels of extracellular potassium (Table XIII). The net effect of ouabain was therefore to decrease the difference in the formation of lactic acid between the control and K-103 cells. It seems likely that the ouabain sensitive portion of glycolysis in the control cells is concerned with the production of energy used for the reaccumulation of potassium ions. Contrary to the effects of ouabain on rat diaphragm (Clausen, 1966) this compound had no effect on the ability of the Novikoff ascites cells to synthesis glycogen. It might be noted that the reversibility of the potassium inhibitions which is observed in the current work (Tables XIX and XX) is also in contrast to the permanent cellular damage to rat diaphragm produced by incubation in a high potassium medium (Clausen, 1968). The different behavior of glycolysis and glycogen synthesis in response to ouabain, and the difference in the time course of the potassium induced inhibition of these processes [i.e. the inhibition of glycolysis is constant but a progressively increasing inhibition of glucose incorporation into glycogen is observed (Table XVI)] indicate that the effects of potassium on these two pathways of glucose metabolism are only indirectly related, if, indeed, they are related at all.

The experiments which have been performed do not identify the specific site or sites at which potassium acts to inhibit glycolysis. However

an effect on the transphosphorylating enzymes of the glycolytic sequence is suggested by the observations that the activities of a number of enzymes concerned with transphorylation are stimulated by potassium ions (Ussing, 1960) and that fructokinase (Hers, 1952) and pyruvic kinase (Kachman et al., 1953) are inhibited by high concentrations of this ion. Inhibitory effects of increased levels of extracellular potassium on glycolysis in the Novikoff hepatoma (Ashmore et al., 1958) and on glucose utilization in Ehrlich ascites cells (Riggs and Walker, 1963) have also been reported.

The Regeneration of ATP in the K-103 Medium

The regeneration of ATP following the addition of glucose to freshly prepared cells involves the formation of ribonucleotide from the free base and the rephosphorylation of AMP to form ATP. What are the effects of changes in the extracellular potassium concentration on these activities? In the absence of glucose the regeneration of ATP from AMP was similar in cells incubated in both the control and K-103 media (Table X). In the absence of a decrease in the level of ATP the incorporation of adenine into the acid soluble fraction was also the same in the control and K-103 media (Table XXVII). The slow regeneration of ATP in the presence of elevated levels of potassium is not, therefore, due to a direct effect of potassium on either of these processes. Following preincubation glycolysis in the K-103 medium is decreased by 15 to 20% (Table XVI). If the sequence of glycolytic events following the addition of glucose to freshly prepared cells in the

K-103 medium is similar to that observed with the control cells, and the identical decrease in high energy nucleotide phosphate levels under both conditions (Figure 3) suggests that this is probably the case, then an inhibitory effect of potassium on the lower, ATP generating portion of glycolysis would result in the observed prolonged recovery period. The consequences of the reduced rate of ATP regeneration are seen in the long lag period of glycogen formation, in the very marked inhibition of protein synthesis in the non-preincubated cells, and in the failure of glucose to stimulate adenine incorporation into the acid soluble fraction for at least 30 minutes. The latter effect would further impede the regeneration of ATP by minimizing the formation of ribonucleotide from the free base.

CLAIMS TO ORIGINAL RESEARCH

1. When glucose is added to freshly prepared Novikoff ascites hepatoma cells the following metabolic events occur:

a) an initial rapid period of glycolysis and glucose utilization followed after 1 minute by a phase lasting 6 to 10 minutes during which both of these activities are decreased;

b) a large accumulation of fructose diphosphate during the first 6 minutes;

c) a lag period of approximately 10 minutes duration in glycogen synthesis and PRPP formation;

d) a lag period in the incorporation of glycine into protein.

e) a lag period of 20 to 25 minutes duration before the rate of adenine incorporation into the acid soluble fraction in the presence of glucose is maximum;

f) a net flow of ions during the first 10 minutes that results in a decrease in the intracellular potassium concentration and an increase in the intracellular sodium concentration;

g) a rapid depletion of ATP and a decrease in total adenine nucleotides;

h) an accumulation of hypoxanthine as the major product of purine catabolism.

It is concluded that the altered metabolic pattern described above is consequent upon the decrease in ATP concentration.

2. Following a preincubation of 5 to 10 minutes the addition of glucose has little effect on the concentration of ATP. Under these conditions the rate of glycolysis is constant between 1 and 40 minutes after the addition of glucose, the accumulation of fructose diphosphate is greatly reduced, the lag periods in glycogen and PRPP synthesis are eliminated, the lag period in protein synthesis is reduced and the velocity of adenine incorporation into the acid soluble fraction is maximum during the first 10 to 15 minutes after the addition of glucose.

3. The addition of mannose, but not of fructose, galactose, or ribose, to freshly prepared cells elicits a decrease in the high energy nucleotide phosphate level which is similar to that caused by glucose.

4. The regeneration of ATP begins approximately 3 minutes after the addition of glucose and requires 40 to 60 minutes to complete.

5. Maximum rates of protein synthesis, glycogen synthesis, and of glucose utilization are supported by ATP concentrations which are only 40 to 60% of the steady state level. The accumulation of potassium ions is initiated when the ATP level is only 20 to 30% of the steady state value.

6. Glycolysis, PRPP synthesis, and the incorporation of adenine into the acid soluble fraction undergo an active phase between 10 and 25 minutes after the addition of glucose to freshly prepared cells. During this period the rates of these processes are considerably greater than the corresponding velocities exhibited by preincubated cells.

7. The initial velocities of adenine and hypoxanthine incorporation into the acid soluble fraction are increased two to threefold by a 10 minute preincubation in the absence of glucose and four to sevenfold by a 20 to 25 minute preincubation in the presence of glucose. Longer periods of preincubation in the presence of glucose result in a decrease in the initial rates of incorporation and the velocities approach those which occur when the preincubation is performed in the absence of glucose.

8. The rate of ATP regeneration is increased in the presence of exogenous adenine and decreased in the presence of hadacidin, the final steady state level being higher in the former and lower in the latter instance than that attained in the control cells.

9. Glycolysis is unaffected by either adenine or hadacidin, but respiration is increased in the presence of adenine and decreased in the presence of hadacidin.

10. In the presence of hadacidin the rate of glycogen synthesis is reduced. Hadacidin does not affect glycogen synthesis if glucose is added following preincubation.

11. Partial replacement of extracellular sodium ions by potassium ions produces reversible inhibitions of glycolysis, glycogen synthesis, and protein synthesis.

12. At increased levels of extracellular potassium ions there is a reduced rate of ATP regeneration following the initial glucose induced depletion. Associated with the lower rate of ATP regeneration under these conditions are a) a longer lag period in glycogen synthesis, b) a longer period before glucose stimulates adenine incorporation into the acid soluble fraction, c) a very marked inhibition in protein synthesis, and d) a longer period of reduced glycolytic activity.

13. Replacement of sodium by rubidium ions causes inhibitions similar to those produced by potassium. When lithium is the substituting cation, protein synthesis, glycolysis, and glycogen synthesis are reduced but the inhibitions are not as great as those produced by either potassium or rubidium.

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