CARBOHYDRATE METABOLISM OF CELLS TRANSFORMED BY POLYOMA VIRUS.

Marcella Mason Broadfoot, B.Sc.

Summary.

1. Nutritional studies on strain L cells.

(a) The amino acids essential for the growth of strain L cells in serum-free medium were found to be: arginine, cystine, glutamine, histidine, isoleucine, leucine, lysine methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. Glutamic acid, asparti acid and glycine were not essential for optimal growth over a four day period. It is suggested that the requirement for proline and serine (which are not present in some other synthetic media) was due to the low cell inoculum used and the short growth period. Conversion was shown to occur between phenylalanine and tyrosine *in vitro* but not between cystine and methionine.

(b) Interaction was shown to exist between isoleucine, leucine, and valine, and between arginine and lysine. Thus not only the concentrations of these amino acids in media are important but also the ratios of their concentrations.

(c) From these studies HBRT 1 medium was evolved, containing the optimal concentrations of these essential

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amino acids in a medium based on Waymouth's MB 752/1 medium (Waymouth, 1959).

2. Environmental factors and respiration.

The respiration of five cell strains (normal human foreskin fibroblast, HeLa, HLM, Y5 and L5178Y strains) was studied with particular reference to influences of environmental factors on cellular respiration.

(a) The respiration of human fibroblast and L5178Y cells declined rapidly after inoculation into new medium. This fall in respiration could be prevented by adding Krebs citric acid cycle intermediates to the medium. Strain L cells have been shown to exhibit similar behavio (Danes and Paul, 1961). The respiration of HeLa, HLM, ar Y5 cells was not affected by change in medium.

(b) In all cells respiration was less in cultures maintained at pH 7.8 than in cultures maintained at 7.4. Respiration was also lower at pH 6.8, with the exception of HLM cells.

(c) All cells exhibited a Crabtree effect (inhibition of respiration by glycolysis).

(d) Respiration was reduced when cultures were maintained in oxygen tensions above or below that obtained in equilibrium with air.

(e) The respiratory rate of all cells was higher in

the presence of 1 per cent. CO_2 than at lower concentrations. Higher concentrations were slightly inhibitory.

3. Carbohydrate metabolism of BHK 21 strain.

Comparisons of carbohydrate metabolism were made between clones of BHK21 strains which had been transform by polyoma virus *in vitro* and clones which had not been transformed. Since 'transformed' cells produced tumour *in vivo*, the use of this system enabled comparisons to b made between cells, capable and incapable of producing tumours *in vivo*, both derived from the same cell. Bot cell types were growing at similar rates in a controlled environment. Six lines of cells were used, of which th were 'normal' and three 'transformed'.

(a) Respiration. The factors previously shown to affect the respiration of cultured cells were found to affect respiration of 'normal' and 'transformed' BHK21 cells in a similar manner.

When respiration was measured under standard conditions, there was no significant difference in the respiratory rate of 'normal' and 'transformed' cells.

(b) Glycolysis. Environmental factors have previous been shown to affect glycolysis (Paul, 1959). Glycolyti rates of BHK21 cells were therefore measured under four sets of standard conditions (pH's 6.8 or 7.4 in an

atmosphere of air/5 per cent. CO_2 or nitrogen/5 per cent CO_2). Under all these environmental conditions, glycolysis was higher in the 'transformed' cells than in the 'normal' cells.

(c) Enzymes. The activities of several enzymes of carbohydrate metabolism were assayed. No glucose-6-phosphatase activity was demonstrated. The activities of lactic dehydrogenase, malic dehydrogenase, and 6-phosphogluconate dehydrogenase were similar in the 'norma and 'transformed' cells. Hexokinase activity was higher in the 'transformed' cell than in the 'normal' cell, a finding which correlates with the increased glycolysis in the 'transformed' cell. Isocitric dehydrogenase was also higher in the 'transformed' cell though this result is not paralleled by an increased respiratory rate.

Glucose-6-phosphate dehydrogenase activity was lower in the 'transformed' cell than in the 'normal' cell. It is suggested that, since both cell types have similar growth rates, this low activity in the 'transformed' cell must be sufficient to provide pentoses for nucleic acid production during growth. Possible reasons for the differences are discussed.

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By

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MARCELLA M. BROADFOOT.

C O N T E N T S

S U M M A R Y.

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INTRODUCTION.

A knowledge of precisely defined differences in metabolism between normal and cancer cells, either qualitative or quantitative, might be of therapeutic importance in the control of cancers and would constitute an advance in our understanding of the carcinogenic process. Over the years many differences in metabolism have been reported but very often these have not been substantiated by subsequent studies.

Differences in carbohydrate metabolism have been most frequently reported. Here too there has been great disagreement; Warburg (1956) on one hand has proposed that impaired respiration was the cause of cancer, while others (Weinhouse, 1956) have stated that the differences lie in glycolytic metabolism.

One reason for the lack of agreement about differences in tumour metabolism lies in the nature of the systems used. The difficulty which has faced all investigators in the field of cancer research has been the search for a suitable system for the comparison of normal and malignant tissues. Early work was carried out on spontaneous tumours and on normal tissues, compared as two groups. Such comparisons have two main limitations.

1. Non-reproducibility of the System.

Normal tissues vary greatly in their metabolism. For example some, such as retina, exhibit high aerobic glycolysis, while others, such as liver, show almost none. Thus comparisons between groups of tissues can only show approximately any marked differences in metabolism. In an ideal system tumours would be compared with their cells of origin.

2. Non-reproducibility of Carcinogenic Conditions.

Spontaneous tumours in laboratory animals are relatively rare. Apart from this disadvantage, to understand the nature of cancer induction it is essential to know the contributing factors. It was therefore important to be able to induce tumours in experimental animals under controlled conditions.

The progress which has been made towards a solution of these difficulties will be considered.

1. Non-reproducibility of the System.

There is a wide variation between individual animals in their susceptibility to tumour induction by known carcinogens. When the use of mice in cancer research was introduced on an extensive scale, it was observed that tumours occurred in certain cages of the colonies more frequently than in others and it was shown (Murray, 1911) that female mice, in whose ancestors cancer of the breast was frequent, were more liable to develop the disease than mice in whose ancestors the disease was rarer. These observations, which led to investigation of the milk factor in carcinogenesis, had widespread effects on the study of cancer generally.

To investigate the milk factor, inbred strains of mice were developed. Such strains allow the investigator to work with animals of reasonably well-known reproducible and constant characteristics. Many strains of such genetically stable mice are now available and are used in all fields of cancer research. It is possible with these strains to predict the susceptibility of generations of mice to spontaneous tumours and to carcinogenesis by a specific compound.

The short life span of small animals such as rats and mice and the fatal effects of tumours would limit their study were it not for an early observation by Hanau and later Jenson (Greenstein 1954). They succeeded

in transplanting a tumour from one rat to another and showed that the tumours produced arose from the transplant and not from the host cells. This meant that experiments were no longer limited by the life-span of one animal, since the tumour being studied could be carried for generations.

The use of transplantable tumours in inbred strains does not solve all the problems inherent in cancer research. Such transplanted tumours may change after several generations. Ehrlich and Apolant (1906) found that an adenocarcinoma, transplanted serially, was sarcoma-like by the fourteenth generation. For biochemical studies three major difficulties are still unresolved.

Tumour tissue as material for study.

Tumours are large populations of a mainly homogeneous cell type but they may not be completely so. Thus cellular and scirrhous breast tumours may have 10 per cent. epithelial tissue in one case, and 90 per cent. in the other.

Owing to the solid formation of most tumours, the state of the cells in various portions of the tumour may be very different. It is common to find necrotic cells in the centre or core of the tumour, due to inadequate blood supply, and a higher proportion of viable cells towards the surface of the tumour (Caspersson, 1950). Tissue slices of such solid tumours are often used in biochemical

studies and great care must then be taken to utilise only viable tissue.

However, the problem is not completely solved by discarding the necrosed portion of the tumour since it has been shown that, on making serial sections of a rat hepatoma cancer nodule after injection of a labelled amino acid into the animal, the specific activity as measured by autoradiography decreases progressively from the periphery to the centre of the tumour, emphasizing the importance of circulation (Zamecnik, et al., 1951).

The use of ascites tumours obviates this difficulty. This term is given to tumour cells which grow freely in suspension in fluid in the peritoneal cavity. They can be collected by aspiration and transplanted very easily, and since they are free-growing, their viability and nutritional environment does not vary as in the solid tumour. With such tumours it has been possible to show that one malignant cell can transmit the tumour; Hosokawa (1950) produced a Yoshida sarcoma with a single cell of the tumour.

Growth rate of tissues.

Tumours are, by definition, growing tissues (mainly fast-growing) while most normal tissues are resting, that is not growing. To compare metabolic patterns in two tissues so different in growth rate may lead to erroneous conclusions.

Many studies have been published in which the DNA content of normal and cancer cells have been compared and an increased DNA content has been found in the cancer cells by Barer (1952), Klein (1951), MacIndoe and Davidson (1952), Menten et al.(1953), Mirsky and Ris (1949), and White et al.(1953). Such results infer that a characteristic of cancer cells is their high degree of polyploidy or aneuploidy. However results have also been published in which the DNA content of cancer cells was very similar to that of normal cells (Davidson et al. 1951; Mark and Ris, 1949; Metais and Mandel, 1950; Price et al. 1950; Thomson and Frazer, 1954).

The DNA content of a resting cell is a constant characteristic (Vendrely and Vendrely, 1956). However at any time the quantity of DNA per cell in a growing population will be higher than this constant, due to DNA production for replication. It is therefore possible that the increase in DNA content found in tumour cells by so many workers is merely a reflection of the greater growth rate of tumour tissue.

In attempts to eliminate the factor of growth rate, a rapidly growing tissue, such as regenerating liver, has been used as the normal control tissue for malignant hepatomas.

Inhomogeneity of normal tissue.

Normal tissues are extremely inhomogeneous in cell type. In liver for example there are at least five different cell types, each of which performs a specified function, and whose metabolic pattern may therefore differ from the other cell types. Environmental factors affecting these cells, and tumour cells, are complex, availability of substrates and hormone action being two such factors.

Differences in metabolism between various cell types, normal and malignant, could be determined if homogeneous populations of cells could be grown in standardised environmental conditions. Such a system can be attained using cell culture techniques.

The techniques of cell culture have been used in cancer research since the early days of its inception. Beebe and Ewing (1906) tried to grow an 'infective canine lymphosarcoma' in the blood of infected and uninfected dogs, while early experiments of Carrel (1925) attempted to show differences in behaviour of normal and cancer cells grown in culture.

Cell culture.

The ideal system in the study of the metabolism of cells is a pure, homogeneous strain of cells growing in a completely controlled environment.

Pure cell strains.

When a section of tissue is explanted, the cells are disaggregated by mechanical or protcolytic means and inoculated into a suitable medium and container. The population of cells which results is not homogeneous though there is a selection in that the cell types most suited for survival under the conditions employed will outgrow more sensitive cells. After a number of generations *in vitro* the cells may become established and will then grow in culture indefinitely. Such cultured cells are designated permament strains. Many strains of cells are now available and their use in research is widespread. A pure cell strain can be obtained by isolating a single cell from which a population of cells is grown - a process referred to as 'cloning'. The homogeneous population of cells which results is a clone of the original strain.

Controlled environment.

The most important factor in the growth of cells *in vitro* is the medium in which they are grown. In the early days of tissue culture, complex natural media were used, such as plasma clots. However it was obvious even then that the benefits of synthetic media were great, since the composition of natural media is unknown and very variable, making it almost impossible to achieve reproducible experimental conditions. As early as 1911 Lewis and Lewis (1911,

1912) began investigations into the essential constituents of the very complex media then used.

A simple salt solution can, with the addition of glucose, maintain survival of cells for a short time. Many of these balanced salt solutions (BSS) have been formulated, derived from the salt solution originally described by Ringer (Gey, 1936, 1945), Sims (1941), Earle (1943), Hanks, (1946). BSS is a solution of several inorganic salts and serves to control the pH of the media and the osmotic pressure as well as providing essential inorganic ions (necessary for many enzyme activities). For prolonged survival however more complex media, containing vitamins and amino acids, are necessary.

The first systematic studies in the search for synthetic media led to the publication by Fischer et al. (1948) of medium V605, and a similar type of medium was independently formed by White (1946). Following on this work, Morgan, Morton and Parker produced Medium 199 in 1950.

These media are still rather complex, containing as well as vitamins and amino acids, many other compounds such as citric acid cycle intermediates and methylnaphthahydroquinone in Fischer's V605 and nucleosides, nucleotides and pentose sugars in Medium 199. The addition of serum to these media is still necessary for prolonged growth. It would be much more satisfactory to dispense with serum

from media since different batches of serum can vary greatly in their growth potential, some being quite toxic.

Parker and Healey (1955) and McQuilken et al. (1957) formulated medium 858 and NCTC 109 respectively, which can sustain the growth of cells without the addition of serum. These media are even more complex than those mentioned above, and contain coenzymes, lipids, and nucleic acid derivatives.

Since then many attempts have been made to simplify the media used and determine the essential components. In 1955 Eagle published a very simple medium in which all the components are necessary for growth. This medium contains concentrations of the components which are necessary for minimal growth rather than optimal growth and must be supplemented by serum for sustained growth.

A medium which will support the growth of Strain L cells without serum was published by Waymouth (1959) - medium MB 752/1. The medium is more complex than Eagle's medium although it is relatively simple compared to the previous media.

Investigations were carried out and are reported in this work on the essential amino acids and their optimal concentrations in an attempt to formulate a simple medium which would sustain the growth of cells in culture without the addition of serum.

There are disadvantages in the use of such *in vitro* systems. Many people consider that when cells are grown *in vitro* for any length of time they are no longer normal.

Changes on explantation.

During the first few days in culture, cells undergo considerable biochemical adaptation to their new environment. Such changes, particularly in carbohydrate metabolism, have been shown by Paul and Pearson (1957). However, normal and malignant cells adapt themselves similarly to the altered environment (Paul and Pearson, 1957; Yamada and Ono, 1958).

Long-term culture.

Cells which have been continuously cultured for some time may develop variations in chromosome number (Hauschka and Leven, 1958), but it is not possible to correlate such changes with cell morphology, metabolism or malignancy as measured by transplantation.

There are many reports of cells from normal tissues which, after generations *in vitro*, became malignant. Thus Firor and Gey (1945), Gey *et al.* (1949, 1952), and Gey (1956) found that strains of rat fibroblasts produced fibrosarcomas on transplantation, while Earle *et al.* (1943, 1950) showed carcinogenic changes in L cells in the presence and absence of methylcholanthrene. However, Sanford *et al.* (1956) reported actual losses of malignancy in tumour cells

which had been cultured for some time, as did Hsu and Klatt (1959).

It is therefore advisable to check periodically on the malignancy of cells in culture by transplantation into suitable hosts.

Correlation of results of *in vitro* systems.

The behaviour of cells in culture may not readily compare with behaviour *in vivo*. Thus Hirschberg *et al.* (1959) found that studies on differential drug toxicity on malignant cells in culture were poorly correlated with the effects of the same drugs *in vivo*. Such differences in effect may be due to metabolism of the drug in the animal before the compound reaches the tumour.

Studies on the effects of chemicals on cells in culture are not complicated by the possibility that the substance does not reach the cell, although it may not be able to penetrate the cell membrane. Studies on metabolism are not confused by possible poor supplies of substrate to the tissue in question or of alterations in pH or oxygen tension which may inhibit or enhance the specific metabolism.

Origin of malignant cells.

There are available many strains of normal cells, and malignant cells cultured from tumours obtained *in vivo*. Although homogeneous populations of these normal and

malignant cells are thus available, the use of these cell systems raises the same major problem as the use of normal and malignant tissues grown *in vivo*. Thus the cell of origin of the malignant cells, and in many cases of the normal cells, is unknown. While the effects of environmental conditions and of the addition of compounds on the metabolism of malignant cells in culture can be rewarding, in order to show aspects of metabolism in which the malignant cell may differ from the normal, adequate normal controls are essential.

The ideal tissue culture system in cancer research would therefore be one in which a pure strain of normal cells, which do not produce tumours on inoculation into suitable hosts, is transformed by a known carcinogenic agent into cells capable of producing tumours *in vivo*.

2. Non-reproducibility of Carcinogenic Conditions.

(a) Chemical Carcinogenesis.

The first observations on tumours which might be induced by chemicals were made in the eighteenth and nineteenth centuries by clinicians; by Potts who in 1775 described cancers in chimney sweeps and related their high occurrence to an effect of soot, and later by Bell in 1876, who suggested that shale oil was responsible for cutaneous cancers in workers of this industry. Since then there have been numerous accounts of chemical sources of human cancer.

These observations, however, could not be tested until Yamagiva and Ichikava (1918) managed to produce a chemically induced tumour in experimental animals. They painted rabbits' ears with coal tar and, after a long latent period, obtained papillomas and carcinomas. Following this advance in the study of carcinogenesis, earlier observations could be confirmed. Thus Passey in 1922 induced skin cancers in mice painted with extracts of soot, while Leitch (1922) confirmed Bell's observations in mice.

Now that an experimental system was available for testing compounds of carcinogenic activity, the search began for active chemicals in coal tar, and in 1933 Cook, Hewett and Hieger isolated and identified 3,4-benzpyrene from coal tar, while in 1934 Cook and Haslewood synthesised

and described the properties of methylcholanthrene, an extremely potent carcinogen.

These experiments concerned tumours produced at the site of application of the carcinogen. However, Yoshida (1934, 1935) induced a tumour of hepatic tissue by the subcutaneous administration of aminoazotoluene.

At the present time many compounds with carcinogenic activity are known and most tumours studied have been induced by chemicals. While many of such compounds induce a wide variety of tumours in the one animal, there are several which affect one organ only. These latter compounds, the azodye group which produce hepatic tumours almost exclusively, and urethane which attacks only the lungs, are most frequently used in experimental carcinogenesis.

Chemical carcinogenesis in vitro. Many studies on the effect of chemical carcinogens on tissue cultured material have been carried out. Lasnitzki (1954) treated prostate organ cultures of mice with 20-methylcholanthrene and found that mitosis was greatly increased and there was much hypertrophy. Sauerteig (1955) attempted to transplant cultures so treated into suitable hosts, but without success. Lasnitzki has also applied 3,4-benzpyrene to lung organ cultures and has described precancerous changes though she was unable to produce malignant cultures.

The effect of carcinogens on cells in culture has also received attention. Creech (1939, 1940) compared the action of carcinogenic and non-carcinogenic related chemicals on mouse fibroblasts and found that the carcinogens increased growth of the cultures 50 per cent. over the non-carcinogens. On the other hand, Earle and Voegtlin (1938) found that the effect of methylcholanthrene on mouse fibroblasts was an initial slowing down of growth proportional to the concentration of carcinogen and time of exposure.

More recently Sachs (1963) has induced transformation of hamster cells with 3,4-benzpyrene and 3-methylcholanthrene, *in vitro*. A transformation similar to that produced by polyoma virus (p. 18) was found, as judged by changes in organisation resulting in a random arrangement of the cells. Tumour production *in vivo* had not been determined.

(b) Viral Carcinogenesis.

In 1911 Rous found that certain forms of sarcoma in fowls could be transmitted by cell-free extracts of the tumour. This led to the discovery of carcinogenic viruses. Such viruses have a remarkable specificity and invariably induce the same tumour whereas, as pointed out previously, most carcinogenic chemicals can induce several types of cancer in different sites.

In 1924 Carrel made the first attempt to transform normal cells in culture with a tumour-producing virus, using blood monocytes and a cell-free filtrate of Rous sarcoma. After some weeks in culture he inoculated the cells into chickens and found that tumours were produced from the cells but that the supernatant culture fluids also had the ability to produce tumours. Coman (1946) used rabbit epidermis and Shope papilloma virus. He did not produce a malignant conversion but showed that benign tumours were produced only by the virus-treated culture and not by the control culture or by the virus alone. The systematic study of tumour viruses was advanced in 1958, when Temin and Rubin produced an accurate tissue culture assay for Rous sarcoma virus, and showed that one virus particle was sufficient to change one cell. They found, however, that at any given time only 10 per cent. of the cells were affected and that this percentage could be altered by changing conditions of culture.

Much work in the last few years has been concerned with polyoma virus (Stewart and Eddy, 1958) and its effects on cells in vitro. Sachs and Winocour (1959) found a genetically homogeneous line of polyoma virus which could induce tumours in mice, hamsters, rats and rabbits. In most of these species the tumours so produced did not release virus but with mice they obtained release

of virus in varying amounts. In the same year Sachs et al. showed that tumour induction in vivo and cytopathogenic effect in vitro were produced by the same virus. (Sachs et al., 1959).

Vogt and Dulbecco found no evidence of cell degeneration in mouse and hamster embryo cultures infected with polyoma virus, but the virus-treated cells gave rise to tumours when injected into the animal while control cells and virus alone did not. They showed that virus release was caused by reinfection of a small proportion of the cells, and concluded that the differences found in the amount of virus released was due to the fact that some transformed cells (e.g. hamster) were more resistant to reinfection than others (e.g. mouse) (Dulbecco and Vogt, 1960).

Sanford et al. (1961) have shown transformation of cloned cells from C_3H mice to tumour-producing cells by polyoma virus.

Stoker and his colleagues have been studying the effect of polyoma virus on fibroblastic cell cultures derived from Syrian baby hamster kidney Strain BHK 21. After infection of a clone of this strain by polyoma virus, colonies of 'transformed' cells could be detected and isolated from colonies of uninfected ('normal') cells by morphological differences in the colonies. The

'transformed' cells could also be characterised by the production of tumours on inoculation into hamster cheek pouch.

The rate of transformation is low but is similar in cloned and uncloned cultures, suggesting that the transformation is not due to populations of mixed, genetically stable, susceptible and unsusceptible cells (Macpherson and Stoker, 1962). The appearance and size of the colonies after some days in culture suggest that there is little delay between infection and transformation (Stoker and Macpherson, 1961).

Although virus cannot be seen in or recovered from the cells, these 'transformed' cells produce tumours when inoculated back into host, even after many generations in culture.

The use of the BHK 21 cell system of 'normal' and 'transformed' cells enables comparisons to be made between two cell populations, both growing rapidly, maintained under standard conditions, both derived from the same parent cell and differing only in their capacity to produce tumours in vivo.

MECHANISM OF ACTION OF CARCINOGENS.

1. Chemical Carcinogenesis.

There are currently three main theories as to the mechanism of action of chemical carcinogens.

(a) The somatic mutation theory.

One of a series of statements on the nature of cancer made by a committee of distinguished scientists as far back as 1938 was "The new property of the cell appears to develop suddenly, becomes a fixed character, and is transmitted to its descendants. It gives evidence of being a somatic mutation." (quoted by Potter, 1964).

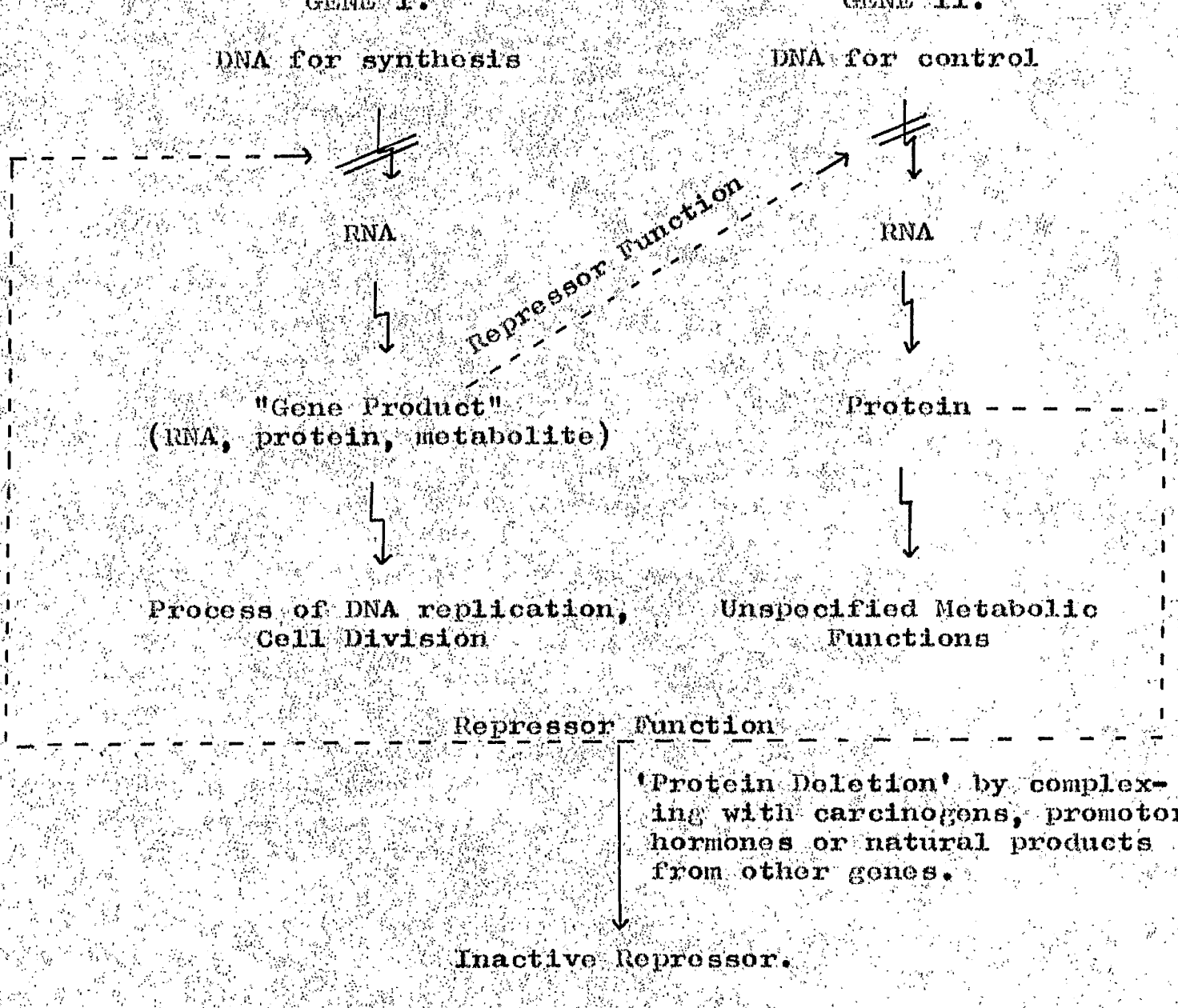
Such a theory requires that the carcinogen reacts with the genetic material of the cell causing an alteration to a form characteristic of the malignant cell. Since the carcinogenic process can be of a progressive nature it has been necessary to consider multiple mutations. Many of the studies in support of the somatic mutation theory emphasised the abnormalities in chromosomal numbers found in tumours (Kit and Griffin, 1958). With the discovery of tumours without large abnormalities in chromosome number (Hauschka, 1961) it has been necessary to assume that, if carcinogenesis is due to mutation, they may affect individual loci and that the aberrations in chromosome number were secondary to the carcinogenic process.

(b) Theories concerned with genetic regulatory mechanisms.

From a study of the kinetics of induction of β-galactosidase in a variety of mutants of E.coli, Jacob and Monod proposed a system of regulation of protein synthesis. They suggested that the rate of formation of a protein was directly related to the rate of formation of messenger RNA and that a 'structural' gene was responsible for the mRNA synthesis. Two enzymes which were simultaneously induced by the inducer for either of them (such as β-galactosidase and galactoside transacetylase) would have a master gene (operator gene) controlling the two structural genes. The complex consisting of the structural genes and the operator gene is called an 'operon'. They suggested that there is another controller, the regulator gene which produces a substance called the repressor, which acts directly on the operator gene to prevent it functioning. It is supposed that the inducing substrate reacts with the regulator to prevent it from repressing the operator gene and thus the consequence of added inducer is increased production of the enzyme. (Fig. 1). (Jacob and Monod, 1961).

Regulation of protein synthesis by this type of mechanism has been thoroughly investigated in bacteria and much evidence supports the theory (Cohen and Monro, 1957; Jacob and Monod, 1961; Monod, 1958; Monod and

FIG. 1. RELEASE OF CELL DIVISION BY FEEDBACK DELETION WITH NO CHANGE IN GENOME IN A RECIPROCATING SYSTEM. (Monod and Jacob, 1961).



Cohen-Bazire, 1953). Inducible and repressible enzymes have now been shown in many animal tissues and cells, particularly in cells in culture.

Thus protein synthesis may be regulated by extra-chromosomal molecules acting on the regulator gene. In the original theory such repressors were not thought to be protein in nature, but probably RNA. In later papers however (Monod et al. 1963; Garen and Garen, 1963) proteins are thought to be more likely as the repressors.

It was suggested that the initial event of carcinogenesis might be brought by the transient action of an agent capable of complexing or inactivating temporarily a genetic locus, or a repressor, involved in the control of multiplication (Monod and Jacob, 1961).

(c) Immunological theory.

This theory postulated by Green was based on work on the effects of non-carcinogenic tumour-inhibiting compounds in tars (such as 3:4-benztetraphene). (Green, 1958)

The basic concept is that all neoplastic cells lack one or more substances (probably protein complexes or their related enzyme systems) which confer tissue and individual identity on the cell. The cell thus becomes more antigenically 'neutral' and does not attract in the same degree the normal immunologically regulated disposal mechanism. This lack of 'identity proteins' constitutes

in itself the neoplastic state.

The discovery of carcinogenic viruses (Rous, 1911) led to the viral theories of carcinogenesis. Rous (1959) denied the somatic mutation theory in favour of the virus theory and chemical carcinogenesis was thought by several investigators to be due to release or activation of a carcinogenic virus within the cell by the chemical carcinogen.

A 'reconciling assumption' has been proposed - that the two theories are not mutually exclusive and that the virus genetical material may cause alteration in the genetic apparatus of the host cell.

2. Viral Carcinogenesis.

Tumour viruses differ considerably in physical and chemical properties. Thus the Rous sarcoma virus, for example, contains RNA and is damaged by ether, while the polyoma virus contains DNA with a rigid shell of protein. It is presumed that the carcinogenic material of the virus is the nucleic acid rather than the protein structure and for the DNA viruses it has indeed been shown that DNA extracted from a tumour virus (rabbit papilloma virus) can initiate tumours (Ito and Evans, 1961; Ito, 1962); while Abel and Crawford (1963) found that the protein shell of polyoma virus has no transforming activity.

These two classes of carcinogenic virus render the 'reconciling assumption' less acceptable. Although both types of virus introduce information for synthesis of proteins which may lead to a change in cell behaviour, it is difficult at present to understand how the RNA introduced by, for example, Rous virus can be passed on to daughter cells.

(a) Rous sarcoma virus.

Another obstacle to the 'reconciling assumption' is that Rous-induced sarcomas continue to produce virus (Temin and Rubin, 1959). If this were always the case in viral carcinogenesis there would be no need for a somatic mutation theory since virus would always be present

to supply the new information.

Since then, it has been found that in stock cultures Rous sarcoma virus is always accompanied by Rous-associated virus (RAV) (Rubin and Vogt, 1962). This RAV differs from Rous virus only in its ability to produce a Rous transformation in the cells. A Rous transformation is characterised as more rapid growth of the infected cells and a change in the morphology of colonies of the cells. The transformation has now been produced by Rous sarcoma virus alone and in this case the transformed cells do not continue to produce virus. However, if the cells are then exposed to RAV they produce not only RAV but also Rous sarcoma virus. Rubin has concluded that Rous sarcoma virus is genetically defective and cannot multiply without the addition of a 'helper' virus, although it is able to induce carcinogenesis.

(b) Polyoma virus.

Of the DNA viruses causing transformation, polyoma virus and SV 40 virus have been most studied, since their action can be investigated *in vitro*. Fibroblasts infected with polyoma virus exhibit three effects. They either show no change and eliminate the virus, or degenerate while producing new virus, or undergo transformation to cells showing loss of contact inhibition and increased transplantability (Vogt and Dulbecco, 1960; Sachs and

Medina, 1961).

When single cells are exposed to virus and immediately isolated in microculture, transformed cells can be produced (Stoker, 1963) showing that the transformation is not due to a carcinogenic product of one cell acting on another cell. Although one virus particle seems to be sufficient to initiate transformation, a very large amount of virus is required suggesting that either the transforming virus particle is a rare variant in the population or simply that there is a very low chance of successful transformation after infection.

Cultures of polyoma transformed hamster fibroblasts may continue to produce virus, but it is possible by cloning to produce populations of transformed cells from which the virus seems to disappear. It has been shown that virus production by transformed cells is due to repeated infection by small numbers of degenerating cells and not to spontaneous release from multiplying infected cultures (Vogt and Dulbecco, 1962).

In the case of DNA carcinogenic virus therefore it would seem that continued presence of the virus is not essential for carcinogenesis in accordance with the 'reconciling assumption' of the somatic mutation theory and the viral theory. However, the possibility cannot be ruled out that the DNA containing virus is similar to

the RNA containing Rous sarcoma virus which is produced by transformed cells when Rous associated virus is present. Vogt and Dulbecco (1962) have not been able to find any virus particles or viral DNA within the transformed cells nor has it been possible to induce virus production by agents which are known to induce virus release in lysogenic cultures of bacteria. On infection of cells transformed by one variant of polyoma, by a second variant, the second variant virus is produced but never the first variant, that is, the transforming virus cannot be recovered by addition of an associated virus. Therefore either the viral DNA is no longer present in the transformed cell, or it is present in a defective form which with the present techniques cannot be recovered. There is a little evidence that the viral DNA may still be present. Sjögren et al. (1961), and Habel (1961) have shown the presence of a viral specific cell antigen in polyoma transformed cells. Animals immunised with active polyoma virus are insusceptible to further transplantation of virus-free transformed cells. This immunity is due to an antigen of the cells and not to a virus particle antigen.

While it is apparent, therefore, that tumour viruses act directly and not simply by selection of pre-existing tumour cells, nor by release of non-viral carcinogens into

the environment, it is not certain whether the continued presence of viral nucleic acid is essential for carcinogenesis. Since it is possible that a large part of the genetic potential of a cell is repressed, in transformation viral DNA could cause derepression of some parts of the genetic potential, producing a change in the balance of the cell; but such a theory would not require the continued presence of the viral DNA. Before one can theorise on the mechanism of transformation by DNA viruses it is vital to prove if the presence of the viral DNA is obligatory for continued tumour growth.

BIOCHEMICAL THEORIES OF CANCER.

There have been three main theories of cancer, from the biochemical viewpoint.

1. Warburg's Hypothesis.

Historically the first of these was Warburg's theory of a respiratory defect which was published in the 1930s. In a review in 1956, Warburg states 'Cancer cells originate from normal body cells in two phases. The first phase is the irreversible injuring of respiration. Just as there are many remote causes of plague - heat, insects, rats - but only one common cause, the plague bacillus; there are a great many remote causes of cancer - tars, rays, arsenic, pressure, urethane - but there is only one common cause into which all the other causes of cancer merge, the irreversible injuring of respiration.

The irreversible injuring of respiration is followed, as the second phase of cancer formation, by a long struggle for existence by the injured cells to maintain their structure, in which a part of the cells perishes from lack of energy, while another part succeeds in replacing the irretrievably lost respiration energy by fermentation energy. Because of the morphological inferiority of fermentation energy, the highly differentiated body cells are converted by this into undifferentiated cells that

grow wildly - the cancer cells.' (Warburg, 1956).

2. Greenstein's Theory of Convergence.

Greenstein measured enzyme levels in normal and malignant tissues and found that the pattern of individual enzyme levels in malignant tissues as a group were much more uniform than in normal tissues. From these results, and data from other laboratories, (Greenstein, 1947, 1956) he suggested that carcinogenesis involved a convergence of enzyme levels from the very distinct, characteristic levels of normal tissues to a common level. In these studies also malignant tissues and normal tissues were considered as groups and no attempt made to correlate enzyme activities in malignant cells with levels in the normal cells of origin.

Both these theories (Warburg's and Greenstein's) were descriptive of differences found between normal and malignant tissues without reference to primary effects of carcinogens as against secondary effects of selection in a given environment. Since 1960 a new class of transplantable tumours has been available - the 'minimal deviation' tumours. These tumours were discovered during a search for liver tumours which differed as little as possible from normal liver (Morris et al, 1960). They are malignant and are histologically highly differentiated,

slow-growing hepatocellular carcinomas containing pyrimidine and amino acid catabolysing enzymes (Ono et al. 1963; Pitot et al. 1963; Jones et al. 1961; Emmelot et al. 1961) in amounts similar to normal liver.

Such tumours disproved Greenstein's hypothesis of convergence as imperative for carcinogenesis, suggesting that the tumours previously studied were so far removed from the original tissues in time and secondary adaptations to environment as to be completely unsuitable for comparative work with normal tissues.

3. The Deletion Hypothesis.

Miller and Miller (1947) found that the azo-dye carcinogens were bound by proteins in normal liver, but not in hepatomas, and suggested that carcinogenesis results from 'a permanent alteration or loss of proteins' and that the 'deletion of a protein may upset a competitive balance of proteins'.

Potter and his colleagues changed the hypothesis to one of catabolic enzyme deletion in which cancer formation was postulated to be due to a loss of one or more catabolic enzymes (Potter, 1948). With this theory he did not deny the Warburg or Greenstein hypotheses. All three theories were compatible with a progressive loss of enzymes during the process of cancer progression, but he

proposed that the malignant change came early in the series, did not necessarily involve conversion to an anaerobic metabolism, and that many catabolic deletions might be related not to carcinogenesis *per se* but to secondary effects of growth rate or environment.

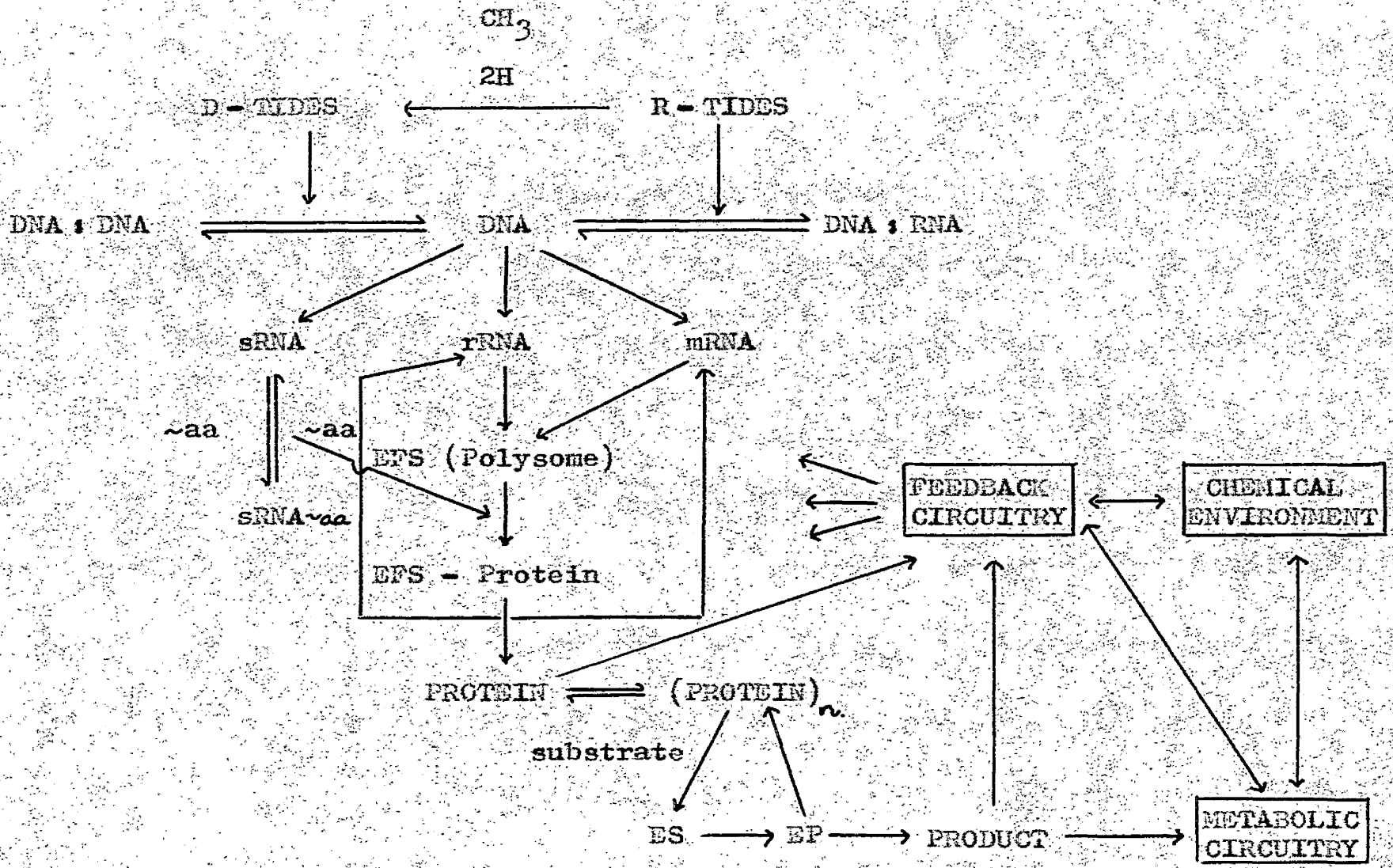
With the introduction of the minimal deviation hepatomas which were found to contain substantial amounts of the catabolic enzymes present in normal liver, levels as high and in some cases higher than normal liver, the theory was restated in similar terms to those of Miller and Miller, with the addition that a similar situation could arise not by loss of an enzyme protein but also by loss of a system which in normal tissues limits growth by a 'self-imposed deficiency of stategic building blocks' (Potter, 1944), thereby introducing the theory of feedback deletion.

Feedback deletion hypothesis. From studies on the minimal deviation hepatomas, it was shown that enzymes which in normal liver could be induced by addition of substrate such as tryptophan pyrrolase, (whose level in normal liver could be increased at least twelve times by the addition of tryptophan), in malignant tumours were not affected by similar additions, the level being similar under all conditions but not necessarily lower than the level in normal liver under maximal conditions. (Pitot

and Morris, 1961). Glucose-6-phosphate dehydrogenase activity of hepatoma 5123 and 7800 were constant during fasting and refeeding in contrast to normal and regenerating liver in which the enzyme activity was increased sevenfold by a 30 per cent. protein diet; but while the enzyme activity in hepatoma 5123 was consistently lower than normal, in hepatoma 7800 activity was consistently very high (Bottomley et al. 1963; Potter and Ono, 1961). All the minimal deviation hepatomas have shown some abnormality in behaviour of substrate-induced enzymes studied, but not all the hepatomas show the same degree of abnormality for each enzyme. The conclusion is drawn that carcinogenesis may include 'a random development of molecular changes, some of which lead to a cell that is able to divide independently of host control mechanisms, but all of which ..... lead to some aberration in mechanisms controlling enzyme levels in the initiated cell'. (Bottomley et al. 1963).

It is envisaged that one or more of the feedback control mechanisms operating in normal cells are destroyed and that this defect in control can be accomplished in one of a number of stages (Fig. 2) of enzyme formation. Such an inhibition of feedback control could be effected by the regulator gene system suggested by Monod and Jacob (1961).

FIG.2. PRESENT CONCEPTS OF GENE EXPRESSION AND MODULATION WITH THE CHEMICAL ENVIRONMENT INDICATED TO SHOW POSSIBLE INTERACTION BETWEEN CELLS. (Potter, 1964).



CARBOHYDRATE METABOLISM OF NORMAL AND MALIGNANT TISSUES.

Comparisons between normal and cancer cells have been made in many aspects of metabolism. In the present work, studies have been made of glycolytic and respiratory rates, and of the activities of enzymes concerned in the metabolism of carbohydrate.

Carbohydrate metabolism.

Warburg measured the amount of glycolysis and respiration in a selection of normal and cancer tissues and, finding that, in the cases studied, glycolysis was much higher and respiration lower than in normal tissues, declared that malignancy was due to impaired respiration. In later experiments he found that cancer tissues could exhibit a respiration level similar to normal tissues, but he maintained his idea of cancer formation, stating that in cancer tissues respiration was impaired, even in these latter examples, since whereas in normal tissues the presence of air causes a changeover of metabolism from glycolysis to respiration (Pasteur effect), in cancer tissues respiration and the presence of air are unable to repress glycolysis (Warburg, 1930).

His theory of cancer was that cancer cells are formed in two stages; in the first step an irreversible lesion of cell respiration occurs, and as a result some of the

cells die, but others become capable of replacing respiration by fermentation as the main energy-producing reaction and are thus changed into cancer cells (Warburg, 1954, 1955, 1956; Warburg et al. 1956). Weinhouse (1956) disagreed. In his view the respiratory rate of tumour tissues was no different from that of normal tissues, but glycolysis was much higher and lactic acid was therefore produced in amounts too large to be removed by oxidation. The argument between the two groups has become heated at times and has continued for years.

Much of the early work on this subject was done on normal tissues as a group, and on malignant tissues as a group, and in some cases respiration was lower in the tumour tissues studied than in the normal tissues. However, the respiratory rate (Q_{O_2}) of a large number of normal tissues ranges from 0.01 to 21 (Burk, 1939; Bywaters, 1936, 1937; Dickens and Weil-Malherbe, 1936, 1941; Fujita, 1928; Kubowitz, 1929; Orr and Strickland, 1938; Rosenfeld and Lasnitzki, 1928; Tamiya, 1927; Warburg and Kubowitz, 1927; Warburg et al. 1924; Warren, 1943), while that of tumour tissues ranges from 0 to 20 (Burk et al, 1948; Crabtree, 1929; DeRoeth, 1957; Dickens, 1936; Dickens and Paley, 1930; Elliott et al. 1935; Kinosita, 1937; Levy et al. 1953; Murphy and Hawkins, 1925; Rosenfeld and Lasnitzki, 1928; Warburg, 1925, 1926, 1956; Warburg et al., 1924; Yushok, 1959.)

The same papers show rates of aerobic and anaerobic glycolysis respectively for normal tissues ranging from 0 to 45 and 0.3 to 88, and for tumour tissues from 0 to 30 and 7 to 83. It is therefore obvious that comparisons should only be made between tumour tissue or cells and their tissues or cells of origin.

MacBeth and Bekesi (1962) found the oxygen consumption and the anaerobic glycolysis of human carcinoma of large bowel, stomach and breast, but not of kidney, invariably exceeded that of comparable normal tissue (aerobic glycolytic rates were not determined).

In the above studies growing tumour tissues were compared with resting normal tissues and the difficulties arising from such a comparison have already been discussed. The use of tissue culture systems supplies both normal and malignant cells growing rapidly.

It has been observed (Paul and Pearson, 1957) that tissues grown in vitro rapidly assumed a glycolytic pattern of metabolism and that this could revert to an aerobic pattern if the cultures were maintained in the same medium for some time. Similar results have been reported by Harris (1958), Munyon and Merchant (1959), Paul (1959). It has since been shown that the glycolytic and respiratory rates of cultured cells vary markedly with the pH of the culture medium (Paul, 1961; Zwartouw and Westwood, 1958);

at a high pH most of the glucose used could be accounted for as lactic acid, while at a lower pH no lactic acid was produced but some was often utilised.

Since the glycolytic rate is different in aerobic and anaerobic conditions (the Pasteur effect (Pasteur, 1879)) and in varying glucose concentrations (Crabtree effect (Crabtree, 1929)) it is obvious that a controlled environment is essential for the study of the glycolytic rates in cells. The respiration of methylcholanthrene-treated subcutaneous mouse fibroblasts (Strain L cells) was also affected by the environment (Danes and Paul, 1961).

It is therefore apparent that comparisons of glycolytic and respiratory rates in normal and cancer cells must be made with cells which are growing at the same rate in a controlled environment.

The metabolism of human embryonic and malignant cells in tissue culture has been studied (Leslie et al. 1957). The average rates of glucose consumption and acid production were higher in embryonic cells than in malignant cells when the basis of reference was total nucleic acid, cell number, protein nitrogen or dry weight.

Woods et al. (1959) have studied glycolysis and respiration of a strain of highly malignant fibroblasts (97 per cent. takes on transplantation) and of a strain of low malignancy (1 per cent. takes), both strains derived

from the same cell. Aerobic and anaerobic glycolysis was three times higher in the highly malignant strain while the respiratory rate was slightly lower (Q O_2 highly malignant 5-10, and of low malignancy 10-15).(Warburg,1956).

As has been stated, the respiratory rate of Strain L cells in culture is affected by environmental factors (Danes and Paul, 1961). The question is then raised, do these factors affect the respiration of all cell strains, of normal and of malignant origin? If indeed they do, then comparisons of respiration can only be made between normal and malignant cells in a controlled environment. The effects of these environmental factors were therefore determindd in a number of cell strains, both of normal and malignant origin.

Comparison of respiration and of glycolysis can most profitably be made in a system in which the malignant cells have been converted by known carcinogens <u>in vitro</u> so that the cell of origin of the malignant cells is known. Such a system is available with baby hamster kidney fibroblasts BHK 21 (MacPherson and Stoker, 1962) in which a population of cells has been grown from one cell. A part of the population has been transformed by polyoma virus into cells which produce tumours on transplantation while another part has been uninfected and can be considered as normal control cells. Comparisons of respiratory and glycolytic rates

were therefore carried out with these cells.

Enzymes.

The enzymes responsible for various steps in the glycolytic and respiratory pathways have been assayed in attempts to pinpoint differences in carbohydrate metabolism between normal and malignant cells.

Suggestions have been made that glycolysis of tumour tissues differed qualitatively from the normal; for example Barr et al. (1928) thought that tumour glycolysis did not involve phosphorylation. LePage (1948) however showed conclusively that phosphorylation of glucose was the first step in tumour glycolysis and that levels of the sugar phosphate were comparable in normal and malignant tissues, while Novikoff et al. (1948) demonstrated the existence of all the glycolytic enzymes in tumour tissues.

A fairly extensive study has been made of the enzymes involved in carbohydrate metabolism in the Novikoff hepatoma in comparison with normal, regenerating, and embryonic liver. The hepatoma contained greatly decreased activity of phosphoglucomutase (Weber and Cantero, 1956) and lactic dehydrogenase; and no detectable glucose-6-phosphatase or fructose-1,6-diphosphatase activity (Weber and Cantero, 1959).

Glucose-6-phosphatase and fructose-1,6-diphosphatase were found to be high in liver and kidney and lower in

other tissues and this could be correlated with the rate of gluconeogenesis of the tissues (Weber and Cantoro, 1955,1959). From the absence of these two enzymes in the Novikoff hepatoma they concluded that during carcinogenesis gluconeogenesis was inhibited. More recently it has been suggested that the Novikoff tumour is not of liver origin but derived from bile duct epithelium - tissue which would not be expected to contain large quantities of these enzymes (Pitot and Potter, 1960). These findings therefore emphasise the importance in cancer research of knowing the exact cell of origin of the tumour.

When enzyme activities were measured in a number of malignant hepatomas found in the search for 'minimal deviation' hepatomas, there was some diversity of enzyme levels. Only one enzyme, phosphoglucomutase, was markedly lower in all 6 of the tumours studied. Glucose-6-phosphate dehydrogenase was higher and lactic dehydrogenase lower in 5 tumours. 6-Phosphogluconate dehydrogenase was higher in 2 cases, lower in the other 5, and glucose-6-phosphatase and fructose-1,6-diphosphatase were absent in the fastest growing tumours and lower than normal in the slow-growing tumours (Weber and Morris, 1963).

In a more comparable system of normal and malignant tissues - that of chorioallantoic membrane and a tumour of the chorioallantoic membrane induced by Rous sarcoma virus - Weber *et al.*(1961) found glucose-6-phosphate dehydrogenase,

6-phospho-gluconate dehydrogenase, fructose-1,6-diphosphatase similar in the normal and cancer tissue, glucose-6-phosphatase not demonstrable in either, and phosphoglucomutase, phosphohexose isomerase and lactic dehydrogenase markedly higher in the sarcoma. The glycolytic activity of the tumour was also shown to be markedly greater than the normal under aerobic conditions and to a lesser extent under anaerobic conditions (Levine et al. 1961).

Beck (1958) estimated the glycolytic enzymes of normal and leukaemic leukocytes. He found no qualitative differences, but the level of hexokinase was low in leukaemic cells. He suggested that this was correlated with the low glycolysis found in leukaemic lymphocytes. Such lymphocytes show a completely reversed picture as compared to most tumour cells in that the normal cell has a high glycolytic rate, higher often than the leukaemic cell.

Scott et al. (1962) studied the changing activities of hexokinase, glucose-6-phosphate dehydrogenase and phospho-gluconate dehydrogenase during carcinogenesis in hamster cheek pouch, induced by dimethylbenzanthracene. They concluded that phosphogluconate dehydrogenase activity most closely correlated with morphological changes from hyperplasia and benign papillomas to malignant papillomas.

Hexokinase, however, is thought to be the rate-limiting

enzyme in glycolysis and Elwood et al. (1963) have found with a variety of hepatomas that the wide diversity of glucose utilisation observed was dependent on hexokinase activity and was correlated with growth rate. The addition of crystalline yeast hexokinase to the Morris 5123 hepatoma which had a low glucose uptake and glycolytic rate increased glycolysis from 5 to 20-fold and oxidation to carbon dioxide from 2 to 5-fold.

Burk and his colleagues also feel that the hexokinase reaction is of great significance in respect to the high glycolytic rate in tumours and have studied the effects of hormones and stress (elevated temperature) on glucose utilisation, by mouse brain and liver and several mouse melanomas and Krebs 2 ascites tumour. They conclude that in the normal tissue glycolysis is controlled by an insulin/anti-insulin mechanism which is absent in anaplastic tumours. They visualise this system acting on the mitochondrial hexokinase (Burk et al., 1960; Woods et al., 1955; Woods et al., 1953; Woods and Hunter, 1959). Wu and Racker (1959) found that in the ascites cells as well as in brain, hexokinase is present mainly in the particles while most other glycolytic enzymes are in the soluble cytoplasmic fraction.

Wenner et al. (1952) have measured the amounts of certain enzymes of the citric acid cycle in various rat

and mouse normal and tumour tissues. They found that citrate synthase, malic, lactic, and isocitric dehydrogenases, fumarate hydratase and oxalacetic decarboxylase are present in comparable amounts in both types of tissue, while aconitate hydrase and α-ketoglutarate oxidase were present in tumour tissue but at a lower level than in the normal tissues studied.

Meister (1950) also found lactic dehydrogenase at the normal level in neoplastic tissues. On the other hand, Hogeboom and Schneider (1950) found isocitric dehydrogenase in mouse hepatoma to be one-third that of normal liver.

With tissue culture cells (HeLa) Barban and Schulze (1956) showed the presence of citrate synthase, aconitate hydrase, and α-ketoglutaric, succinic, malic, and lactic dehydrogenases and fumarate hydratase.

Objects of the present study.

1. Preliminary investigations were designed to formulate a simple synthetic medium containing optimal concentrations of ingredients, capable of sustaining the growth of Strain L cells without the addition of any natural supplements.

2. Environmental factors had been shown to affect the respiratory rate of Strain L cells in culture. Comparisons were therefore made on the effect of these factors on the

respiratory rates of a number of cell strains in culture. The relative uniformity of the patterns found permitted a comparison to be made of normal and cancer cells under controlled conditions.

3. Respiratory and glycolytic rates of baby hamster kidney fibroblasts BHK 21 transformed in culture by polyoma virus to cells capable of producing tumours *in vivo* were compared with the rates in BHK cells which had not been transformed and did not produce tumours *in vivo*.

4. Some of the enzymes involved in carbohydrate metabolism were assayed in transformed and untransformed ('normal') BHK 21 cells in order to pinpoint the gross differences found between the two sets of cells.

MATERIALS AND METHODS.

1. Cell Culture.

(a) Cell strains.

The cell strains used are given in Table 1. All cell strains, with the exception of Strain L5178Y which grew in suspension, were grown as monolayers in flat-sided bottles.

(b) Media.

The media used are shown in Table 1 and given in detail in Appendix A. The basis of all these media was Hank's balanced salt solution, BSS (Hanks, 1949) (Appendix A) but two different buffer systems were used.

1. Bicarbonate/carbonic acid:

This buffer was attained by adding 0.8 M.sodium bicarbonate to the medium to give pH 7.4 (as indicated by phenol red in the medium). The medium was then gassed with 5 per cent. carbon dioxide in air and left in an incubator at 37°C for some hours. More sodium bicarbonate was added to restore the pH to 7.4. This procedure was repeated until equilibration with 5 per cent. CO_2 in air was complete and the pH of the medium remained at 7.4 at 37°C.

2. 0.016 M Tris-(hydroxy-methyl)-amino-methane and 0.005 M. citric acid.

For this system Tris/citrate buffered BSS (Appendix A)

TABLE 1.

CELL STRAINS AND CULTURE CONDITIONS USED IN PRESENT STUDIES.

Cell Strain	Origin	Culture Medium	Percentage Supplement: C.S.	H.S.	B.P.	Tr.
HeLa.	Adult human cervical carcinoma (Gey et al.1952)	Eagles (1955)*	5	5	0.5	-
HLM.	Human foetal liver (Leslie et al.1956)	Waymouth's MB752/1 (1959)	5	-	-	-
L. (adapted to grow without serum).	Mouse subcutaneous fibroblast Clone 929 (Sanford et al.1949)	Waymouth's MB752/1 (1959)	-	-	-	-
Y5.	Chinese hamster skin fibroblasts (Ford & Yerganian 1958)	Eagles (1955)*	5	-	-	-
L5178Y	Mouse lymphoma (Fischer, 1958)	Fischer (1958)	10	-	-	-
BHK 21	Baby hamster kidney fibroblasts.	Eagles (1955)*	10	-	-	0.1
----	Normal human foreskin fibroblasts.	Eagles (1955)*	5	-	0.5	-

C.S. = Calf Serum. H.S. = Human Serum. B.P. = Bactopeptone (amino acid hydrolysate).
Tr. = Tryptose (amino acid hydrolysate).

*Concentration of amino acids in Eagles' Medium (1955) were raised ten times in all cultures.

was used in making up the medium. The gas phase was air.

The calf serum used was obtained from the Institute of Virology, Glasgow, while the Blood Transfusion Centre, Glasgow, supplied the human serum. Bactopeptone and Tryptose were supplied by Difco.

(c) Routine culture.

Cells were inoculated at a density of 10^5/ml. in 10 ml. medium (4 oz. bottles), 20 ml. medium (20 oz. bottles) or 50 ml. medium (Roux flasks). The culture medium was changed after three or four days and after seven days the cells were removed from the glass with trypsin (1/250 Difco) at a concentration of 0.5 per cent. (w/v) in BSS or 0.25 per cent (w/v) in 0.1 M. sodium chloride and 0.01 M. sodium citrate.

Trypsin treatment. The medium was removed from the culture vessel and 5-10 ml. trypsin solution added. After two or three minutes the trypsin was removed and the vessels were left at 37°C until the cells were seen to be detached from the glass. This usually took about five minutes. The cells were then suspended in medium.

In some studies cells were obtained in suspension by scraping them off the glass surface using a glass rod covered with rubber tubing.

(d) Estimation of cell number.

Before inoculation into fresh cultures, or experimental

assays, the number of cells present in the suspension was determined. Electronic machines have been developed which count single cells in suspension rapidly and accurately. In these studies a Ljungberg celloscope and a Coulter cell counter (Model D, Coulter Electronics Limited) were used.

These electronic counters are based on the principle that cells are poor electrical conductors as compared with salt solutions. In operation a dilute suspension of the cells in a solution of 0.12 M. sodium chloride, 0.055 M. citric acid and 0.04 per cent. formalin (filtered through sintered glass) was drawn through a minute hole conducting an electric current between two platinum electrodes. Each cell passing through the hole displaced an equal volume of electrolyte thereby causing a change in the electric current and producing a pulse. These pulses were amplified and counted.

A constant volume of the suspension was drawn through the aperture by a reduced pressure system connected to a column containing mercury. One section of the column was calibrated to contain 0.5 ml. and at each end of the section were electrodes which activated and deactivated the counter as the mercury passed them.

Electronic cell counters can only be used when it is possible to obtain a single cell suspension. Cells that tended to clump together therefore could not be counted in

this way and in these cases counts were made with a Fuchs-Rosenthal haemocytometer.

Method. The supporting ridges of the haemocytometer were moistened. A coverslip was placed on top and pressure was applied until Newton's rings were visible.

The tip of a pasteur pipette containing the cell suspension was placed at an angle of 45° to the edge of the coverslip and the chamber filled with suspension, care being taken not to flood the slide.

The grid of a Fuchs-Rosenthal haemocytometer contains 16 large squares each containing 16 small squares. Cell counts were made of five large squares. The sum of these counts equalled the number of cells per µl.

Such cell counts were simplified in many cases by prior dilution of cells with a staining solution of 0.1 per cent. crystal violet in 0.1 M. citric acid.

Tests for viability on cell suspensions were made by mixing a drop of 0.1 per cent. naphthalene black with a drop of the suspension on a slide. The proportion of unstained cells indicated the viability of the culture.

2. Chemical Assays.

Reagents used were of British Drug Houses Analar grade, except where otherwise stated.

(a) General.

DNA-Phosphorus:

A sample of the cells was washed with BSS and then extracted twice with 2 ml. portions of N perchloric acid at 70°C for 20 minutes with repeated shaking (Paul, 1958). DNA-P was estimated on 2 ml. of the combined extract by the method of Ceriotti (1952).

Reagents: 1. 3.4mM. Indole (0.4 g. of indole was dissolved in 1 ml. of absolute alcohol and this solution was added to 1 litre of deionised water with vigorous shaking).

2. Concentrated hydrochloric acid.

3. Chloroform - May and Baker laboratory reagent grade (Analar grade tends to form phosgene, which interferes with the reaction).

Procedure. 2 ml. of the combined extract were added to a boiling tube, containing 1 ml. of indole solution and 1 ml. of concentrated hydrochloric acid. The mixture was shaken well and the tube placed in a boiling water bath for 10 minutes. After cooling the tubes in cold water, the reaction mixture was extracted three times with 3 ml. portions of chloroform and the extracts discarded. The

tubes were then centrifuged at very low speed (about 500 r.p.m. in the MSE refrigerated centrifuge) for a few minutes to remove any emulsion that might have formed, and the density of the yellow colour was measured on a Unicam SP 600 spectrophotometer at 490 mμ.

Pure DNA was used as a standard. Standards containing 3 μg. DNA-P/2 ml. and blanks of 2 ml. perchloric acid were estimated. The range of the method is 0.5 to 5.0 μg DNA-Phosphorus.

Protein:

Protein was estimated in the enzyme extract prepared by freezing and thawing of cells (page 135). Protein nitrogen was measured by Nessler ammonia determination of a sulphuric acid digest (Paul, 1958).

Reagents. 1. 1g. Selenium dioxide was dissolved in 100 ml. 50 per cent. (v/v) sulphuric acid.

2. Nessler reagent: a solution of 3.5 g. gum acacia in 750 ml. water was added to a solution of 4 g. potassium hydroxide and 4 g. mercuric iodide and the volume adjusted to 1 litre.

3. 2 N Sodium hydroxide.

Procedure. 0.5 ml. selenium dioxide was added to the sample in a digestion tube and the mixture digested on an electric rack for 2 hours. This digestion mixture was then cooled and diluted with distilled water to contain about 10 μg. nitrogen per ml.

To 2 ml. of this solution were added 2 ml. of Nessler reagent and 3 ml. of NaOH. After 15 minutes the yellow colour was measured on a Unicam SP 600 spectrophotometer at 490 mμ.

Standards of ammonium chloride were used, containing 10 μg. nitrogen per ml. and blanks in place of test solutions were carried right through the digestion and estimation.

The values for nitrogen content were converted to protein quantities assuming that 16 per cent. of protein is nitrogen.

(b) Studies on Glycolysis.

Glucose determination:

Protein in the medium was precipitated by the addition of 0.1 ml. of medium to 1 ml. of 0.4 N perchloric acid; glucose in the supernatant was then estimated in the presence of glucose oxidase, peroxidase and o-dianisidine (Keilin and Hartree, 1945; Huggett and Nixon, 1957). Glucose is oxidised to gluconic acid and the hydrogen peroxide thus released oxidises o-dianisidine to a red-coloured product whose optical density is measured at 436 mμ.

Reagents. A pack supplied by C.F. Boehringer, Mannheim was used.

(a) Glucose oxidase - peroxidase mixture - glucose oxidase (250 μg/ml) and peroxidase (40 μg/ml) in 0.12 M. phosphate buffer, pH. 7.0.

(b) o-Dianisidine hydrochloride, 10 mg/ml.

These reagents were stored at -10°C. A reaction mixture containing 1 ml. (b) to 150 ml. (a) was made up. This mixture could be kept for one week at -10°C.

(c) Glucose standard - 91 µg glucose/ml.

Procedure. 0.2 ml. of test solution was added to 5 ml. of reaction mixture. After incubation at 25°C for 20 minutes, the optical density was measured in the Unicam SP 600 spectrophotometer at 436 mµ. Standards (c) and blanks containing perchloric acid were also estimated.

Lactic and Pyruvic acid determinations:

Protein in the medium was removed by treatment with zinc sulphate and sodium hydroxide.

Reagents. 1. 0.35 M. Zinc sulphate (10 per cent w/v).
2. 0.5 N Sodium hydroxide.

Procedure. 2 ml. of medium were added to 5 ml. of zinc sulphate and 5 ml. sodium hydroxide in a centrifuge tube and left for 10 minutes. The precipitated protein was then centrifuged down at 5,000 g. for 20 minutes. Lactic acid and pyruvic acid were estimated in the supernatant.

Estimation of lactic acid (Hullin and Noble,1953):

Reagents. 1. 0.48 M. Copper sulphate (12 per cent w/v).
2. 0.8 M. Copper sulphate (20 per cent.w/v).
3. 0.088 M. p-hydroxydiphenyl reagent -

1.5 g. of recrystallised p-hydroxydiphenyl were dissolved in 10 ml. 5 per cent. NaOH and diluted to 100 ml. with water.

4. Solid calcium hydroxide.

5. Concentrated sulphuric acid (ordinary grade). (Since some batches of analar grade failed to give colour development with standard solutions of lactate).

Procedure. 1 ml. of deproteinised medium was added to a centrifuge tube containing 1 ml. of 20 per cent. copper sulphate and the volume made up to 10 ml. with water. To this was added 1 gm. of solid calcium hydroxide; the mixture was shaken, allowed to stand for 30 minutes and then centrifuged. This copper-lime treatment breaks down α-keto acids and prevents their estimation along with lactic acid. 1 ml. of the supernatant liquid was transferred to a glass-stoppered test tube containing 0.05 ml. 12 per cent. copper sulphate. A number of such tubes were shaken by attachment to a microid flask shaker in an ice-bath and 6 ml. of concentrated sulphuric acid were added in drops to each. The tubes were quickly stoppered and placed in a water bath at 60 ±1°C for 30 minutes. After cooling to room temperature in an ice-water mixture, 0.1 ml. p-hydroxydiphenyl reagent was added and the characteristic purple colour developed at 28 ± 2°C for 20 minutes. Excess reagent was then destroyed by 90 seconds' immersion in a boiling water bath.

A calibration standard containing lithium lactate equivalent to 100 µg. lactic acid/ml. was carried through the estimation. Optical densities were determined on a Unicam SP 600 spectrophotometer at 560 mµ.

Estimation of α keto acids expressed as pyruvic acid. (Leslie and Paul, 1954).

Reagents. 1. Dinitrophenyl hydrazine reagent - 100 mg. 2-4, dinitrophenyl hydrazine were dissolved in 100 ml. 2 N HCl.

2. 0.2 M. Trisodium phosphate.

3. 1.5 N NaOH.

4. Chloroform.

Procedure. 1 ml. of dinitrophenylhydrazine reagent was added to 4 ml. deproteinised medium and the mixture was allowed to stand for 10 minutes at 25°C. The hydrazones thus formed were extracted in three successive volumes of 3 ml. chloroform and the extracts transferred to another tube. The combined chloroform extract was shaken with 5 ml. trisodium phosphate solution to extract the hydrazones from the chloroform. 4 ml. of the trisodium phosphate layer were pipetted into a third tube and the characteristic red colour developed by the addition of 2 ml. of 1.5 N NaOH.

Standards containing 45 µg. pyruvic acid/ml. were carried through the estimation and optical densities were determined with a Unicam SP 600 spectrophotometer at 445 mµ.

The range of this estimation is 5-50 µg. α-keto acid expressed as pyruvic acid.

(c) Enzyme Assays.

Lactic Dehydrogenase (Cabaud and Wroblewski, 1958).

Lactic dehydrogenase activity was assayed by following the reduction of pyruvic acid to lactic acid in the presence of $NADH_2$. Pyruvic acid in the reaction mixture was determined spectrophotometrically as the yellow hydrazone.

Reagents. 1. 0.023 M. Pyruvic acid in 0.044 M di-potassium hydrogen phosphate (buffered substrate). pH 7.8

2. 0.01 M. $NADH_2$.

3. 0.054 M. 2-4-Dinitrophenyl hydrazine (0.1 per cent. w/v).

4. 0.4 N NaOH.

Procedure. 0.01 ml. of $NADH_2$ solution and 0.1 ml. buffered substrate were dispensed into small tubes. 0.01ml of cell extract was added and the tubes were incubated in a 37°C water bath for 30 minutes. 0.1 ml. 2-4-dinitrophenylhydrazine wasthen added and the tubes allowed to stand at room temperature for 20 minutes. 1 ml. of 0.4 N NaOH was added and after 5 to 10 minutes the optical density of the yellow colour was measured with a Unicam SP 600 spectrophotometer at 550 mµ. Controls were prepared by adding 2-4-dinitrophenylhydrazine before the

enzyme extract. A standard containing 120 µg. pyruvic acid was included with each set of assays.

Lactic dehydrogenase activity of the cells was expressed as µ moles of pyruvic acid utilised per minute.

Glucose-6-Phosphatase: (Campbell, 1962).

The activity of glucose-6-phosphatase was estimated by measuring the glucose released from glucose-6-phosphate. The glucose liberated was determined by the glucose oxidase method, using a pack supplied by Boehringer (page 54).

Reagents. 1. 0.12 M Glucose-6-phosphate in 0.1 M. citrate/NaOH buffer, pH. 6.5.

2. 0.37 M. Trichloracetic acid (TCA).

Procedure. 0.05 ml. buffered substrate and 0.05 ml. cell extract were mixed in a centrifuge tube and incubated in a 37°C water bath for 90 minutes. The reaction was terminated with 0.1 ml. TCA which precipitates the proteins. Glucose was estimated in the supernatant. Controls, in which the protein was precipitated before incubation, were carried through the estimation.

In many of the assays of glucose-6-phosphatase, because of the small volumes involved, the enzyme reaction was terminated, not by TCA, but by diluting the reaction mixture with 5 ml. of the buffered enzyme mixture of the Boehringer glucose estimation.

Hexokinase (Eagle et al., 1958).

Hexokinase is involved in the phosphorylation of glucose to glucose-6-phosphate. The activity of the enzyme can therefore be assayed by determining the disappearance of glucose from the reaction mixture.

Reagents. 1. 0.05 M. ATP.

2. 0.1 M Magnesium chloride.

3. 0.5 M Sodium fluoride.

4. 0.1 M Tris (hydroxymethylamino-methane) buffer, pH 7.7.

5. 0.02 M Glucose.

Substrate. This consisted of a mixture of -

0.2 ml. ATP.

0.1 ml. $MgCl_2$.

0.1 ml. NaF.

0.5 ml. Tris buffer.

0.5 ml. Glucose.

Procedure. 20 µl of substrate and 20 µl of cell extract were mixed in small tubes in a 37°C water bath. The reaction was allowed to proceed for exactly 10 minutes. 2.5 ml. Buffer/enzyme mixture of the Boehringer estimation (Glucose, p.54) was added at this time to terminate the reaction. A control was carried out for each assay, 20 µl of cell extract being added after the dilution of the substrate by the buffer/enzyme mixture.

After incubation for 20 minutes at 37°C the optical density of the coloured solution was determined at 436 mμ in an SP 600 spectrophotometer.

The period of incubation was shortened from the time given by Eagle et al. (1958) - one hour - because of non-linearity over long periods.

In a few experiments on hexokinase activity, the method of DiPietro and Weinhouse (1960) was used (page 62).

The remaining enzyme activities were all measured by following the change in concentration of nicotinamide-adenine dinucleotide (NAD) or nicotinamide-adenine dinucleotide phosphate (NADP) between the oxidised and reduced state. This change can be followed spectrophotometrically since the reduced forms of the nucleotides have a distinct absorption band with a maximum at 340 mμ; a band which is absent from the spectrum of the oxidised forms. Thus any enzyme which requires either NADP or NAD, or the reduced forms of these coenzymes, can be assayed by measuring the change in optical density with time at 340 mμ.

This technique was first used by Glock and McLean (1953) and is a very accurate and sensitive method of assay. It can be used to measure the activity of a large number of enzymes by direct estimation, in the case of enzymes requiring these cofactors, and indirectly, by

including an enzyme (requiring NAD or NADP) which acts on the product of the enzyme action being studied. An example of this indirect method is the assay for hexokinase (DiPietro and Weinhouse, 1960). By adding an excess quantity of glucose-6-phosphate dehydrogenase, which requires NADP, the formation of glucose-6-phosphate can be followed by the reduction of NADP, during the subsequent dehydrogenation, the rate of the reaction being dependent on hexokinase activity.

Hexokinase (DiPietro and Weinhouse, 1960).

Reagents. 1. 0.045 M Magnesium chloride.

2. 0.018 M ATP.

3. 0.6 M Glucose.

4. 0.044 M Sodium glycylglycine, pH 7.5.

5. Glucose-6-phosphate dehydrogenase, (70 units/mg).

6. 0.0045 M NADP.

Procedure. All reagents were warmed to 37°C. Two quartz cuvettes (1 cm. light path) were filled with:

0.4 ml. Magnesium chloride.

0.4 ml. NADP.

0.4 ml. ATP.

0.4 ml. Glucose.

0.8 ml. Sodium glycylglycine.

0.001 ml. Glucose-6-phosphate dehydrogenase.

The cuvettes were placed in position in the spectrophotometer which was warmed by a continuous flow of water at 37°C.

The reaction was initiated by the addition of 0.002ml of cell extract to the 'test' cuvette. The change in optical density at 340 mμ was recorded against time for about 10 minutes.

Glucose-6-phosphate Dehydrogenase (Glock and McLean, 1953).

Glucose-6-phosphate dehydrogenase activity was measured by the rate of formation of $NADPH_2$ during the oxidation of glucose-6-phosphate to 6-phosphogluconolactone.

Reagents. 1. 0.02 M Glucose-6-phosphate.

2. 0.001 M. NADP.

3. 0.3 M Magnesium chloride.

4. 0.15 M Tris (hydroxymethylaminomethane) and 0.3 M maleic acid in 0.16 M NaOH, pH. 7.6.

Procedure. All the reagents were warmed to 37°C. To two quartz cuvettes were added:

2.1 ml. Tris/maleic acid/NaOH buffer.

0.1 ml. NADP.

0.1 ml. Magnesium chloride.

0.1 ml. Glucose-6-phosphate.

The reaction was initiated by the addition of cell

extract to one cuvette. The change in optical density was followed at 340 mμ on a Beckman DB recording spectrophotometer at 37°C.

6-Phosphogluconate Dehydrogenase (Glock and McLean 1953).

6-Phosphogluconate dehydrogenase catalyses the oxidative decarboxylation of 6-phosphogluconate to ribulose-5-phosphate by way of a phosphoketogluconic acid. NADP is required in the reaction and the enzyme can therefore be assayed spectrophotometrically.

Reagents. 1. 0.1 M Magnesium chloride.
2. 0.25 M. Glycylglycine, pH 9.0.
3. 0.0026 M NADP (2 mg/ml).
4. 0.05 M 6-Phosphogluconate.

Procedure. To two quartz cuvettes were added:
0.5 ml. Magnesium chloride.
0.5 ml. Glycylglycine.
0.1 ml. NADP.
0.1 ml. 6-Phosphogluconate.
1.1 ml. water.

The reaction was started by the addition of cell extract. The change in optical density was measured against time on the Beckman DB recording spectrophotometer at 340 mμ at 37°C.

Isocitric Dehydrogenase. (Kornberg and Pricer, 1951).

Isocitric dehydrogenase catalyses the oxidation of

isocitric acid to oxalosuccinic acid in the presence of NAD or NADP. NADP specific isocitric dehydrogenases have been isolated from swine heart and from yeast, and NAD specific enzymes have been found in several animal tissues (Ochoa, 1948; Kornberg and Pricer, 1951; Plaut and Sung, 1954). The further decarboxylation of oxalosuccinic acid to α-ketoglutaric acid is catalysed by the same enzyme (Moyle and Dixon, 1956; Siebert et al. 1957).

Reagents. NADP-specific enzyme

1. 0.5 M Potassium phosphate buffer, pH 7.0.
2. 0.1 M Magnesium chloride.
3. 0.025 M NADP.
4. 0.005 M Isocitrate.

Procedure. To two quartz cuvettes were added:

0.2 ml. Potassium phosphate buffer.
1.0 ml. Magnesium chloride.
0.02 ml. NADP.
0.1 ml. Isocitrate.
1.68 ml. distilled water.

The reaction was initiated by the addition of cell extract to one cuvette and the change in optical density was measured against time on the Beckman DB recording spectrophotometer at 340 mμ and 37°C.

The NAD dependent isocitric dehydrogenase was assayed similarly except that 0.02 ml. 0.05 M NAD and 0.02 ml.

0.025 M AMP were added. Negligible amounts of this enzyme were found.

Malic Dehydrogenase (Wroblewski, 1955).

This enzyme was assayed by the reduction of oxalacetic acid to malic acid in the presence of $NADH_2$. Since oxalacetic acid is unstable in solution, it was formed immediately prior to the enzyme assay from aspartic acid and α-ketoglutaric acid by the action of glutamic oxalacetic transaminase. (The reagents used were supplied in a pack by C.F. Boehringer, Mannheim.)

Reagents. 1. 0.042 M Aspartate in 0.1 M phosphate buffer, pH. 7.4.

2. 0.06 M Sodium α-ketoglutarate.

3. 0.012 M $NADH_2$

4. Glutamic oxalacetic transaminase, (1 mg protein/ml).

Procedure. The reagents were added to a quartz cell in the volumes -

2.75 ml. Aspartate.

0.05 ml. Sodium α-ketoglutarate.

0.05 ml. $NADH_2$.

0.05 ml. Glutamic oxalacetic transaminase.

The cell was placed in position in the spectrophotometer at 37°C and left for 5 minutes to allow transamination to occur beteen aspartate and α-ketoglutarate. The

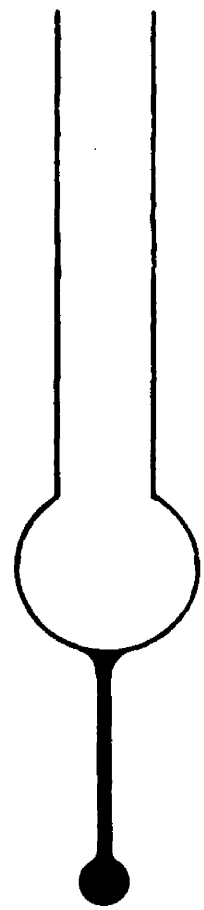
cell extract was then added to initiate the malic dehydrogenase assay and the extinction value at 340 mμ was measured against time.

3. Cartesian Diver Technique.

This technique of measuring oxygen uptake of living cells was first described by Linderstrøm-Lang (1937). Five years later comprehensive accounts, by Linderstrøm-Lang (1942) of the theory of the diver, and by Holter (1942) of the practical details, were published.

The cartesian diver, as used in these experiments, is a small glass vessel consisting of a long neck, open at one end, and blown into a bulb at the other (Fig. 3). Inside this bulb is placed a suspension of cells. As the cells respire they take up oxygen from the air in the diver and produce carbon dioxide. Since in the neck of the diver, as described by Holter, there is a solution of sodium hydroxide which reacts with carbon dioxide producing sodium bicarbonate, there is a change in the gas volume of the diver during respiration. The diver is immersed in liquid in a vessel connected to a manometer; as the gas volume within the diver decreases, liquid is drawn in, thereby increasing the density of the diver. By measuring the increase in negative pressure required to bring the diver up from its resting

FIG. 3.



CARTESIAN DIVER

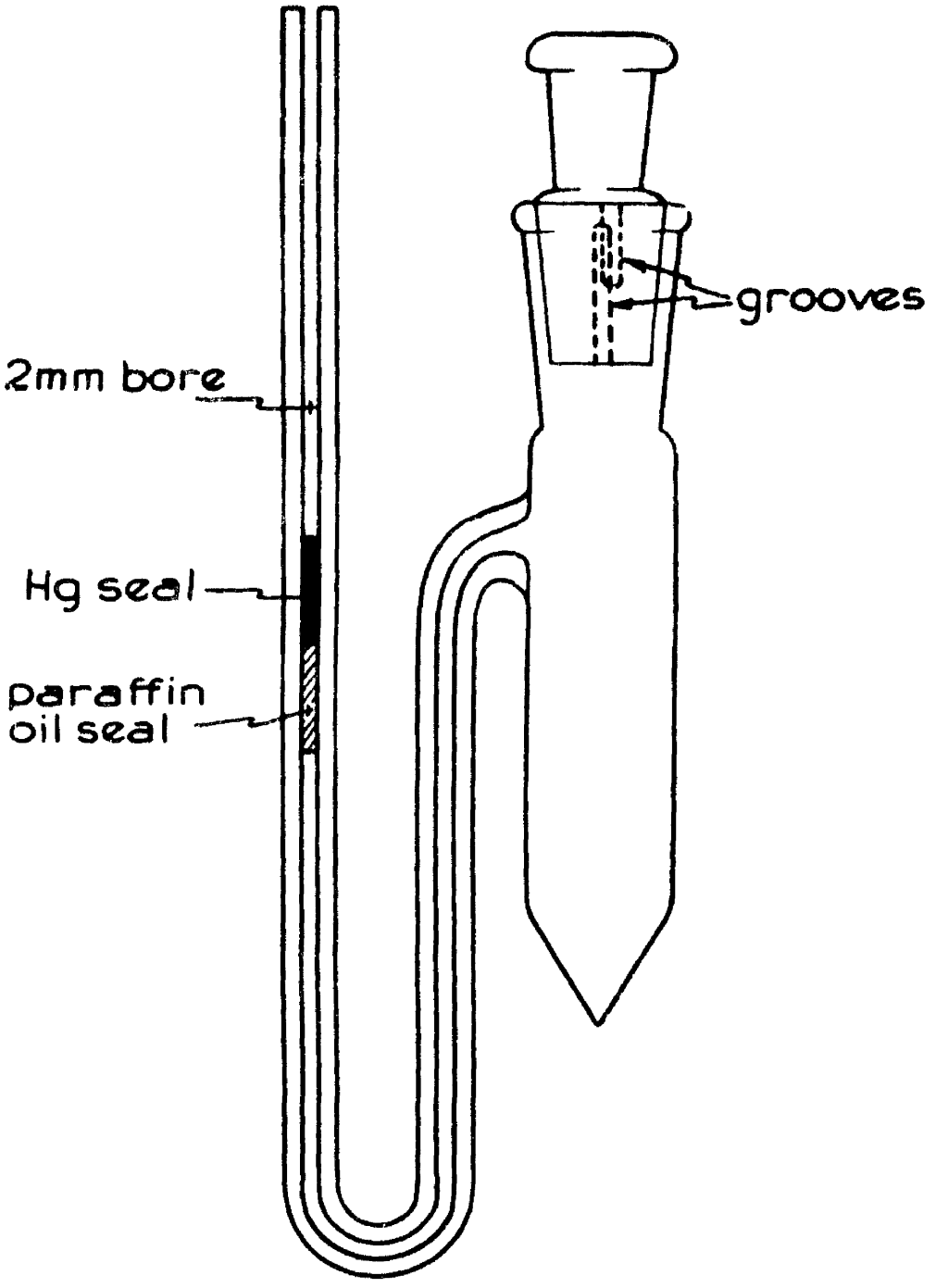
position at the bottom of the vessel to a predetermined level over a period of time, the rate of respiration of the cells can be determined.

The liquid in which the diver floats in the flotation vessel (Fig. 4) must meet certain requirements. The gases contained in the diver should be insoluble in the liquid and water loss from the reaction mixture should be negligible. The liquid must also be of such a viscosity as to allow free movement of the diver.

In practice an aqueous salt solution of suitable density is used as flotation medium and a seal, of a substance which does not permit passage of the gas, is placed in the neck of the diver.

In the technique as described by Holter, a sodium nitrate/sodium chloride solution of density 1.3 gm/ml. was used and the diver contained three seals; an absorption seal which absorbed the gas produced in the reaction, thus changing the buoyancy of the diver; a paraffin seal which prevented loss of water from the diver; and a mouth seal which was a means of adjusting the weight of the diver. The absorption seal absorbed the carbon dioxide produced in the reaction so that respiration was being measured in an atmosphere lacking carbon dioxide. Since carbon dioxide is essential for the growth of mammalian cells (Harris, 1954) it would be desirable to

FIG. 4.



Flotation vessel

(PAUL & DANES 1961)

measure respiration in an atmosphere containing the gas.

To this end Danes and Kieler (1958) altered the absorption seal of the original diver from sodium hydroxide to 0.15 M sodium bicarbonate and obtained partial buffering of carbon dioxide in the gas phase.

The use of 'carbon dioxide buffers' in manometric techniques was introduced by Pardee (1949). He found that diethanolamine, which forms readily dissociable compounds with carbon dioxide, had a capacity for the gas sufficient for the tensions usually found in respiration studies. However, to use this solution to obtain 5 per cent. carbon dioxide in the gas phase required a large reservoir of the buffer solution, which was impossible in the neck of a cartesian diver.

Boell et al. (1939) showed that carbon dioxide readily passed through the oil seal in the cartesian diver. On the basis of these two observations Paul and Danes (1961) developed a technique, in which the carbon dioxide produced in the diver passes through the neck seal and into a 'carbon dioxide buffer'. This allowed the measurement of oxygen uptake in the presence of a constant level of carbon dioxide.

This technique was used in the present work. The apparatus is described below.

Cartesian Diver Apparatus.

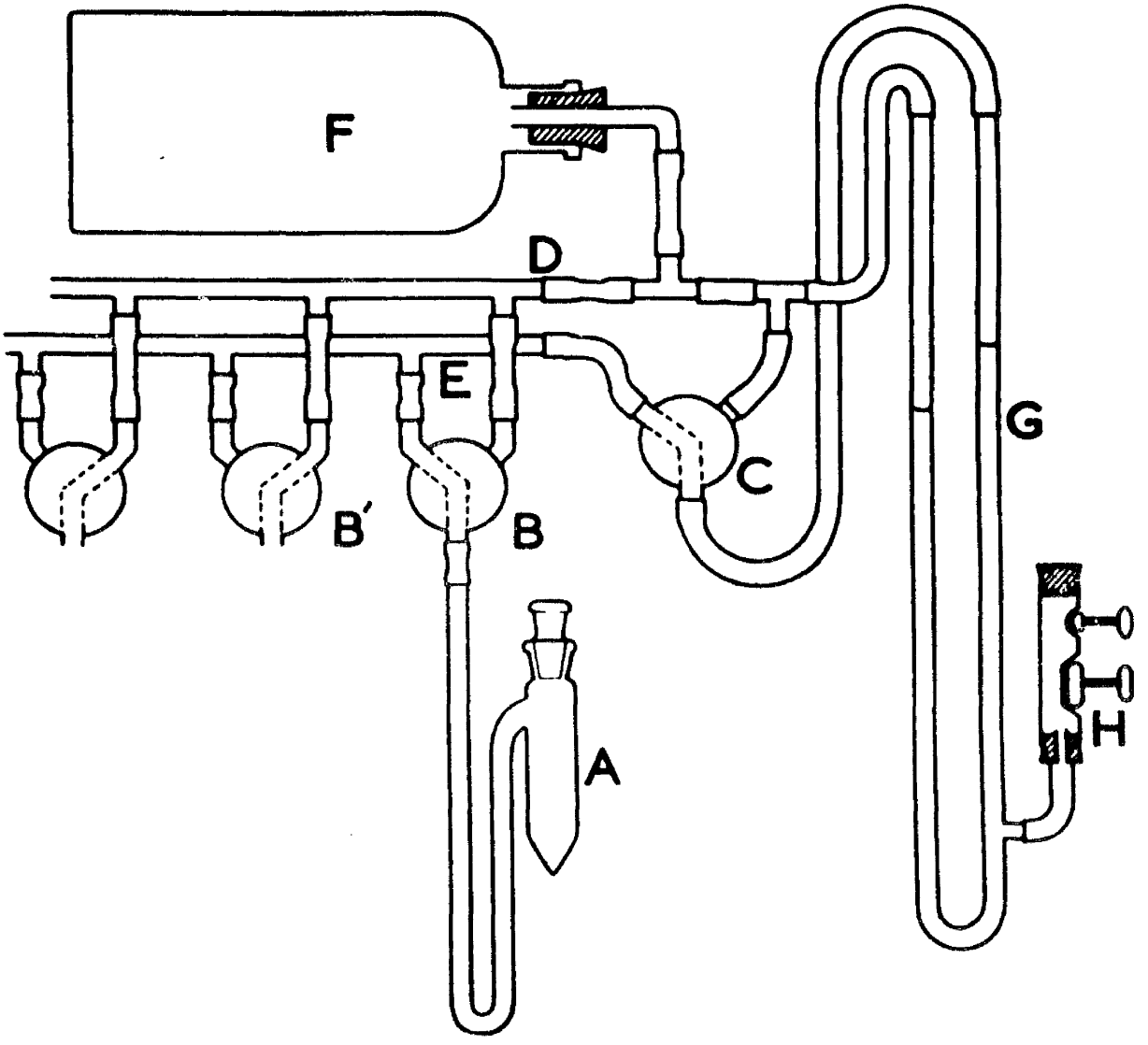
The apparatus consisted of a large glass tank filled with water and equipped with an electrical stirrer. Heat was generated by two electric bulbs, and the temperature was controlled by a mercury/toluene regulator at 37.5°C., ± 0.05°C.

In this tank were suspended the flotation vessels (8 in all) connected, through a double manifold system, to a large air bottle immersed in the water and on the other side to the manometer. Use of this double manifold and the air bottle enabled the flotation vessels to be maintained separately under constant pressure except during the short time when a manometer reading was being taken. It was important that a constant pressure was maintained since the high solubility of carbon dioxide in the flotation medium meant that the equilibrium between the gas and liquid phases was profoundly affected by applied pressure. (Fig. 5).

The Manometer.

The manometer was 150 cm. high and was filled with an appropriate solution. At first Brodie's solution was used (0.8 M sodium chloride with sodium taurocholate added to decrease the surface tension). During the respiration experiments, evaporation of water from this solution was found to give rise to blockages of the connecting tubes

FIG. 5.



Outline drawing of closed constant-pressure system for Cartesian diver manometry. *A*, flotation vessel; *B*, *B'*, stopcocks, flotation vessel to manifolds; *C*, stopcock, manifolds to manometer; *D*, manifold to air bottle; *E*, manifold to manometer; *F*, air bottle (4 l); *G*, manometer; *H*, pressure regulator.

(PAUL & DANES 1961)

in which it condensed. Ethyl lactate was therefore substituted. This was more viscous but worked satisfactorily if a longer time was allowed for each reading.

The Flotation Vessel.

This was a Quick-Fit test-tube, tapered at the bottom and with a U-tube attached to one side (Fig. 4). A small groove was bored halfway up the neck of the tube and halfway down the ground glass stopper, to permit filling and sealing of the completely filled vessel.

The flotation vessel was filled with flotation medium and sealed with the stopper. A little medium was drawn off the open end of the U-tube and a seal of paraffin oil placed on top of the flotation medium. A seal of mercury was then deposited on top of the oil; this prevented loss of carbon dioxide from the flotation vessel.

Flotation Medium.

A solution containing 30 per cent. diethanolamine (v/v) was cleaned by passage through activated charcoal. 50 gms. of potassium iodide were dissolved in 100 mls. of this solution (total volume approximately 150 ml.) and 0.1 ml. of 1 per cent. phenolphthalein was added as indicator. The specific gravity of the medium was measured (1.4 gm/ml.).

The medium was equilibrated with the desired carbon dioxide-air mixture by the method of Krebs (1951). One

third of the total volume was saturated with carbon dioxide at atmospheric pressure and added to the remaining two thirds. The appropriate gas mixture was then bubbled through the medium, using a sintered glass distributor, for two hours at 37.5°C.

The Cartesian Diver.

The diver (Fig. 3) was made of glass obtained from Jena (East Germany), since this glass has a constant density. The thick-walled Jena glass tubing was drawn out into capillary tubing with an internal diameter of about 1 mm., and a wall-thickness of 0.1 mm. approximately. A bulb was blown out at one end and the tube cut to leave a neck length of 10 mm. A solid drop of glass was then attached to the bulb and drawn out to give a thin tail of glass with a solid drop at the bottom end. This tail and bottom drop lowered the centre of gravity of the diver and allowed it to remain upright when placed in flotation medium. The amount of glass in the bottom drop was adjusted so that the diver sank slowly in medium of density 1.4 gm/ml.

When this had been accomplished, the diver was weighed. Divers of weight between 10 and 22 mg. were used, the volume of these being approximately 10 µl.

Pipettes used in charging the diver were made from fine capillary glass tubing as described by Holter (1942).

Those used in adding the cell suspension were calibrated to dispense 2 µl.

Neck Seals. (Fig. 6).

Only one neck seal was required. The material used in this seal had to prevent loss of water from the reaction mixture and allow diffusion of carbon dioxide into the carbon dioxide buffered flotation medium. Dibutylphthalate was found to be suitable for this purpose (Paul and Danes, 1961).

The thickness of the neck seal proved to be a limiting factor in the equilibration of the system and therefore as thin a seal as possible was used, but one which withstood changes in pressure during manometric measurements.

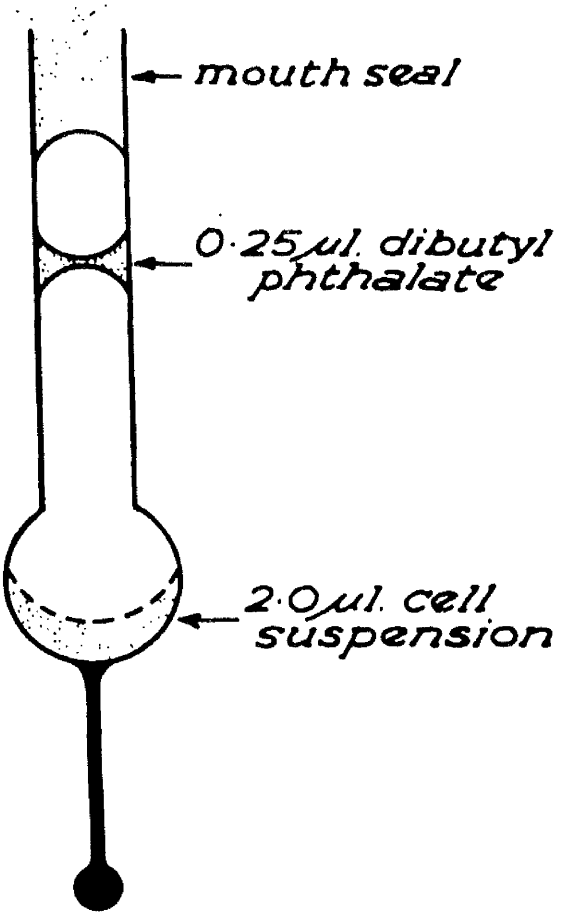
Cell Suspension.

HERT 1 Medium (page 84) equilibrated with 5 per cent. carbon dioxide in air, adjusted to pH 7.4 with sodium bicarbonate, and containing 11 mM glucose, was used as test medium for measuring respiration, except where otherwise stated. Cells were suspended in respiration medium at a concentration of 5×10^3 cells/µl and 2 µl. of this were added to the diver.

Charging of the Diver.

To reduce the time required for equilibration, the divers were filled at 37.5°C. and gassed with the carbon

IG. 6.



Cartesian diver filled with cell suspension (2.0 µl) as a bottom drop, dibutyl phthalate seal (0.25 µl) and a mouth seal (flotation medium).

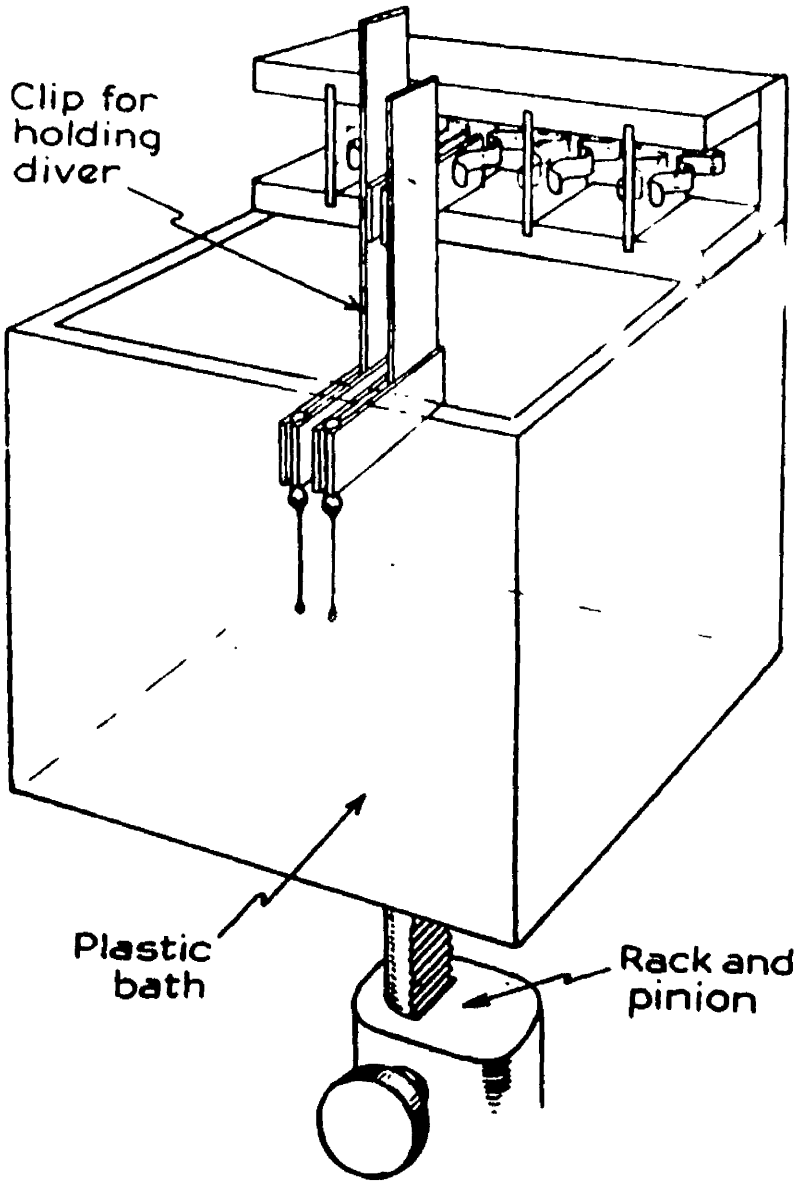
(PAUL & DANES. 1961)

dioxide/air mixture used in the respiration and flotation media. To do this Paul and Danes (1961) added the cell suspension to the divers under water at 37.5°C. in a gassing chamber (Fig. 7) and bubbled the appropriate gas mixture into the diver before and after placing the neck seal. When the pipette used for gassing the diver was being removed, water was occasionally drawn into the diver, and during manipulations became part of and broke the oil seal.

To avoid this difficulty, in later experiments, a lid, with small holes to allow passage of the tip of the pipette into the diver, was fitted to the chamber and water at 37.5°C. was added to the chamber to a level below the mouth of the diver. The gas mixture being used was then bubbled through water and the gassing chamber for some time before and during the charging of the divers, to ensure high humidity.

When the divers had been charged with 2 µl. of cell suspension, the dibutylphthalate seal was placed in the neck of the diver in the position shown in Fig. 6. A seal of water was added (the mouth seal) and the diver was transferred into the flotation vessel. Using a fine pipette the water in the mouth seal was rinsed out and replaced by flotation medium; gas was then removed

IG. 7.



Apparatus for filling and gassing six divers under water at the same time.

(PAUL & DANES 1961)

from the air space between the neck and mouth seals until the diver sank very slowly to the bottom of the flotation vessel. Warmed flotation medium was added to fill the vessel, and the stopper placed in position with the grooves in both the stopper and the vessel communicating. Excess medium was allowed to escape, the vessel was sealed by turning the stopper, and the stopcock on the manifold was connected from the flotation vessel to the air bottle.

After equilibration for an hour, the negative pressure required to readjust the divers to the same level was determined every twenty minutes for several hours.

Calculation of Results.

A straight line graph is obtained when the change in pressure is plotted against time. From this graph the change in pressure in centimetres per hour is calculated (Δp).

The gas volume of the diver is obtained from the equation -

$$V = \frac{g_D + V_{oil}\,\varphi_{oil} + V_W\,\varphi_W - V_{oil}\,\varphi_M - V_W\,\varphi_M \frac{g_D/\varphi_M}{g_l}}{\varphi_M}$$

where g_D is the weight of the empty diver in mgms., V_{oil} V_W denote the volumes and φ oil, φ_W, φ_M, and φ_{gl}. the densities of the dibutylphthalate, the aqueous part of the diver charge, the flotation medium, and the diver glass respectively. (Holter, 1942).

From these two calculations the oxygen consumption ΔV is found from the equation -

$$\Delta V = \frac{V \times \Delta P}{P_o} \cdot \frac{273 + t^o}{273} \quad \mu ls/hour.$$

where V = gas volume of diver.

ΔP = change in pressure in cm/hour.

P_o = barometric pressure of manometer fluid.

t^o = temperature, i.e. 37.5°C. (Holter 1942).

As the number of cells present was known, the oxygen consumption per hour per million cells was determined.

R E S U L T S

1. Nutritional Studies on Strain L Cells.

Initial experiments were designed to develop a minimal, completely synthetic, growth medium for cells in culture. The aim was to eliminate uncontrolled differences in growth rate and metabolism which might arise from different batches of natural substances, such as calf serum. In recent years completely synthetic media have been developed for the Strain L cell (Sanford et al., 1948) such as Medium 858 (Healey et al.,1955) and Medium NCTC 109 (McQuilken et al., 1957). These are very complex media and contain unknown constituents since some of the components are relatively impure - for example Coenzyme A (75 per cent. pure) andNADP (80 per cent. pure). A medium published more recently, Waymouth's medium MB 752/1 (Waymouth, 1959) is simpler in composition and still allows growth of strain L cells without added serum. The present experiments were undertaken with the object of defining the minimal essential components of Waymouth's medium MB 752/1, in the absence of serum or other natural supplements.

A component of Waymouth's medium was considered to be non-essential if, in its absence from the growth medium, the increase in cell number over four days was comparable to the increase in cell number on complete Waymouth's medium.

The cells used in these experiments were strain L cells (Sanford et al., 1948) clone 929, derived from mouse subcutaneous fibroblasts, adapted to grow in Waymouth's medium without serum.

The amino acid requirements of strain L cells growing in serum-free Waymouth's medium were first determined.

The concentrations of amino acids used (Table 2) were those of medium MB 752/1 modified with regard to cystine, isoleucine, leucine, methionine and valine (Paul and Mendelsohn, 1959). Stock solutions of amino acids were made up at either x 500 or x 250, depending on the solubility of the amino acid. From these, test 'mixed stock solutions', each deficient in one amino acid, were made, and a control solution containing all the amino acids. These solutions were x 2.5 final concentration.

Test 'mixed stock solutions' were sterilised by autoclaving except stock glutamine solution which was sterilised separately by filtration since it is unstable under autoclave conditions. It was added to the 'mixed stock solutions' aseptically.

Other stock solutions used were:

1. Waymouth's vitamin stock (100 x final concentration).
2. 20 per cent. glucose (w/v).
3. Penicillin - 2,000 units/ml.
4. Balanced salt solution (BSS) - (Hanks, 1949).

The cells used were strain L cells adapted to grow in Waymouth's MB 752/1 medium without serum.

TABLE 2.

CONCENTRATIONS OF AMINO ACIDS USED IN EXPERIMENTS TO DETERMINE THE AMINO ACIDS ESSENTIAL FOR THE GROWTH OF STRAIN L CELLS.
(Based on Waymouth's Medium MB 752/1).

	mM
L-Arginine HCl.	0.36
L-Aspartic acid.	0.46
L-Cystine.	0.1
L-Glutamic acid.	1.02
Glutamine.	2.38
Glycine.	0.66
L-Histidine HCl.	0.80
L-Isoleucine.	0.76
L-Leucine.	0.09
L-Lysine HCl.	1.42
L-Methionine.	0.1
L-Phenylalanine.	0.30
L-Proline.	0.22
L-Serine.	0.47
L-Threonine.	0.64
L-Tryptophan.	0.20
L-Tyrosine.	0.22
L-Valine.	0.21.

Basal Medium: this was prepared fresh for each experiment and contained:

1 ml. vitamin stock solution.

1 ml. glucose stock solution.

1 ml. penicillin solution.

57 ml. BSS.

All media were equilibrated with 5 per cent. carbon dioxide in air and were adjusted to pH 7.4 with 6.6 per cent (w/v) sodium bicarbonate.

Procedure.

Aliquots of 0.8 ml. of test 'mixed stock solution', deficient in one amino acid, were pipetted into sterile test-tubes, and 1.2 ml. of cell suspension in basal medium was added to each - care being taken to ensure an equal distribution of cells in the basal medium. The tubes were then gassed with a 5 per cent. carbon dioxide and air mixture, stoppered, and incubated in a sloping position at 37°C.

After four days the cells were removed from the glass by treatment with trypsin and counted (Methods, page 49).

The results of such an experiment are shown in Table 3. These results suggested that strain L cells, under these conditions, required all the amino acids of Waymouth's medium MB 752/1 with the exception of glutamic acid, aspartic acid, and glycine. The possibility existed however that

TABLE 3.

THE EFFECT ON THE GROWTH OF STRAIN L CELLS OF OMITTING AN AMINO ACID FROM THE CULTURE MEDIUM.

AMINO ACID MISSING FROM THE CULTURE MEDIUM.	NUMBER OF CELLS x 10^{5} PER TUBE AFTER FOUR DAYS' GROWTH.
Control (no amino acids missing),	37, 35.
L-Arginine,	8, 10.
L-Aspartic acid,	20, 13.
L-Cystine,	9, 8.
L-Glutamic acid,	32, 36.
Glutamine,	9, 8.
Glycine,	39, 35.
L-Histidine,	10, 10.
L-Isoleucine,	9, 9.
L-Leucine,	10, 13.
L-Lysine,	10, 13.
L-Methionine,	9, 8.
L-Phenylalanine,	7, 6.
L-Proline,	9, 16.
L-Serine,	19, 16.
L-Threonine,	8, 8.
L-Tryptophan,	13, 12.
L-Tyrosine,	12, 10.
L-Valine,	9, 9.
Inoculum,	10.

when all three of these amino acids were omitted from the medium the cells might be unable to grow.

Mixed stock solutions were therefore prepared as in the previous experiment, omitting glutamic and aspartic acids and glycine alone, in pairs, and together. The experimental details were the same and Table 4 shows that the three amino acids could be omitted from the medium without affecting growth.

The conversion of phenylalanine to tyrosine (by hydroxylation) is known to occur *in vivo* (Moss and Schoenheimer, 1940). It seems from Table 4 that such a conversion also takes place *in vitro* in these cells, since in the absence of tyrosine growth was unimpaired if phenylalanine was present but in the absence of both amino acids no growth occurred.

Both cystine and methionine are essential for the growth of strain L cells and no interconversion exists, since the absence of either of these amino acids inhibited growth nearly as much as their combined absence.

In vivo serine is readily converted to glycine and formate (Shemin, 1946) by a reversible reaction (Kisliuk and Sakami, 1955) and the one carbon unit of formate can be transformed into many compounds important for growth, such as the nucleoside thymidine. Since serine was shown (Table 3) to be essential for optimal growth, while glycine

T A B L E 4.

EFFECT ON GROWTH OF STRAIN L CELLS OF OMITTING, SEPARATELY AND TOGETHER:-

1. Aspartic acid, Glutamic acid, and Glycine.
2. Cystine and Methionine.
3. Tyrosine and Phenylalanine.

AMINO ACID OMITTED FROM THE CULTURE MEDIUM.	NUMBER OF CELLS x 10^4 PER TUBE AFTER FOUR DAYS' GROWTH.
Control (no amino acids missing),	51, 56.
1.	
Aspartic acid,	51, 53.
Glutamic acid,	35, 49.
Glycine,	40, 43.
Aspartic acid + Glutamic acid,	63, -.
Aspartic acid + Glycine,	54, 50.
Glutamic acid + Glycine,	47, 46.
Aspartic acid + Glutamic acid + Glycine,	53, 46.
2.	
Cystine,	16, 21.
Methionine,	19, -.
Cystine + Methionine,	12, 15.
3.	
Tyrosine,	42, 35.
Phenylalanine,	18, 16.
Tyrosine + Phenylalanine,	11, -.

Inoculum, 10.

was not, test-tube cultures were set up as described previously, omitting serine but adding formate or thymidine (at a final concentration of 0.2 mM). The results of this experiment (Table 5) show that such additions do not overcome the requirement of the cells for serine. The requirement would thus seem to be for serine itself and not for its breakdown products, suggesting that in these cells the conversion between the three compounds is not reversible.

Waymouth's medium MB 752/1 (1959) however does not contain serine while the glycine concentration is the same as that used in these experiments. The inoculum used in these studies was low and the requirement for serine may be due to a leakage of serine into the medium. Such a leakage would not affect cultures with a high inoculum. In this respect Eagle (1962) has found a requirement for serine when cloning cells.

Proline has not been found to be an essential amino acid in *in vivo* studies, though it is present in many proteins in low concentrations. Collagen contains a fairly high concentration of proline and it is interesting therefore that Strain L cells, which being of fibroblastic origin would synthesise collagen *in vivo*, require proline. Eagle (1955) found no requirement for proline with Strain L cells but in his experiment growth was measured after 15 days in proline-free medium. It is possible there-

TABLE 5.

THE EFFECT ON GROWTH OF L CELLS IN SERUM-FREE MEDIUM OF OMITTING SERINE, IN THE PRESENCE AND ABSENCE OF ADDED THYMIDINE OR FORMATE.

SUBSTANCES OMITTED AND ADDED TO CULTURE MEDIUM.	NUMBER OF CELLS/TUBE x 10^4 AFTER FOUR DAYS' GROWTH.
None (control).	63, 63.
-Serine.	28, 14.
-Serine + Thymidine.	25, 25.
-Serine + Formate.	31, 28.

fore that, like serine, this amino acid may leak out from the Strain L cells until an equilibrium is reached between the interior and exterior of the cell.

From these initial experiments a medium was formulated (HERT 1) containing:

L-Arginine HCl	-	0.36 mM
L-Histidine HCl	-	0.80
L-Isoleucine	-	0.76
L-Leucine	-	0.09
L-Lysine HCl	-	1.42
L-Methionine	-	0.10
L-Phenylalanine	-	0.30
L-Proline	-	0.22
L-Serine	-	0.47
L-Threonine	-	0.64
L-Tryptophan	-	0.20
L-Valine	-	0.21
Glutamine	-	2.38
L-Cystine	-	0.06
L-Tyrosine	-	0.22

Vitamins (Waymouth's MB 752/1).

Thiamine	-	0.03 mM
Ca Pantothenate	-	0.003
Pyridoxine HCl	-	0.003
m-Inositol	-	0.005
Nicotinamide	-	0.008
Vitamin B_{12}	-	0.00015
Folic acid	-	0.0008
Biotin	-	0.00008
Riboflavin	-	0.003

Glucose - 22.2 mM.

Having established the minimal amino acid requirements for the growth of strain L cells, further experiments were undertaken to establish the optimum concentrations of each amino acid.

To determine this, stock solutions were made up as described previously, omitting glutamic acid, aspartic acid, and glycine, as well as the amino acid being tested. 50 mM solutions of each amino acid were prepared and 5 mM, 0.5 mM, and 0.05 mM solutions made by serial dilution.

Trypsin has been shown to have a deleterious effect on cells *in vitro* when the culture medium does not contain serum (Hebb and Hu, 1960). For this reason, in the previous experiments, the cells were removed from stock culture flasks by scraping. This treatment, however, tends to give a suspension with clumps of cells. There may therefore be differences in cell numbers between aliquots of such a suspension. To circumvent this difficulty a slightly different technique was adopted.

Cells in the stock cultures were removed by treatment with trypsin (page 49) and counted. 10^5 cells were inoculated into each test-tube in 2 ml. of HERT 1 medium containing 2 per cent. calf serum. After 24 hours the medium was removed, the tubes washed out with 2 ml. of BSS, and the test solutions added, giving final concentrations of the amino acid being tested of 0, 0.01, 0.1, 1.0, and 10.0 mM. The results of these experiments are shown in Table 6.

TABLE 6.

EFFECT OF DIFFERENT CONCENTRATIONS OF ESSENTIAL AMINO ACIDS ON GROWTH OF STRAIN L CELLS IN SERUM-FREE MEDIUM.

TEST AMINO ACID.	NUMBER OF CELLS x 10^4/TUBE AFTER FOUR DAYS' GROWTH Concentration of Test Amino Acid.					CONTROLS
-------	0	0.01mM	0.1 mM	1.0 mM	10.0mM	
Serine,	17, 15	19, 19	22, 23	25, 23	13, 13	26
Arginine,	8, 18	10, 11	20, 29	31, 22	7, 6	22
Lysine,	10, 12	11, 12	18, 25	19, 17	14, 8	26
Phenylalanine,	7, 7	16, 30	22, 30	24, 27	14, 16	
Histidine,	20, 17	37, 42	57	74, 63	20, 18	54
Proline,	68, 64	57, 54	70, 68	48, 48	35, 41	52 51
Methionine,	20, 22	40, 50	48, 45	41,45,39	30,26,28	46
Threonine,	27, 27	35,35,35	50,38,33	39,33,29	33,39,31	50
Tryptophan,	16, 18	24, 44	32, 38	32, 27	13, 17	

The optimal concentrations found from these experiments are given below and also the concentrations in HERT 1 and Waymouth MB 752/1.

	mM	HERT (mM)	MB 752/1 (mM).
Serine	0.1 - 1.0	0.5	-
Arginine	0.1 - 1.0	0.4	0.36
Lysine	0.1 - 1.0	1.3	1.42
Phenylalanine	0.1 - 1.0	0.3	0.3
Histidine	1.0	0.8	0.8
Proline	0.1	0.2	0.44
Methionine	0.1	0.1	0.34
Threonine	0.01 - 10.0	0.6	0.6
Tryptophan	0.1	0.2	0.2

Of the amino acids studied in this way, only with Histidine and Proline does there seem to be a relatively sharp optimal concentration.

A competitive interrelationship between leucine, isoleucine, and valine has been demonstrated in bacteria (Dien et al. 1954). In this work it was shown that the inhibition of growth caused by a high concentration of one of these amino acids could be prevented by increased concentrations of the other two.. Such an interaction was now sought in strain L cells.

Leucine free of isoleucine was used so that the concentrations of both were accurately known.

Preparation of isoleucine-free leucine.

1 gm. of leucine was dissolved in 50 ml. of water and heated to boiling point. Excess copper carbonate was added (about 2 gms.) and the mixture was removed from the heat. The resulting precipitate was filtered and washed several times with water. The filtrate was then gassed for an hour with hydrogen sulphide and filtered to remove excess copper. The filtrate was concentrated to 25 ml. under pressure, at which concentration leucine crystallises. Recrystallisation was carried out twice with water. On occasions colloidal copper sulphide was present in the supernatant; it was removed by passing the suspension through a thin layer of activated charcoal.

Interaction studies.

200 mM stock solutions of valine, leucine and isoleucine were made up and from these dilutions were made, giving stock solutions of 100, 10, 1.0, 0.1 mM.

Basal medium.

30 ml. mixed amino acid stock (x5) (- leucine, isoleucine and valine).
1.5 ml. vitamin stock solution.
1.5 ml. glucose stock solution.
70.5 ml. BSS.

Procedure.

64 test-tubes were prepared, each containing 5×10^4 cells in 2 ml. of MERT 1 and 2 per cent calf serum. After

24 hours this medium was removed. The cultures were washed with 2 ml. of BSS and 0.4 ml. of basal medium was added to each tube. 0.4 ml. of the appropriate stock solutions of leucine, isoleucine and valine were then added to give all possible combinations of the three amino acids at concentrations of 0.01, 0.1, 1.0, and 10.0 mM (see Table 7).

There does appear to be some interaction between the three amino acids in these cells (Table 7) though not to a marked extent. When the leucine concentration is kept at 0.1 mM, greatest growth occurs when isoleucine concentration is 0.1 mM and valine 10 mM. If under these conditions the isoleucine concentration is raised by a factor of ten to 1.0 mM, the optimal concentration of valine is lowered by the same factor to 1 mM. Maximum growth in this experiment was in 10 mM leucine, 10 mM isoleucine, and 1 mM valine.

One part of Table 7 was then examined separately, in more detail, by keeping the concentration of one amino acid, isoleucine, constant, at 1 mM, and varying the concentrations of the other two from 0.08 to 10 mM. The experimental procedure was the same as that described previously and the results are shown in Table 8. This experiment also showed some interaction between leucine and valine when the isoleucine concentration was constant.

TABLE 7.

EFFECTS ON GROWTH OF STRAIN L CELLS IN SERUM-FREE MEDIUM OF VARYING THE CONCENTRATIONS OF THREE INTERACTING AMINO ACIDS.

CELL NUMBER x 10^{-4}/TUBE AFTER FOUR DAYS' GROWTH.

Isoleucine conc.	0.01 mM				0.1 mM				1.0 mM				10.0 mM			
Valine conc.	0.01,	0.1,	1.0,	10.0	0.01,	0.1,	1.0,	10.0	0.01,	0.1,	1.0,	10.0	0.01,	0.1,	1.0,	10.0
Leucine conc. 0.01 mM.	33,	54,	43,	51	26,	87,	66,	64,	37,	76,	92,	78,	30,	118,	84,	135
0.1 mM.	32,	58,	41,	50	55,	61,	86,	122	39,	108,	115,	26,	27,	80,	71,	52
1.0 mM.	32,	44,	27,	33	26,	107,	83,	104	38,	114,	115,	118,	40,	106,	85,	89
10.0 mM.	24,	24,	24,	30	23,	142,	134,	123	33,	85,	145,	126,	23,	61,	162,	100

TABLE 8.

EFFECT OF VARYING THE CONCENTRATIONS OF LEUCINE AND VALINE, WITH ISOLEUCINE CONCENTRATION CONSTANT AT 1 mM., ON GROWTH OF STRAIN L CELLS.

CELL NUMBER x 10^{-4}/TUBE AFTER FOUR DAYS' GROWTH				
LEUCINE CONCEN-TRATION	VALINE CONCENTRATIONS.			
	0.08 mM	0.4 mM.	2.0	10.0 mM
0.08 mM.	106, 96, 108	110, 114, 124	- 107, 121	92, 126, 86
0.4 mM.	80, 70, 100	- 89, 79	64, 95, 40	- 86, -
2.0 mM.	94, 101, 126	- 124, 126	78, 124, 132	125, - 97
10.0 mM.	27, 31, 31	133, 143, 176	150, 110, 129	104, - 121

The scatter of results and the relatively small differences make comprehension difficult, but it is clear, for example, that a high concentration of leucine with a low concentration of valine is inhibitory, while lowering the leucine concentration or raising the valine concentration increases growth.

These experiments showed that not only the concentrations of leucine, isoleucine and valine were important, but also their ratios in synthetic media.

Arginine/Lysine.

A similar experiment was designed to investigate possible interaction between arginine and lysine in the concentration range of 0.01 to 10 mM. Some type of imbalance is also evident with arginine and lysine (Table 9); thus growth is less in high (10 mM) arginine, low lysine (0.01 mM) and in high arginine (10 mM), high lysine (10 mM) than it is in 10 mM arginine and 0.1 or 1.0 mM lysine or in 1 mM arginine with all four lysine concentrations.

The inhibition of utilisation of one amino acid by another may be due to a competition for the enzymes involved in peptide formation or other reactions - in this respect Dien et al. (1954) found that the keto acid or peptide of valine had a greater activity in reversing the toxicity of isoleucine than had the free amino acid.

TABLE 9.

EFFECT OF VARYING THE CONCENTRATIONS OF ARGININE AND LYSINE, ON THE GROWTH OF STRAIN L CELLS IN SERUM-FREE MEDIUM.

CELL NUMBER x 10^4/TUBE AFTER FOUR DAYS' GROWTH				
LYSINE CONCEN-TRATION.	ARGININE CONCENTRATION			
	0.01 mM	0.1 mM	1.0 mM	10.0 mM
0.01 mM.	40, 42, 37	55, 107, 98	110, 86, 107	65, 82, 69
0.1 mM.	36, 53, 48,	84, 65, 110	143, 164, 95	102, 118, 58
1.0 mM.	34, 36, 42	94, 142, -	162, 138, -	- 123, 67
10.0 mM.	35, 32	110, 97, 85	128, 115, 95	59, 45, 58

The medium HERT 1 which was formulated as a result of those studies was capable of sustaining growth of strain L cells without the addition of serum. With other, more exacting, cell strains, which were used in later studies, serum was still necessary for long term culture.

2. Environmental Factors and Respiration.

Using a modified cartesian diver technique for measuring respiration (Paul and Danes, 1961), Danes and Paul (1961) found that the respiratory rate of strain L cells could be profoundly altered by varying the culture or experimental conditions. The studies to be described were undertaken with other cell strains to provide comparative data.

The cell strains used were:

1. Normal human foreskin fibroblast. These cells were obtained from the Genetics Department, University of Glasgow, and had been cultured for 6-8 months *in vitro*. Respiration of these cells was obtained only in the first set of experiments. Thereafter the cell line was lost.

2. Strain HeLa (Gey *et al.*, 1952). This strain became established from cultures of epidermoid carcinoma of human cervix and has now been grown in culture for 12 years.

3. Strain HLM (Leslie et al., 1956). Human foetal liver (parenchymal) cells.

4. Strain Y5 (Ford and Yerganian, 1958). This strain was derived from Chinese hamster skin fibroblasts in experiments designed to study chromosomal variations after long-term culture. It still retains the normal

chromosomal picture of Chinese hamster cells which have 11 relatively distinct pairs of chromosomes.

5. Strain L5178Y (Fischer, 1958) - mouse lymphoma cells which can be carried *in vitro* or as an ascites tumour. In the present studies they were grown *in vitro*.

Danes and Paul (1961) found that, after inoculation into fresh medium, L cells showed a much lower respiratory rate and, after three days in culture, the respiratory rate had risen again. This fall in respiration could be prevented by the addition of citric acid cycle intermediates and was attributed to a leakage of small molecules from the cells. The effect of such conditions was then studied in the cell strains mentioned above.

Procedure.

Test-tube cultures containing 10^5 cells/ml. were set up in 2 ml. of three different media:

1. Fresh medium (medium in which cells had not grown).
2. Fresh medium containing citric acid cycle intermediates (Solution A, page 228).
3. Conditioned medium - medium in which cells had grown for four days, with the glucose concentration and pH adjusted to original values.

The gas phase in these cultures was air and the pH was 7.4. Cultures were taken 24, 48, and 72 hours after

inoculation. The cells were removed from the glass by scraping (except for the L5178Y strain which grows in suspension). After counting, the cells were suspended in respiration medium to give a final concentration of 5×10^6 cells/ml. 2 µl. of this suspension were used per diver and respiration measurements were carried out as described on page 75.

Results.

The respiratory rates found under these conditions are shown in Table 10, which also includes the rates found for the strain L cell by Danes and Paul (1961), for comparison.

The HeLa, HLM, and Y5 strains showed no fall in respiration after inoculation into fresh medium, and the addition of citric acid cycle intermediates had very little effect. HeLa cells actually showed a fall in respiration after culture in conditioned medium or in fresh medium after 72 hours, which suggested the accumulation during growth of substances inhibitory to respiration.

The L5178Y cell resembled the L cell in that the addition of Solution A prevented the fall in respiration found after inoculation into fresh medium. The reason for this fall in respiration has been attributed to a leakage of citric acid cycle intermediates from the cells.

TABLE 10.

INFLUENCE OF MEDIUM ON RESPIRATION.

Cell strain	Duration of culture. hrs.	Oxygen uptake µl/hr/cell x 10^6		
		Fresh medium	Conditioned medium	Fresh medium + solution A
HeLa	24	15.00	7.53	12.20
	48	10.50	8.21	10.90
	72	55.50	4.69	4.40
HLM	24	7.50	6.74	7.14
	48	8.39	6.45	8.25
	72	10.03	7.02	9.73
L5178Y	24	0.47	0.83	0.88
	48	0.43	0.92	1.34
Y5	24	3.8	-	4.1
	48	4.0	4.8	3.9
	72	3.9	5.25	5.1
Human Skin Fibroblasts	24	1.06	0.70	8.07
	48	1.45	1.25	8.11
	72	1.80	1.30	8.52
L (Danes and Paul 1961)	24	2.20	5.15	7.39

Conditioned medium - medium in which cells have been grown with glucose concentration and pH adjusted.

Solution A - citric acid cycle intermediates (page 228).

When the concentration of these in the medium is increased, either by growth of cells in the medium or by the addition of the compounds to fresh medium, an equilibrium is attained and respiration remains high.

The human skin fibroblast exhibited low respiration in fresh and conditioned media and the addition of citric acid cycle intermediates to the medium resulted in a high rate of respiration, suggesting that in these cells continued growth did not compensate for the low level of intermediates in the cell.

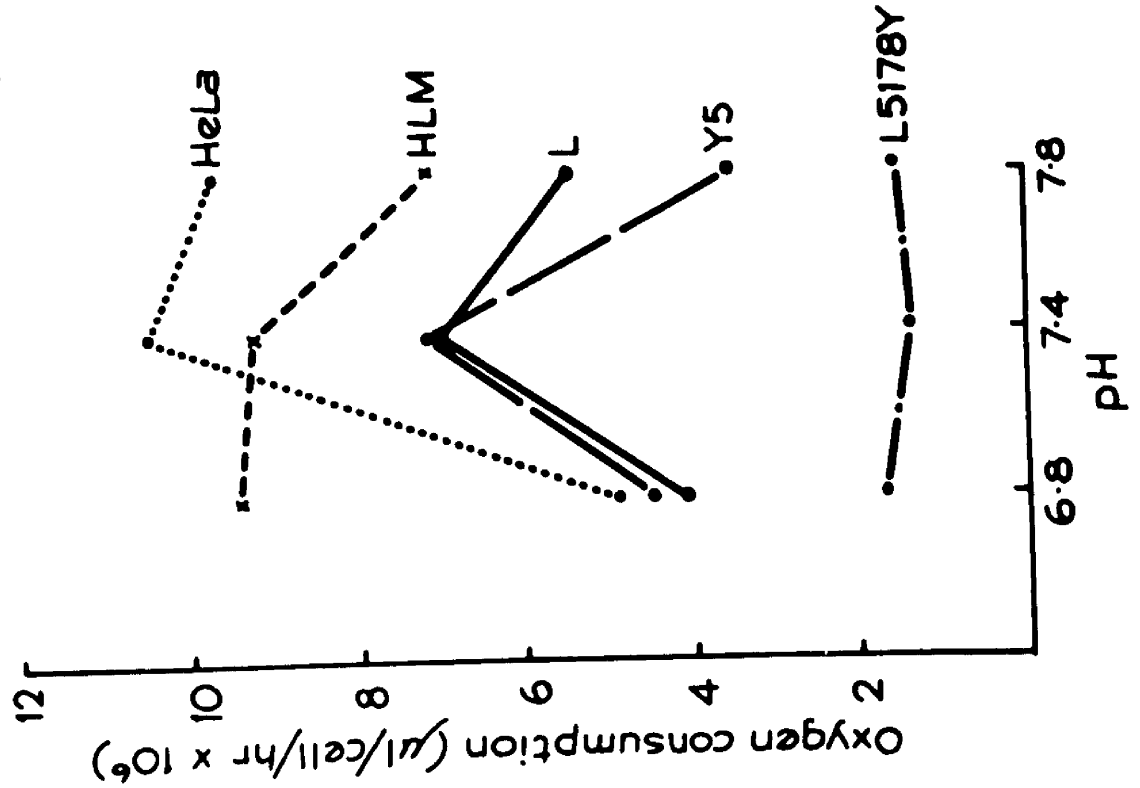
In all experiments on other variables affecting respiration, these results were taken into consideration and conditions used which gave a high respiratory rate, i.e. in strains where the respiratory rate fell after inoculation into fresh medium, Solution A was added or the cells were taken after 72 hours in culture.

Effect of hydrogen ion concentration.

Respiration of cultures grown in media of pH 6.8, 7.4 or 7.8 was measured after 72 hours in culture. Cultures were set up in test-tubes with an inoculum of 10^5 cells/ml. in media of the three pH VALUES. Respiration was measured in medium of pH 7.4.

The results of such experiments are given in Fig. 8. All strains showed maximal respiration at pH 7.4 and the respiratory rate decreased more or less rapidly

FIG. 8.


HeLa
HLM
L
Y5
L5178Y
6·8
7·4
7·8
pH
2
4
6
8
10
12
Oxygen consumption (μl/cell/hr x 10⁶)

in both acid and alkaline conditions.

It has been shown (Zwartouw and Westwood, 1958; Broda et al., 1959; Paul, 1959) that cells grown at an alkaline pH produce much more lactic acid from a given amount of glucose than do cells grown at an acid pH, i.e. that there is a gradual transition from a more glycolytic to a more oxidative pattern as the pH is lowered. The precise nature of this phenomenon is not known but does not seem to be directly related to respiration, since the respiratory rate was found to be highest at pH 7.4 and decreased in both acid and alkaline conditions.

Change of pH in the range studied had very little effect on the respiratory rate of strain L5178Y cells. The respiratory rate of these cells was extremely low and it is possible that small differences in respiratory rate at this level were below the sensitivity of the technique.

Effect of oxygen tension.

Three sets of cultures were set up in test-tubes as in previous experiments in medium which had been equilibrated with -

(a) 95 per cent nitrogen and 5 per cent carbon-dioxide.

(b) air, and 5 per cent carbon dioxide.

(c) 95 per cent oxygen and 5 per cent carbon dioxide, respectively.

The cultures were gassed with the appropriate gas mixture. After 48 hours in culture (72 hours in the case of the Y5 cells), respiration was measured in air/5 per cent. carbon dioxide, as in previous experiments (Table 11).

All strains showed maximum respiration in air/5 per cent carbon dioxide and considerably lower respiration in high or low oxygen tensions. When cells are grown in a medium equilibrated with 95 per cent. oxygen they at first grow and respire rapidly and exhibit a high glycolytic rate (Brosemer and Rutter, 1961; Rueckert and Mueller, 1960), and then die suddenly. This toxicity is thought to be due to accumulation of peroxides (Liberman and Ove, 1958), in the medium.

Effect of glucose concentration - Crabtree effect.

Inhibition of respiration by high glucose concentrations was first described by Crabtree (1929) in sarcoma tissue and was not found in the normal tissues then studied. However, it has since been described in normal tissues which exhibit aerobic glycolysis (Este et al., 1957; Rosenthal, 1940).

Cells from stock cultures (to which Solution A (page 228) had been added if necessary) were suspended in HERT medium containing glucose concentrations ranging from 0 to 88 mM., and the respiration measured. The

TABLE 11.

RESPIRATORY RATES AFTER 48 HOURS' GROWTH IN VARIOUS OXYGEN TENSIONS (72 HOURS IN THE CASE OF Y5 STRAIN).

Oxygen uptake µl/hour/cell x 10^6			
Cell strain	Percentage oxygen in gas phase		
	0	20	95
HeLa	7.60	11.00	4.70
HLM	5.83	8.24	6.45
L5178Y	0.7	1.1	0.32
Y5	2.9	3.5	0.88
L (Danes and Paul 1961)	3.35	8.04	1.69

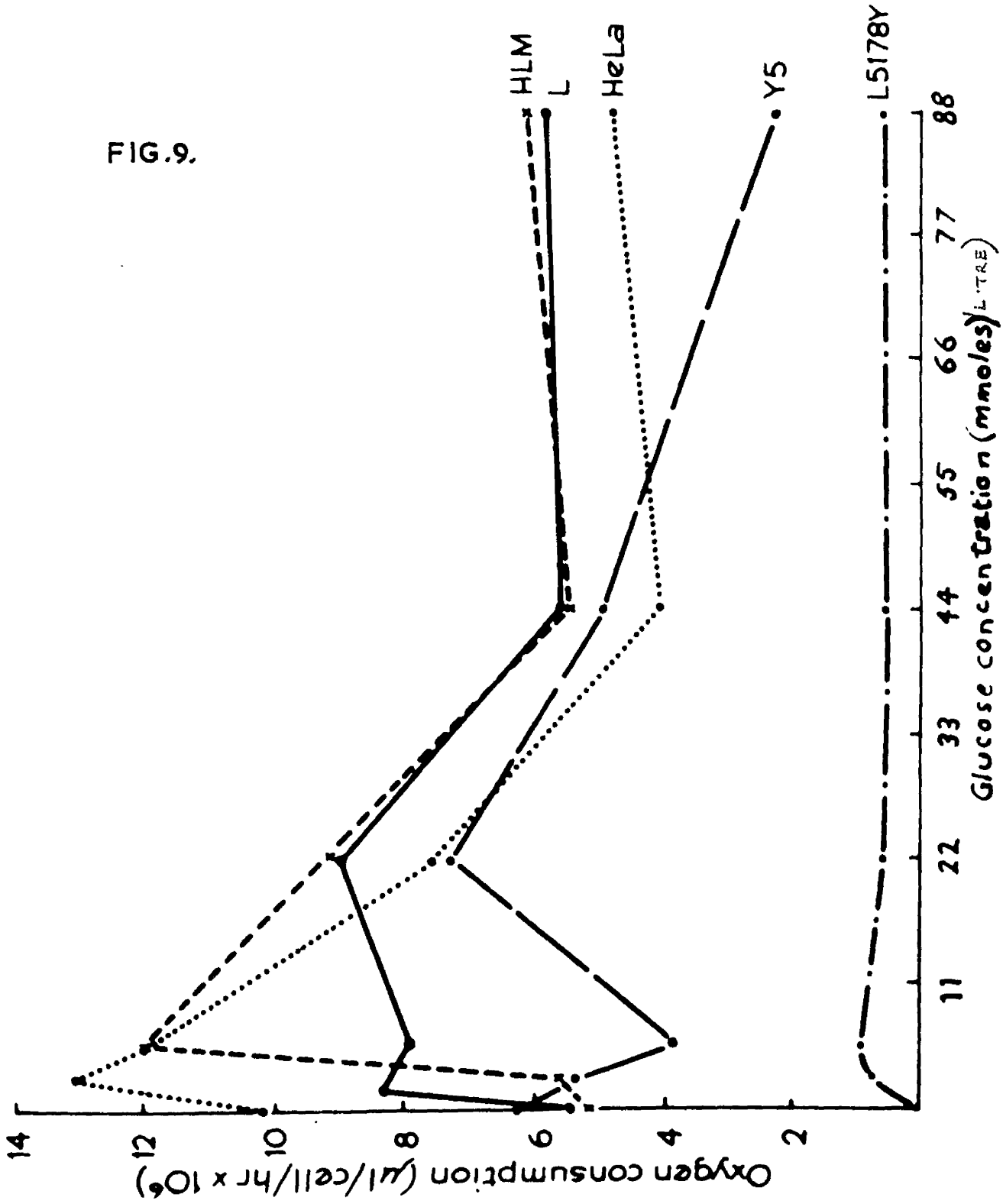
results are shown in Fig. 9.

All strains (with the possible exception again of L5178Y strain, which again showed extremely low respiration) exhibited a Crabtree effect at high glucose concentrations. However, at lower glucose concentrations there were two interesting observations:

1. Respiratory rate of strains HeLa and HLM increased with the addition of glucose to the medium (L strain - Danes and Paul, 1961 - behaved similarly). This increase could be explained by an initial lack of substrates for the citric acid cycle, and the Y5 strain which did not show this initial rise might contain a larger pool of substrates.

2. The Y5 strain which showed a decrease in respiratory rate from 0 to 5 mM. glucose then showed an increased rate up to 22 mM. glucose followed by a gradual inhibition of respiration with increased glucose. This truncated pattern was also shown, to a lesser extent, by strain L cells (Danes and Paul, 1961) - included in Fig.9 and it was suggested that this pattern might be associated with the observed leakage of citric acid cycle intermediates. However the continued high level of respiration of Y5 strain cells after inoculation into fresh medium and the lack of effect of Solution A on respiration (Table 10) did not indicate any leakage of

FIG.9.


HLM
L
HeLa
Y5
L5178Y
Oxygen consumption (μl/cell/hr x 10⁶)
14
12
10
8
6
4
2
11
22
33
44
55
66
77
88
Glucose concentration (mmoles/LITRE)

citric acid cycle intermediates in the Y5 strain cells.

Effect of carbon dioxide tension.

Cells from stock cultures were used (in the case of 'leaky' cells - page 99 - Solution A - page 228 - had been added to stock cultures, or the cells had been grown for a few days without change of medium to ensure a high respiratory rate). Respiration was measured in medium equilibrated to pH 7.4 with four gas mixtures:

(a) air with carbon dioxide removed by prolonged exposure to potassium hydroxide in a desiccator.

(b) air - i.e. 0.03 per cent carbon dioxide.

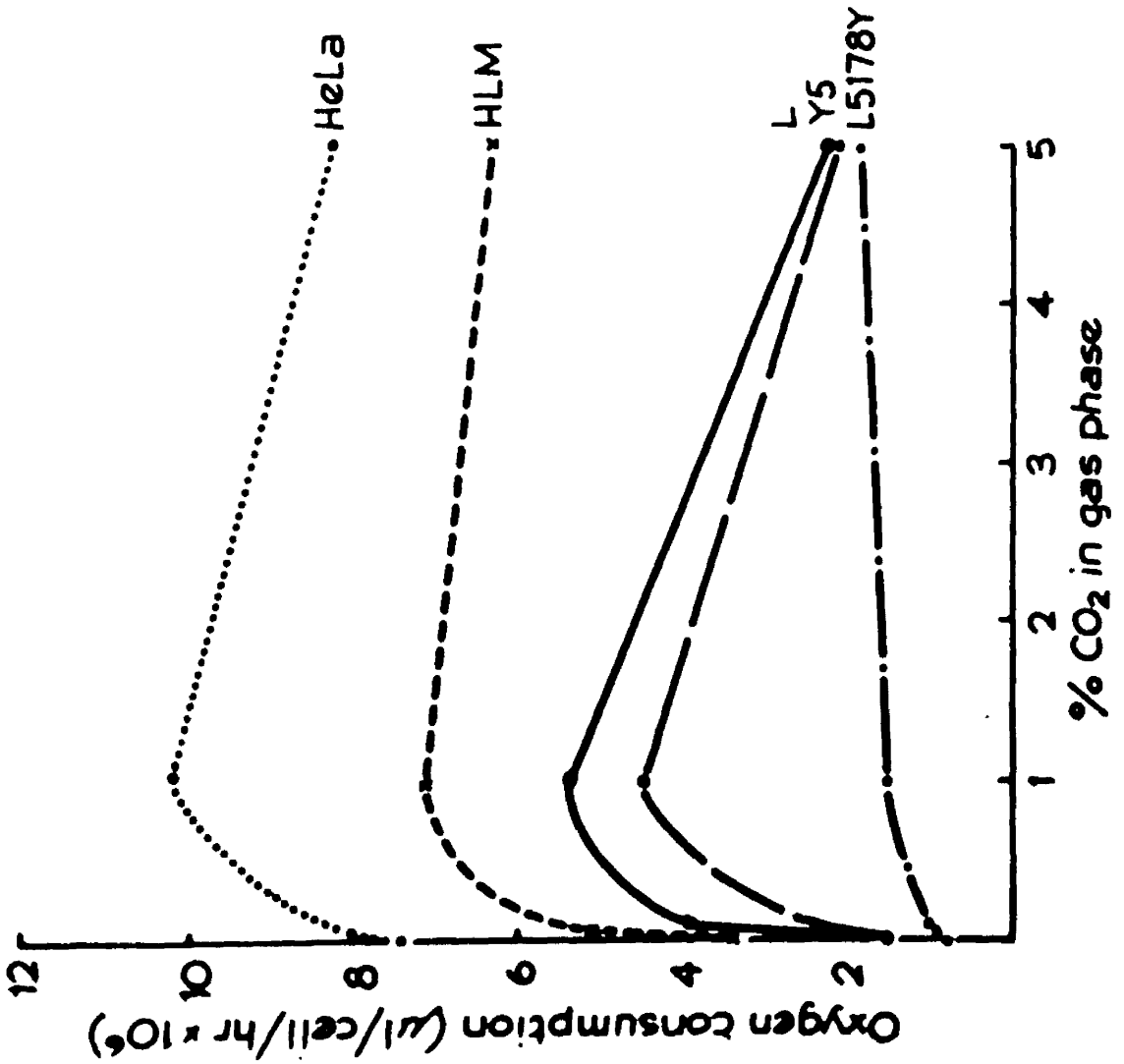
(c) air/1 per cent. carbon dioxide.

(d) air/5 per cent. carbon dioxide.

The flotation medium in which the divers were suspended was equilibrated with the appropriate mixture of carbon dioxide and air.

Fig. 10 shows that the respiratory rate of HeLa, HLM, and Y5 strains was highest in 1 per cent carbon dioxide / air mixture as had been found in the L strain (Danes and Paul, 1961). There was little difference in respiration in 1 per cent and 5 per cent. carbon dioxide in air in the L5178Y strain, although in common with the other strains, respiration was lowered in an atmosphere lacking carbon dioxide.

FIG. 10.


HeLa
HLM
L
Y5
L5178Y
1
2
3
4
5
% CO_2 in gas phase
12
10
8
6
4
2
Oxygen consumption (μl/cell/hr × 10^6)

3. Respiration Studies on Strain BHk21.

After infection of baby hamster kidney fibroblasts strain 21 (BHk21) by polyoma virus *in vitro*, colonies of 'transformed' cells could be detected and isolated from colonies of uninfected or 'normal' cells by morphological differences in the colonies, since the 'transformed' cells did not exhibit parallel alignment in monolayers. "Transformed' cells could also be characterised by the production of tumours after inoculation into hamster cheek pouch (Macpherson and Stoker, 1962). Several of such 'normal' and 'transformed' clones have been maintained in culture and were used in these studies.

'Normal' clones: C13, C13A (hereafter referred to as A cells), C13C (C cells). These clones do not produce tumours in hamsters and exhibit parallel alignment in monolayers.

"Transformed" clones: C13TC11 (TC11 cells), C13Y (Y cells), C13Z (Z cells). These clones do produce tumours in hamsters and do not exhibit parallel alignment in monolayers.

(a) C13 and TC11 cells. (Macpherson and Stoker, 1962). A population of clone C13 of strain BHk21 cells was exposed to polyoma virus. The 'transformed' cells which developed were cultured and designated TC11 cells. Another population of C13 clone was cultured without infection by polyoma virus.

(b) A and Y cells; C and Z cells (Stoker, 1962).

A population of C13 cells was exposed to polyoma virus. After 7 days' growth three well-separated colonies with normal morphology and two of the colonies with the random arrangement of 'transformed' cells were marked and removed separately from the culture. Each colony was grown to a population of about one million cells and then cloned. The apparently normal clones were designated A, B, C, and the transformed colonies were designated Y and Z (Stoker, 1962).

Clones A, C, Y, and Z were used in these studies.

A study of the respiration of the two types of cell in this system is important in consideration of Warburg's theory of damaged respiration (Warburg, 1956). In such a system the environment can be much more closely controlled than <u>in vivo</u> - an important factor as shown by the previous experiments. In contrast to many previous comparative studies, there is here no doubt as to the normal counterpart of the malignant cell. Also, in culture, the growth rates of the two cell types are similar; hence the effect of growth on respiratory rate is eliminated.

Several details in the previous experimental methods were reconsidered with respect to these cells.

(1) Effect of proteolytic enzymes.

It has been found that cells which have been removed from glass by the action of proteolytic enzymes such as trypsin take some hours to recover when inoculated into serum-free medium (Hebb and Hu, 1960). Since the respiration medium contained no serum and measurements were carried out only a few hours after removal of the cells from glass, trypsin was not used in the previous respiration experiments. In these studies scraping the cells from glass gave a monocellular dispersion.

After scraping however the BHk21 cells were obtained as a suspension of clumps of cells on which accurate cell counts were difficult to obtain.

Respiration was therefore measured in TC11 cells which had been treated with trypsin to determine whether in these cases the enzyme action had a deleterious effect. Table 12 showed that after treatment with 0.5 per cent. trypsin in citrate (page 49) respiration was considerably depressed.

The clones of the BHk21 cell strain were therefore scraped off the glass as in previous experiments. Great care was taken to break up the clumps of cells as much as possible and counts were made of cells stained with crystal violet (0.1 per cent. in 0.1 M citric acid) in

TABLE 12

RESPIRATION RATES OF C13TC11 CELLS WHICH HAD BEEN REMOVED FROM GLASS SURFACE BY SCRAPING OR BY TREATMENT WITH TRYPSIN.

Method of removing cells from glass surface.	Oxygen uptake $\mu l\ O_2$/hour/cell x 10^6
Scraping	6.1
Trypsin in citrate	4.5

a haemocytometer slide (page 51).

(2) Hydrogen ion concentration of culture medium.

It has been found that growth (Paul, 1959) and respiration (page101) vary to a marked degree with the pH of the growth medium (the optimum pH being 7.4). It is necessary therefore to maintain a constant pH during the growth of cells prior to respiratory measurements.

The BHK21 cells strain, particularly the 'transformed' clones, produces relatively large quantities of acid during growth, leading to a fall in pH of medium buffered with bicarbonate/carbonic acid which was used in previous experiments. The pH of the growth medium of these cells can be maintained for some days when the medium is buffered with Tris/citrate (page 219)(Paul, 1962, unpublished). Respiration of cells grown in Tris/citrate buffered medium was therefore compared with that of cells grown in bicarbonate/carbonic acid buffered medium (Table 13).

Since cells grown in Tris/citrate buffered medium showed a lower respiratory rate than those grown in bicarbonate buffer, the latter buffer system was used in all respiratory studies, and to ensure a relatively constant pH during culture the cells in stock culture were fed the day prior to and following the setting up of the experiment, and respiration was measured after 48 hours'

TABLE 13.

THE EFFECT OF GROWTH IN MEDIUM, BUFFERED WITH BICARBONATE/CARBONIC ACID, OR WITH TRIS/CITRATE, ON RESPIRATION OF C13TC11 CELLS.

Time in Test Medium, (hours)	OXYGEN UPTAKE µl Oxygen/hour/cell x 10^6	
	Bicarbonate/ Carbonic Acid Buffer	Tris/citrate Buffer
24	1.7	2.1
48	2.5	1.3
72	2.7	1.7

Respiration was measured in medium buffered with Bicarbonate/carbonic Acid.

culture in experimental conditions.

At the same time it was apparent that the medium in which respiration was measured did not affect the respiratory rate (Table 14) since respiration of cells, taken from the same culture and suspended in HERT (page 84) Hank's BSS (page 218) or Tris/citrate buffered BSS (page 227) containing the same concentration of glucose (11 mM) and equilibrated with 5 per cent. carbon dioxide in air, was very similar.

Therefore Tris/citrate buffered BSS containing glucose (11 mM) and equilibrated with the appropriate carbon dioxide/air mixture and sodium bicarbonate to pH 7.4 (page 47) was used during measurement of respiration.

Effect of environmental conditions on respiration of BHK21 cells.

The effects on C13 and TC11 clones of the BHK21 strain of environmental conditions which had been found to affect the respiration of a number of cell strains in tissue culture were determined.

(1) The effect of culture medium on C13 and TC11 cells.

Three different culture media were used:

(a) Fresh medium (medium in which cells had not been grown),

(b) Fresh medium supplemented with citric acid cycle intermediates (Solution A, page 228 - Fresh medium + Solution A.

(c) Conditioned medium - medium in which cells had been grown for 4 days with the glucose concentration and pH adjusted to original values.

TABLE 14.

THE EFFECT OF VARYING THE MEDIUM IN WHICH RESPIRATION WAS MEASURED ON THE RESPIRATION OF C13 CELLS. ALL MEDIA WERE EQUILIBRATED WITH 5 PER CENT. CARBON DIOXIDE AND CONTAINED 11 mM. GLUCOSE.

Respiration Medium.	Oxygen uptake, $\mu l\ O_2$/hour/cell x 10^6
HERT 1.	2.06
B.SS. (Hanks, p. 218).	2.00
Tris/citrate BSS (p. 219).	2.06

The gas phase in these cultures was air and the pH 7.4. The experimental details are as given on page 99. Tris/citrate buffered BSS was used as respiration medium. Respiration was measured after 48 hours in experimental media without change of media.

The respiratory rates of Cl3 cells in all three types of media were similar (Table 15). TCll cells showed a slightly higher respiration in supplemented medium than in fresh medium, but this rise was very small in comparison with that found for strain L cells (Danes and Paul, 1961) and for L5178Y strain (page 100) Respiration after culture in conditioned medium was similar in both cell types to that found after culture in fresh medium. These cells do not seem therefore to leak citric acid cycle intermediates into the medium.

These two experiments on the influence of culture medium on Cl3 and TCll cells were not carried out simultaneously and the state of the cells prior to the experiments, particularly the pH of the stock cultures, is not known. This factor may account for the difference in respiratory rate found between the Cl3 and TCll cells. Such high respiration of the TCll cells was never repeated.

(2) Effect of carbon dioxide tension.

It was previously found, page106, that the respiratory

T A B L E 15

INFLUENCE OF MEDIUM ON RESPIRATION OF C13 AND TC11 CELLS.

Cell Strain	Oxygen Uptake $\mu l\ O_2$/hour/cell x 10^6		
	Fresh Medium	Conditioned Medium	Fresh Medium + Solution A.
C13	5.5	5.75	5.6
TC11	8.2	8.1	9.0

Cells were grown in test media for 48 hours.

Conditioned medium - medium in which cells had been grown for 4 days with glucose concentration and pH adjusted.

Solution A - citric acid cycle intermediates, (page 228).

rate of all cell strains studied was highest when respiration was measured in a gas phase of 1 per cent. carbon dioxide in air. Similar experiments were carried out on the Cl3 and TCll cells and in these cells also respiration was higher when measured in 1 per cent. carbon dioxide in air than it was in air from which carbon dioxide had been removed or in air/5 per cent. carbon dioxide (Fig. 11).

The details of these experiments, which were not carried out on the Cl3 and TCll cells simultaneously, are given on page 106.

(3) Effect of oxygen tension.

Sets of cultures were set up as in previous experiments (page 98) in medium which had been equilibrated with -

(a) 95 per cent. nitrogen + 5 per cent. carbon dioxide.

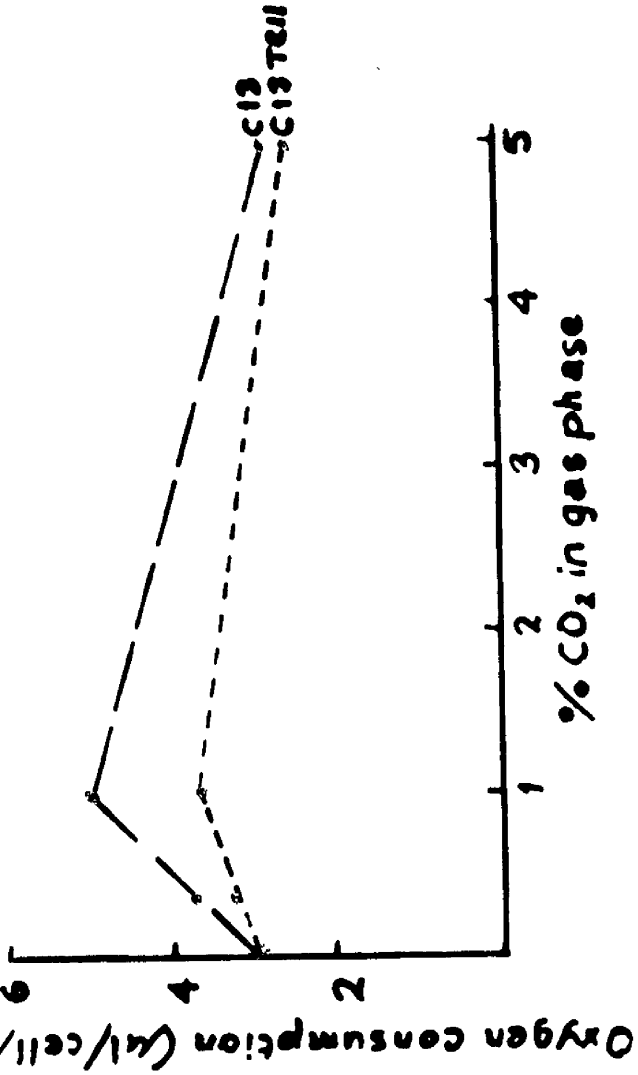
(b) air/5 per cent. carbon dioxide.

(c) 95 per cent. oxygen + 5 per cent. carbon dioxide,

and the cultures were gassed with the appropriate gas mixture. After 24 hours, medium was renewed and the cultures re-gassed. After 48 hours the respiration of the cells grown under these conditions was measured in air/5 per cent. carbon dioxide.

Respiration was higher in air/5 per cent. carbon

FIG. 11.


C13
C13 Tell
1
2
3
4
5
% CO_2 in gas phase
6
4
2
Oxygen consumption (μl/cell/hr x 10^6)

dioxide than in either 95 per cent. nitrogen or 95 per cent. oxygen and 5 per cent. carbon dioxide (Table 16) as found previously with other cell strains (page 103).

(4) Effect of glucose concentration.

C13 and TC11 cells were suspended in Tris/citrate buffered BSS containing glucose in a concentration range of 0 to 88 mM, in the case of the TC11 cells, and 0 to 22 mM. for C13 cells, and respiration measured.

Both the 'normal' and 'transformed' cell type (Fig 12) exhibit a Crabtree effect of decreased respiration caused by increased glucose concentration, after an initial rise in respiration, which may be due to the production of substrates for the citric acid cycle from glucose.

From these experiments it seemed that the two cell types, 'normal' C13 cells and 'transformed' TC11 cells, showed the same general picture of respiration, a pattern compatible with that found for the other cell strains, as judged by the effect of culture conditions on respiratory rates.

The main interest, however, in these two cell types was a comparison of respiration under standard conditions. To this end respiration was measured in the three sets of cells described previously, i.e. C13 ('normal') and TC11 ('transformed'), A ('normal') and Y ('transformed'),

FIG.12.

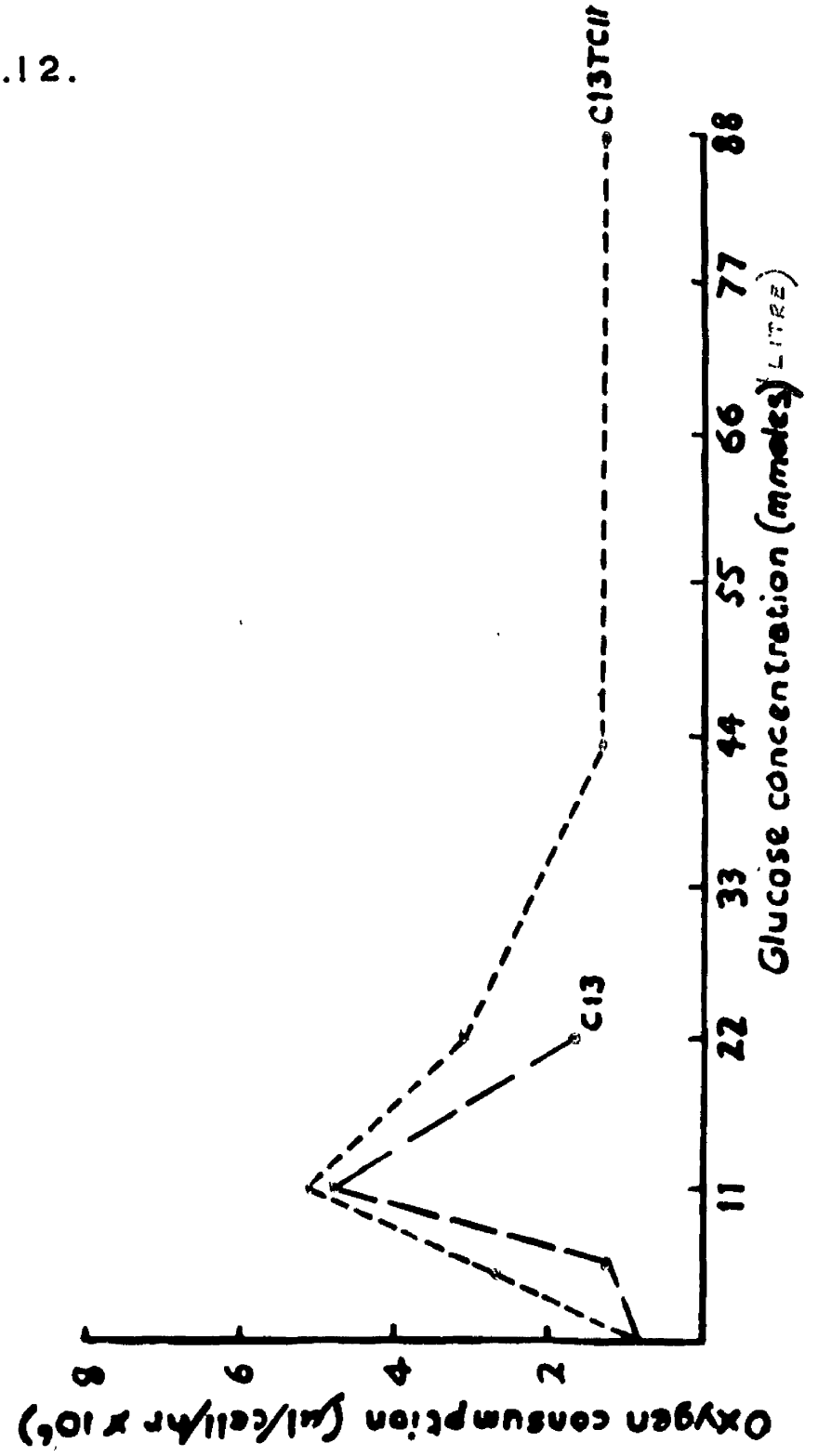

C13TCII
C13
Glucose concentration (mmoles/LITRE)
11
22
33
44
55
66
77
88
2
4
6
8
Oxygen consumption (µl/cell/hr x 10⁶)

TABLE 16

RESPIRATORY RATES OF C13 AND OF TC11 CELLS AFTER 48 HOURS' GROWTH IN VARIOUS OXYGEN TENSIONS

Cell Strain	Oxygen Uptake µl/hour/cell x 10^6		
	Percentage oxygen in Gas Phase		
	0	20	95
C13	2.02	4.1	-
TC11	2.1	3.9	2.4

C ('normal') and Z ('transformed').

Procedure.

Stock cultures were fed and after 24 hours trypsinised (page 49). Test cultures were set up in 4 oz. bottles, containing 10^5 cells per ml. in 10 ml. Eagles and 10 per cent. calf serum + 0.1 per cent. tryptose (page 49) buffered to pH 7.4 with sodium bicarbonate and 5 per cent. carbon dioxide in air (page 47). The cultures were fed and gassed after 24 hours and the cells scraped off the glass after 48 hours. A sample was counted using a haemocytometer slide (page 51) and a sufficient number of cells removed for respiration measurements. The remainder of the cells were analysed for DNA (page 52).

Respiration was measured in Tris/citrate buffered BSS (page 219) with 11 mM. glucose and sufficient sodium bicarbonate was added to give a pH of 7.4 when the medium was equilibrated with 1 per cent. carbon dioxide in air.

The respiratory rates under these conditions are shown in Tables 17, 18, and 19. There was no significant difference in respiration between the 'normal' and 'transformed' cells when respiration was expressed in terms of cell number but when the basis of reference was DNA content the Z ('transformed') cell exhibited lower respiratory rates than C ('normal') cells (Table 19). This can be explained by the hyperploid nature of the Z cell (page 137).

T A B L E 17

RESPIRATORY RATE OF C13 AND TC11 STRAIN CELLS.

Cell Strain	Oxygen consumption	
	µl O_2/hr/cell x 10^6	µl O_2/hr/µg DNA-P
C13	3.0 2.14 4.84 3.54 1.25 2.17 3.46 4.8 5.0	4.9 2.02 5.2 2.3 1.87 1.55 2.74 4.5 4.5
Mean ± St.E.	3.34 ± 0.42	3.29 ± 0.43
TC11	1.74 2.55 2.74 2.99 3.85 6.0 3.9	1.29 1.58 3.1 5.98 4.65 4.4 7.8
Mean ± St. E.	3.4 ± 0.48	4.11 ± 0.79.
	N.S. (P > 10%)	N.S. (P > 10%)

T A B L E 18

RESPIRATORY RATE OF C13A and C13Y STRAIN CELLS.

Cell Strain	Oxygen consumption	
	µl O_2/hr/cell x 10^6	µl O_2/hr/µg DNA-P
C13A	1.8	1.28
	2.4	1.3
	1.23	1.46
	1.39	1.58
	1.43	1.72
	1.17	1.59
	1.2	1.0
Mean ± St.E.	1.52 ± 0.12.	1.42 ± 0.08.
C13Y	1.27	0.96
	1.22	1.02
	0.917	1.87
	0.99	2.07
	1.3	1.67
	1.24	1.41
	1.27	1.73
Mean ± St.E.	1.17 ± 0.06	1.53 ± 0.15.
	10% > P > 5%	N.S. P ≫ 10%

T A B L E 19

RESPIRATORY RATE OF C13C and C13Z STRAIN CELLS.

Cell Type	$\mu l\ O_2/hr/cell \times 10^6$	$\mu l\ O_2/hr/\mu g$ DNA-P
C13C	2.38	2.11
	2.88	2.42
Mean	2.63	2.26
C13Z	2.94	1.54
	2.77	1.48
Mean	2.85	1.51

4. Glycolysis Studies on Strain BHk21.

Warburg and his colleagues (Warburg, 1930) observed that tumour tissues almost invariably had a higher rate of aerobic glycolysis than normal cells. They attributed this to an irreversible lesion in the respiratory pathway in the malignant cell.

Since then it has been found that carbohydrate metabolism may be profoundly altered by environmental conditions. For instance, Paul and Pearson (1957) showed that carbohydrate metabolism of chick embryonic liver changed from an aerobic metabolism to a glycolytic pattern within a few hours after explantation. After some days glycolysis decreased and respiration increased in these cultures. Harris (1957, 1958) found that metabolism in culture of freshly disaggregated tissue was glycolytic in type during rapid growth and became aerobic as stationary phase was reached. This phenomenon has been found also in cell strains (Paul, 1959; Munyon and Merchant, 1959). A change of pH in the cultures may partially explain these findings. Thus Zwartouw and Westwood (1958) showed that a much larger amount of glucose is converted to lactic acid and pyruvic acid in alkaline conditions.

More glucose is utilised by cells growing in anaerobic conditions than in aerobic. The first

description of this was made by Pasteur (1879), working with a yeast capable of both aerobic and anaerobic existence. Numerous studies have since established that respiratory inhibition of glycolysis is a constant occurence in cells capable of energy production by both these pathways.

Thus, apart from the necessity for a suitable system inwhich the glycolysis of malignant cells is being compared with that of normal cells of origin, it is also essential to adhere to standard conditions of pH, oxygen tension, time of incubation and growth phase of the cells.

In these experiments glycolysis was measured by estimating the quantity of glucose utilised by a number of cells in a given time and the amounts of lactic and pyruvic acids liberated.

Since glycolysis varies with oxygen tension and pH, a comparison of glycolysis in 'normal' and 'transformed' strains of BHk21 cells was carried out at two oxygen tensions and two pH's in the four possible combinations.

Procedure.

The cell types used were the 'normal' and 'transformed' baby hamster kidney fibroblast BHk21 strain (page 107). The medium was Eagles (1955) with 10 per

107

cent calf serum and 0.1 per cent. tryptose (page 49) in Tris/citrate buffered BSS (page 219) with 11 mM glucose. The medium was equilibrated with air/5 per cent. carbon dioxide or nitrogen/5 per cent. carbon dioxide to two pH's. Thus four test media were used:

1. pH 6.9 equilibrated with air/5 per cent. CO_2.
2. pH 6.9 " " nitrogen/5 per cent. CO_2.
3. pH 7.4 " " air/5 per cent. CO_2.
4. pH 7.4 " " nitrogen/5 per cent. CO_2.

Preparation of media: Medium (Eagles and calf serum and tryptoso) was prepared at twice the final concentration and mixed in equal proportions with BSS buffered with Tris/citrate and with sufficient sodium bicarbonate or hydrochloric acid to give the appropriate pH when the medium was equilibrated with air or nitrogen containing 5 per cent. CO_2. (Table 20).

Rapidly growing cells were trypsinised and suspended in test media and an inoculum of 2×10^6 cells in 2 ml. medium set up in 25 ml. conical flasks. The cultures were gassed with the appropriate gas mixture and were maintained in a shaking water bath at 37°C. for 17 hours. The suspension was then centrifuged, and the cell-free medium was deproteinised (pages 54, 55). Initial samples of the media were also assayed. After deproteinisation the media were stored at -10°C. until

T A B L E 20

BUFFERED BSS USED IN GLYCOLYSIS EXPERIMENTS.

	pH 6.9	pH 7.4
Tris/citrate buffered BSS	100 ml.	100 ml.
6.6 per cent. $NaHCO_3$	-	4.8 ml.
0.5NHCl.	1 ml.	-
4.0 M NaCl.	7.2 ml.	4 ml.
Distilled water	1.6 ml.	1 ml.

estimations of glucose, lactic acid and keto acids were performed (pages 54-58).

Results.

Tables 21, 22, and 23 show the glucose utilised and the lactic and pyruvic acid produced by three BHk21 cell strains which had been 'transformed' by polyoma virus (namely, C13TC11, C13Y, C13Z strains) each compared with its 'normal' untransformed counterpart (C13, C13A and C13C respectively).

There seems no doubt that glycolysis was much higher in the 'transformed' cell than in the 'normal' cell at either pH 6.9 or 7.4 under aerobic or anaerobic conditions, as judged by the increased utilisation of glucose and production of lactic and pyruvic acid. These results confirm Warburg's views on glycolysis in tumour cells, though from previous results of respiratory measurements there is no evidence of the impaired respiration which he suggested was the cause of the high glycolysis.

In all cell strains, glycolysis was higher at pH 7.4 than at 6.9, as had been described by Zwartouw and Westwood (1958), and Paul (1959).

TABLE 21: GLUCOSE UTILISATION, LACTIC AND PYRUVIC ACIDS PRODUCED BY C13 AND TC11 STRAIN CELLS DURING 17 HOURS.

Cell Strain	Conditions of culture	Glucose utilised µmoles/10^6 cells		Lactic Acid produced µmoles/10^6 cells		Pyruvic Acid produced µmoles/10^6 cells.	
		No. of Observ-ations	Mean ± St.E.	No. of Observ-ations	Mean ± St.E.	No. of Observ-ations	Mean ± St.E.
C13	pH 6.9 Air/5% CO_2	4	0.48 ± 0.03	4	1.01 ± 0.16	4	0.68 ± 0.02
	pH 6.9 Nitrogen/ 5% CO_2	4	0.94 ± 0.033	4	2.10 ± 0.4	4	0.83 ± 0.019
	pH 7.4 Air/5% CO_2	4	2.57 ± 0.049	4	6.36 ± 0.96	4	1.94 ± 0.05
	pH 7.4 Nitrogen/ 5% CO_2	4	2.93 ± 0.026	4	6.26 ± 0.97	4	1.98 ± 0.01
TC11	pH 6.9 Air/5% CO_2	4	0.81 ± 0.06	3	0.099 ± 0.02	3	0.22 ± 0.005
	pH 6.9 Nitrogen/ 5% CO_2	4	1.05 ± 0.078	4	0.37 ± 0.03	4	0.28 ± 0.005
	pH 7.4 Air/5% CO_2	4	4.68 ± 0.18	4	7.87 ± 0.23	4	1.55 ± 0.03
	pH 7.4 Nitrogen/ 5% CO_2	4	5.52 ± 0.12	4	13.33 ± 0.78	4	1.50 ± 0.03

TABLE 22: GLUCOSE UTILISED AND LACTIC AND PYRUVIC ACIDS PRODUCED BY A AND Y STRAIN CELLS DURING 17 HOURS.

Cell Strain	Conditions of culture	Glucose utilised µmoles/10^6 cells		Lactic Acid produced µmoles/10^6 cells		Pyruvic Acid produced µmoles/10^6 cells.	
		No. of Observ-ations	Mean + St.E.	No. of Observ-ations	Mean + St.E.	No. of Observ-ations	Mean + St.E.
A	pH 6.9 Air/5% CO_2	12	0.77 ± 0.078	12	2.26 ± 0.144	12	1.03 ± 0.263
	pH 6.9 Nitrogen/ 5% CO_2	12	0.70 ± 0.081	12	2.82 ± 0.231	12	1.32 ± 0.257
	pH 7.4 Air/5% CO_2	12	1.50 ± 0.098	11	4.05 ± 0.286	10	2.39 ± 0.332
	pH 7.4 Nitrogen/ 5% CO_2	12	1.65 ± 0.104	11	4.66 ± 0.238	11	2.43 ± 0.308
Y	pH 6.9 Air/5% CO_2	12	1.31 ± 0.098	12	2.64 ± 0.274	12	2.79 ± 0.517
	pH 6.9 Nitrogen/ 5% CO_2	11	1.36 ± 0.163	11	3.03 ± 0.244	11	2.50 ± 0.485
	pH 7.4 Air/5% CO_2	12	2.75 ± 0.153	11	5.24 ± 0.564	12	8.61 ± 0.202
	pH 7.4 Nitrogen/ 5% CO_2	11	2.79 ± 0.099	11	4.96 ± 0.510	10	7.84 ± 0.234

TABLE 23: GLUCOSE UTILISED AND LACTIC AND PYRUVIC ACIDS PRODUCED BY C AND Z STRAIN CELLS DURING 17 HOURS.

Cell Strain	Conditions of culture	Glucose utilised µmoles/10^6 cells		Lactic Acid produced µmoles/10^6 cells		Pyruvic Acid produced µmoles/10^6 cells	
		No. of Observ-ations.	Mean ± St.E.	No. of Observ-ations	Mean ± St.E.	No. of Observ-ations	Mean ± St.E.
C	pH 6.9 Air/5% CO_2	4	0.48 ± 0.045	4	0.45 ± 0.07	4	0.42 ± 0.028
	pH 6.9 Nitrogen/ 5% CO_2	4	1.09 ± 0.049	4	1.49 ± 0.15	4	0.73 ± 0.028
	pH 7.4 Air/5% CO_2	4	1.14 ± 0.051	4	1.75 ± 0.055	4	0.41 ± 0.002
	pH 7.4 Nitrogen/ 5% CO_2	4	1.59 ± 0.08	4	2.19 ± 0.04	4	0.85 ± 0.038
Z	pH 6.9 Air/5% CO_2	4	2.00 ± 0.1	4	2.92 ± 0.205	4	0.32 ± 0.03
	pH 6.9 Nitrogen/ 5% CO_2	4	2.42 ± 0.08	4	3.25 ± 0.335	4	0.40 ± 0.03
	pH 7.4 Air/5% CO_2	3	3.04 ± 0.025	4	5.26 ± 0.185	4	0.68 ± 0.02
	pH 7.4 Nitrogen/ 5% CO_2	4	3.55 ± 0.19	4	5.61 ± 0.165	4	0.78 ± 0.023

5. Enzyme Studies.

Evidence has been presented showing that glycolysis occurred to a much greater extent in BHK21 cells which had been 'transformed' by polyoma virus than in untransformed cells, while respiration was similar in both. Attempts were therefore made to determine the nature of the differences in carbohydrate metabolism between the two types of cell. To this end assays were made of the activity of some of the enzymes concerned.

The following enzymes were assayed:

1. Hexokinase.
2. Glucose-6-Phosphatase.
3. Lactic Dehydrogenase.
4. Isocitric Dehydrogenase.
5. Malic Dehydrogenase.
6. Glucose-6-Phosphate Dehydrogenase.
7. 6-Phosphogluconate Dehydrogenase.

The question arises as to the best basis for expression of enzyme activities - cell number, DNA content, or protein content.

The estimation of enzyme activity on a basis of cell number does not take into account the synthesis of cell material during growth and division. It would therefore be more enlightening to base activity on the quantity of

some cellular constituent. DNA content of cells is often used since the DNA content of the resting cell is a constant and increased DNA content reflects growth of the population. Enzyme activities may also vary with the nutritional state of the cell (Freshney, 1964), as does protein content of the cells as a whole. Enzyme activities based on protein content of cells should therefore give more constant values over a number of experimental determinations. However, it is feasible that different enzymes may be affected to varying degrees by nutritional factors.

In this study enzyme activities have been based on all three quantities:- cell number, DNA content, protein content. It would seem that any significant differences between the 'normal' and 'transformed' cells are apparent no matter ON which of the three quantities the enzyme activities are based. With the C and Z set of cells in particular however, small differences in activity of an enzyme based on cell number were completely removed on a basis of DNA content due to the hyperploidy of the Z cell.

Expression of enzyme activity.

The activities of enzymes assayed by chemical methods (lactic dehydrogenase, glucose-6-phosphatase and hexokinase) were expressed as μmoles of substrate used or product formed per minute. With the enzymes which were assayed by the change in reduced or oxidised form of a co-factor

the following system was used.

A quantity of cell extract was used, such that a linear graph of % transmission against time was obtained. Using this graph, the change in optical density per minute was calculated from the time, in minutes, required to reduce the % transmission from 95 to 85. The increase in optical density in one minute was converted to μmoles of NADP reduced per minute using the molecular absorbancy for NADP = 6.22×10^6/mole (Horecker and Kornberg, 1948).

Since the cell number, DNA and protein contents per cell were known, units of enzyme activities were based on all these quantities.

The differences in enzyme activities between the two types of cell ('normal' and 'transformed') were calculated for each experiment and these values for a number of experiments were tested for statistical significance, using Students t-test.

Experimental Procedure.

In each experiment a 'transformed' subline was compared with its 'normal' counterpart, e.g. C13 with TC11, A with Y cells, and O with Z cells.

Roux flask cultures were set up with 5×10^6 cells in 50 mls. Eagles medium (Eagles, 1955) plus 10 per cent. calf serum and 0.1 per cent. tryptose in Tris/citrate buffered salt solution (page 219). The pH of the cultures

was 7.4 and the gas phase was air. The cultures were fed on the third day and on the seventh day the cells were removed from the glass by treatment with trypsin in citrate (page 49). The cells were suspended in BSS (without phenol red) and a sample of this suspension was stained with crystal violet and counted in a haemocytometer slide (page 51). In some cases the cells were also tested for viability with nigrosin stain (page 51).

The cells were spun down at 5,000 *g* for 5 minutes and washed with BSS (- phenol red). They were then suspended in 0.9 per cent. sodium chloride (w/v) at a concentration of 10^8 cells per ml.

A cell extract was prepared by rapidly freezing the suspension in a solid carbon dioxide/absolute ethanol mixture and thawing rapidly in 37°C. water. This extraction was performed three times. The extracted sample was then centrifuged at 2,000 *g* for one hour at 2°C. and the supernatant stored in an ice/water mixture until used. The enzymes were assayed as soon as possible after extraction and always on the same day, to avoid any possible loss of enzyme activity with time.

Protein in the extract was determined by the Nessler method (page 53) and DNA-P in the residue by the method of Ceriotti (page 52).

The DNA and protein contents per cell found for these three sets of cell lines are given in Table 24.

TABLE 24.

COLLECTED VALUES OF DNA-P CONTENT AND PROTEIN CONTENT.

	Cell Type	No. of Observ-ations	Mean Value, $\mu g/10^6$ Cells	St. E.	% P
DNA-P	C13	7	1.49	0.10	> 10
	TC11	6	1.38	0.22	n.s.
	A	18	1.34	0.11	> 10
	Y	19	1.59	0.10	n.s.
	C	11	1.05	0.08	
	Z	10	1.74	0.21	0.5 > P > 0.1
Protein	C13	7	100.0	17.7	> 10
	TC11	6	114.0	26.9	n.s.
	A	18	70.0	7.5	
	Y	19	112.0	9.9	0.5 > P > 0.1
	C	8	93.0	16.2	
	Z	8	191.0	20.1	0.5 > P > 0.1

DNA.

The DNA content of C13 and TC11 cells, and of A and Y cells, is not significantly different, but Z cells contain much more DNA than the corresponding 'normal' cells - C cells.

A higher DNA content has been shown in many tumours but this is not obligatory for malignancy since many tumours have a normal DNA content per cell (page 6). Another possible reason for the high DNA content of the Z cells is the observed increase in DNA in cells which have been grown in culture for some time. There is evidence that cells maintained for long periods in tissue culture tend to develop chromosomal abnormalities and that after some generations in vitro tri- and tetraploid cells predominate (Levan and Biesele, 1958; Hsu and Klatt, 1959). This chromosomal variation has been thought to be correlated with the onset of malignancy (Levan and Hauschka, 1953; Levan and Biesele, 1958). Hsu and Klatt (1959) have found however that chromosomal variation in the Novikoff hepatoma was actually associated with loss of malignancy.

Protein.

The protein content per cell varies with the nutritional state of the cell, being lower in starved cells (Froshney, 1964). These determinations were therefore

carried out on cells which would be expected to be in the same nutritional state. Protein values for all six strains of cells showed that the C13, TC11, Y and C strains contain similar amounts of protein per cell; the A cells have less, and the Z cells much more protein per cell.

Taking into consideration the DNA results, it would seem that the Z cells are hyperploid and much larger cells altogether.

Results.

Hexokinase. Tables 25, 26, 27 and 28 show a significantly higher hexokinase activity in the 'transformed' cell strains TC11, Y and Z than in the 'normal' strains C13, A and C, when activities are based on cell number. The TC11 and Z strain cells exhibit at least twice the activity found in the C13 and C cells, both strains of 'transformed' cells having activities of about 0.5 to 0.6 x 10^{-2} μmole NADP/minute/10^6 cells, while activity in the 'normal' strains falls in the range 0.2 to 0.3 x 10^{-2}. When these values are based on the content of DNA and protein in the cells, the 'transformed' cells still show an increased hexokinase activity, although the differences are smaller.

The 'normal' strain A cell has a much higher activity than the other two 'normal' strains - about

TABLE 25.

ACTIVITY OF HEXOKINASE IN C13 AND TC11 STRAIN CELLS, EXPRESSED AS µmoles GLUCOSE UTILISED PER MINUTE/MILLION CELLS; PER MINUTE/µg PROTEIN; AND PER MINUTE/µg DNA-P.

µmoles Glucose x 10^2/min/cell x 10^6		µmoles Glucose x 10^4/min/µg Protein		µmoles Glucose x 10^2/min/µg DNA-P	
C13	TC11	C13	TC11	C13	TC11
0.372	0.678	0.283	0.588	0.375	0.650
0.286	0.557	0.319	0.756	0.322	0.638
0.431	0.538	0.224	0.254	0.283	0.280
0.515	0.627	0.350	0.297	0.305	0.325
0.223	0.683	0.392	1.010	0.182	0.829
0.290	0.594	0.479	0.913	0.183	1.607
0.274	0.526	0.451	0.963	0.192	2.727
$P < 0.1\%$		$P = 1\%$		$10\% > P > 5\%$	

TABLE 26

ACTIVITY OF HEXOKINASE IN A AND Y STRAIN CELLS, EXPRESSED AS μmoles OF GLUCOSE UTILISED PER MINUTE/ MILLION CELLS; PER MINUTE/μg PROTEIN; AND PER MINUTE/μg DNA-P.

μmoles Glucose utilised x 10^2/min/cell x 10^6		μmoles Glucose x 10^4/min/μg Protein		μmoles Glucose x 10^2/min/μg DNA-P	
A	Y	A	Y	A	Y
0.689	0.829	0.468	0.437	0.434	0.294
0.515	0.700	0.851	0.672	0.300	0.224
0.750	0.935	0.493	0.437	0.297	0.330
0.627	0.773	0.885	0.375	0.381	0.342
0.566	0.918	0.510	0.683	0.255	0.328
0.364	0.655	0.588	0.571	0.333	0.304
0.302	0.694	0.498	0.596	-	-
P < 0.1%		P > 10% n.s.		P > 10% n.s.	

TABLE 27.

ACTIVITY OF HEXOKINASE IN A AND Y STRAIN CELLS, DETERMINED BY THE METHOD OF DiPIETRO AND WEINHOUSE (1960), EXPRESSED AS µmoles NADP REDUCED PER MINUTE/MILLION CELLS; PER MINUTE/µg PROTEIN; AND PER MINUTE/µg DNA-P.

µmoles NADP $\times 10^2$/min/cell $\times 10^6$		µmoles NADP $\times 10^4$/min/µg Protein		µmoles NADP $\times 10^2$/min/µg DNA-P	
A	Y	A	Y	A	Y
0.896	1.051	1.49	1.33	0.824	0.992
0.864	1.056	1.62	1.25	0.760	0.976
0.864	1.080	1.44	1.22	0.739	1.120
0.912	1.192	1.82	1.76	0.843	1.288
0.984	1.200	1.95	1.57	0.968	1.392
0.672	0.792	0.944	0.75	0.502	0.776
0.634	0.864	0.944	0.94	0.565	0.784
0.704	0.976	1.20	0.93	0.720	0.811
$P < 0.1\%$		$5\% > P > 2.5\%$		$0.5\% > P > 0.1\%$	

TABLE 28.

ACTIVITY OF HEXOKINASE IN C AND Z STRAIN CELLS, EXPRESSED AS µmoles GLUCOSE UTILISED PER MINUTE /MILLION CELLS; PER MINUTE/µg PROTEIN; AND PER MINUTE/µg DNA-P.

µmoles Glucose utilised x 10^2/min/cell x 10^6		µmoles Glucose x 10^4/min/µg Protein		µmoles Glucose x 10^2/min/µg DNA-P	
C	Z	C	Z	C	Z
0.263	0.689	0.174	0.426	0.255	0.283
0.249	0.616	0.179	0.322	0.174	0.266
0.286	0.728	0.179	0.372	0.180	0.300
0.189	0.302	0.232	0.272		
0.218	0.756	0.361	0.386	-	-
0.269	0.302				
0.085	0.414	-	-	-	-
0.073	0.459				
P < 0.1%		2.5% > P > 1%		0.5% > P > 0.1%	

0.6 to 0.8 units, and the corresponding 'transformed' strain Y cell about 0.8 to 1 units. As determined by Eagle's method, this increase in hexokinase activity is statistically significant when units of activity are expressed in terms of cell number but no significant difference is observed when units are based on DNA or protein content. When hexokinase activity is measured by the more sensitive method of DiPietro and Weinhouse, hexokinase activity in the Y cell is significantly higher when units are based on cell number or DNA content, but lower when based on protein content.

The strains A and Y set of cells showed several differences in activities of enzymes from the other two sets of strains. In several cases an increased enzyme activity in one strain of the set, based on cell number (and similar to increases found in the comparable strains of the other two sets of cells) disappears when activity is based on DNA or protein content (e.g. isocitric dehydrogenase, p.52).

Apart from this abnormality of the A/Y set of cells which cannot be explained by significantly different DNA and protein content per cell, it is apparent that hexokinase is higher in the 'transformed' cell than in the 'normal' cell, particularly when units are based on cell number. An increased hexokinase activity was similarly

shown in tumours of the hamster cheek pouch (Scott et al., 1962). Beck (1958) and DiPietro et al. (1962) have shown in their systems that hexokinase is the rate-limiting enzyme in glycolysis. Thus a higher activity of hexokinase might be expected to be present in more actively glycolysing tissues.

Elwood et al. (1963), studying a number of hepatomas, found a wide range of glucose utilisation rates which were related to hexokinase activity levels and correlated with the growth rates. They found that, in a low glycolysing tumour, the Morris 5123 hepatoma, hexokinase activity was lower than in normal liver and that the addition of yeast hexokinase increased the lactic acid production.

In measurements of enzymic activities of cells in culture (HeLa strain) the rate-limiting enzyme appeared to be dependent on the type of serum used. Wu (1959) found phosphofructokinase rate-limiting when the cells were grown in horse serum (20 per cent.) while in horse serum (20 per cent.) plus human serum (10 per cent.) the reaction catalysed by glyceraldehyde -3-P dehydrogenase appeared to be rate-limiting. In the present experiments calf serum (10 per cent.) was used, ~~and under such conditions hexokinase would appear to be rate-limiting~~.

Glucose-6-Phosphatase. No glucose-6-phosphatase activity was found in these cell strains. A similar absence has been found by Porske et al. (1957) in Chang liver cells, and by Lieberman and Ove (1958b) in cultures of human appendix, liver, lung, and HeLa strain cells (carcinoma of the cervix, Gey et al. 1952). They found a marked similarity in the levels of the enzymes they studied and suggested that growth in vitro leads to a rapid, nongenetic loss of specialised functions.

Pitot (1960) showed glucose-6-phosphatase activity in only three of the ten rat hepatomas he studied, while Abraham et al. (1961) demonstrated the presence of the enzyme in normal liver but not in hepatoma.

Weber et al. (1961) on the other hand did not find activity in chorioallantoic membrane nor in a Rous virus induced sarcoma of the chorioallantoic membrane.

Lactic Dehydrogenase (Tables 29, 30). In the C13 and TC11 set of cells no difference in lactic dehydrogenase activity was found. The results for the other two sets of cells showed a similar picture except that in the C,Z set, when enzyme activity was based on DNA content, lactic dehydrogenase was significantly higher in the C cell. This result can be accounted for by the fact that the Z cells contain a much higher quantity of DNA per cell (page 137) and are probably tetraploid.

TABLE 29.

ACTIVITY OF LACTIC DEHYDROGENASE IN C13 AND TC11 STRAIN CELLS, EXPRESSED AS µmoles PYRUVIC ACID UTILISED PER MINUTE/MILLION CELLS; PER MINUTE/µg PROTEIN; and PER MINUTE/µg DNA-P.

µmoles Pyruvic Acid utilised x 10^{2}/min/cell x 10^{6}		µmoles Pyruvic Acid x 10^{4}/min/µg Protein		µmoles Pyruvic Acid x 10^{2}/min/µg DNA-P	
C13	TC11	C13	TC11	C13	TC11
0.519	0.442	-	-	-	-
0.463 0.554	0.593 0.599	0.240 0.376	0.282 0.283	0.304 0.318	0.309 0.312
0.650 0.650 0.673	0.770 0.644 0.690	0.457 0.507 0.457	0.553 0.487 0.429	0.529 - -	0.790 - -
P > 10% n.s.		P > 10% n.s.		P > 10% n.s.	

TABLE 30

ACTIVITY OF LACTIC DEHYDROGENASE OF A AND Y, AND C AND Z STRAIN CELLS, EXPRESSED AS µmoles PYRUVIC ACID UTILISED PER MINUTE/MILLION CELLS; PER MINUTE/µg PROTEIN; AND PER MINUTE/µg DNA-P.

µmoles Pyruvic Acid utilised x 10^2/min/cell x 10^6		µmoles Pyruvic Acid, x 10^4/min/µg Protein		µmoles Pyruvic Acid, x 10^2/min/µg DNA-P	
A	Y	A	Y	A	Y
0.656	0.715	-	-	-	-
0.752 0.758 0.750 0.764	0.720 0.707 0.724 0.707	-	-	-	-
P > 10% n.s.					

C	Z	C	Z	C	Z
0.437 0.466 0.486	0.524 0.604 0.463	0.290 0.335 0.305	0.324 0.316 0.237	0.424 0.326 0.308	0.215 0.148 0.189
P > 10% n.s.		P > 10% n.s.		2.5% > P > 1%	

Meister (1950) and Wenner et al. (1952) measured lactic dehydrogenase activity in a wide range of normal and cancer tissues and found no marked difference; while Kubowitz and Ott (1943) isolated crystalline lactic dehydrogenase from rat liver, and Jensen sarcoma, and found them qualitatively the same.

Weber and his colleagues have measured the activities of many of the enzymes involved in carbohydrate metabolism in a number of tumour systems - mainly comparing hepatomas and the corresponding normal livers. They found that lactic dehydrogenase was higher in Rous induced sarcoma than in the normal tissue (Weber et al., 1961a), but in hepatomas enzyme activity was much lower than in normal liver (Weber et al., 1961b; Weber and Cantero, 1959) - in a study of seven hepatomas they found a significant decrease in activity in six and an increase of lactic dehydrogenase in one.

Isocitric Dehydrogenase (Tables 31, 32, 33). A comparison of isocitric dehydrogenase activity in the 'normal' and 'transformed' cells of the three sets does not give a uniform picture. In the C13/TC11 set the 'transformed' cell (TC11) has a very significantly increased isocitric dehydrogenase activity when activity is expressed in terms of cell number, protein or DNA. With the C/Z set the same pattern emerges except that on a basis of DNA the

T A B L E 31.

ISOCITRIC DEHYDROGENASE ACTIVITY IN C13 AND TC11 CELLS; EXPRESSED AS µmoles NADP REDUCED/MINUTE/MILLION CELLS; PER MINUTE/µg PROTEIN; and / MINUTE/µg DNA-P.

µmoles NADP reduced/ min/cell x 10^6		µmoles NADP/ min/µg Protein		µmoles NADP/ min/µg DNA-P	
C13	TC11	C13	TC11	C13	TC11
0.748	0.981	0.0082	0.0133	0.653	1.626
0.585	0.820	0.0064	0.0110	0.520	1.088
1.801	2.926	0.0093	0.0138	1.176	1.512
1.961	3.023	0.0133	0.0141	1.160	1.560
0.751	1.399	0.0131	0.0205	0.608	1.677
0.547	1.479	0.0114	0.0224	-	-
0.769	1.158	0.0125	0.0211	-	-
0.5% > P > 0.1%		0.5% > P > 0.1%		1% > P > 0.5%	

T A B L E 32.

ISOCITRIC DEHYDROGENASE ACTIVITY IN A AND Y CELLS; EXPRESSED AS µmoles NADP REDUCED/MINUTE/MILLION CELLS; PER MINUTE/µg PROTEIN; and PER MINUTE/µg DNA-P.

µmoles NADP reduced/ min/cell x 10^6		µmoles NADP/ min/µg Protein		µmoles NADP/ min/µg DNA-P	
A	Y	A	Y	A	Y
1.897	1.198	-	-	-	-
1.624	1.182	-	-	-	-
1.720	1.744	0.0288	0.0144	2.288	1.528
1.320	1.568	0.0254	0.0163	0.992	1.504
1.367	2.192	0.0286	0.0176	1.104	1.264
1.246	1.056	-	-	0.912	0.560
1.069	1.056	-	-	0.856	0.667
0.740	1.440	0.0118	0.0126	1.152	1.504
1.013	1.440	0.0166	0.0125	1.224	1.176
1.945	2.176	-	-	2.304	2.272
2.042	2.208	-	-	2.160	2.224
1.833	2.192	-	-	1.872	2.368
1.833	2.048	-	-	2.144	2.128
0.375	0.483	0.0384	0.0384	0.342	0.459
0.402	0.630	0.0464	0.0461	0.350	0.584
0.342	0.598	0.0352	0.0416	0.291	0.621
0.381	0.602	0.0474	0.0560	0.350	0.648
0.498	0.666	0.0608	0.0544	0.451	0.774
0.315	0.406	-	-	-	-
0.318	0.474	-	-	-	-
0.286	0.446	-	-	-	-
5% > P > 2.5%		P > 10% n.s.		P > 10% n.s.	

T A B L E 33.

ISOCITRIC DEHYDROGENASE ACTIVITY IN C AND Z CELLS; EXPRESSED AS μmoles NADP REDUCED PER MINUTE/MILLION CELLS; PER MINUTE/μg PROTEIN; and PER MINUTE/μg DNA-P.

μmoles NADP reduced/ min/cell x 10^6		μmoles NADP/ min/μg Protein		μmoles NADP/ min/μg DNA-P	
C	Z	C	Z	C	Z
1.384	2.368	0.0091	0.0146	1.344	0.976
1.432	3.088	0.0104	0.0162	0.992	1.344
1.216	2.128	0.0077	0.0109	0.768	0.880
1.024	2.448	0.0125	0.0221	-	-
0.944	3.648	0.0155	0.0186	-	-
0.576	0.976	-	-	0.547	0.621
0.512	1.312	-	-	0.542	0.696
0.624	1.744	-	-	0.595	0.448
0.5% > P > 0.1%		2.5% > P > 0.1%		P > 10% n.s.	

increase in activity is not statistically significant. This can be explained by the polyploidy of the Z cell type (page 137). However, although the increase in isocitric dehydrogenase activity in the Y cell as compared with the A cell is significant when expressed in terms of cell number, there is no increase in activity in terms of DNA or protein.

Wenner et al. (1952) found comparable levels of isocitric dehydrogenase in tumours and normal tissues based on weight of acetone-dried powder.

Any increase in isocitric dehydrogenase activity in the 'transformed' cell however is not correlated with increased respiratory rate, suggesting that this enzyme is not rate-limiting in the Krebs cycle.

Malic Dehydrogenase (Tables 34, 35, 36). All three sets of cells showed no significant difference in malic dehydrogenase activity when expressed in terms of DNA or of protein. When the activity was measured on a cell number basis, however, the Y cells ('transformed') had a significantly higher activity than the A cells, but the other two sets of 'normal' and 'transformed' cells showed no difference in activity.

Potter (1946) found lower activity of malic dehydrogenase in a rat hepatoma than in normal liver, but Wenner et al. (1952), working on a number of normal and cancer tissues could find no appreciable difference.

T A B L E 34

MALIC DEHYDROGENASE ACTIVITY IN C13 AND TC11 STRAIN CELLS; EXPRESSED AS µmoles $NADH_2$ OXIDISED PER MINUTE/MILLION CELLS; PER MINUTE/µg PROTEIN; and PER MINUTE/µg DNA-P.

µmoles $NADH_2$/ min/cell x 10^6		µmoles $NADH_2$/ min/µg Protein		µmoles $NADH_2$/ min/µg DNA-P	
C13	TC11	C13	TC11	C13	TC11
42.88	33.76	0.474	0.456	-	-
64.00 61.12	44.00 42.56	0.331 0.416	0.208 0.202	42.08 36.48	22.88 22.24
75.20 55.36 58.88	66.40 72.80 53.60	1.320 1.152 0.976	0.984 1.112 0.984	61.12	80.00
34.56	34.56	-	-	-	-
P > 10% n.s.		P > 10% n.s.		P > 10% n.s.	

TABLE 35

MALIC DEHYDROGENASE ACTIVITY IN A AND Y STRAIN CELLS; EXPRESSED AS µmoles $NADH_2$ OXIDISED PER MINUTE/MILLION CELLS; PER MINUTE/µg PROTEIN; and PER MINUTE/µg DNA-P.

µmoles $NADH_2$/min/cell x 10^6		µmoles $NADH_2$/min/µg Protein		µmoles $NADH_2$/min/µg DNA-P	
A	Y	A	Y	A	Y
34.88	42.88	-	-	-	-
30.72	43.52	-	-	-	-
24.32	37.76	0.477	0.389	32.32	36.16
30.40	40.32	0.515	0.403	23.36	35.36
23.20	34.24	-	-	17.12	18.08
22.40	32.32	-	-	18.08	20.32
30.40	42.72	0.656	0.342	25.28	19.84
50.72	104.80	0.816	0.920	79.20	108.32
68.80	84.80	1.136	0.728	84.00	77.60
P < 0.1%		P > 10% n.s.		P > 10% n.s.	

T A B L E 36

MALIC DEHYDROGENASE ACTIVITY IN C AND Z STRAIN CELLS; EXPRESSED AS μmoles $NADH_2$ OXIDISED PER MINUTE/MILLION CELLS; PER MINUTE/μg PROTEIN; and PER MINUTE/μg DNA-P[1].

μmoles $NADH_2$/ min/cell x 10^6		μmoles $NADH_2$/ min/μg Protein		μmoles $NADH_2$/ min/μg DNA-P	
C	Z	C	Z	C	Z
31.04	35.04	0.205	0.216	30.08	14.32
32.32	50.88	0.232	0.267	22.72	22.08
29.12	48.96	0.182	0.251	18.40	20.00
100.00	109.44	1.224	0.984	-	-
75.20	152.80	1.240	0.768	-	-
28.16	26.40	-	-	26.88	16.80
26.08	42.88	-	-	27.52	22.72
10% > P > 5%		P > 10% n.s.		P > 10% n.s.	

Glucose-6-Phosphate Dehydrogenase (Tables 37, 38, 39). In all three sets of 'normal' and 'transformed' cells, the 'transformed' lines showed a statistically significant decrease in glucose-6-phosphate dehydrogenase, however the results were expressed.

Other published results of glucose-6-phosphate dehydrogenase activity in normal and neoplastic tissues have not reported this decrease. While Weber and his colleagues found similar values in normal and neoplastic tissue induced by Rous virus (Weber et al. 1961a) and in a hepatoma compared with normal liver (Weber et al., 1961b), they found in a study of seven hepatomas, glucose-6-phosphate dehydrogenase was significantly higher than the normal in six, and Scott et al. (1962) found a similar increase in a chemically induced tumour of the hamster cheek pouch.

Studying substantial numbers of normal and malignant tissues, Glock and McLean (1954) found similar values for glucose-6-phosphate dehydrogenase activity, though the range of values is considerable (8 to 730 units).

The decrease in this enzyme which is involved in the formation of pentose sugars from glucose via the hexose monophosphate shunt, was unexpected. However in this system both cell types are growing at the same rate and therefore no increase in pentose formation due to in-

T A B L E 37.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY IN C13 AND TC11 STRAIN CELLS, EXPRESSED AS µmoles NADP REDUCED PER MINUTE/ MILLION CELLS; PER MINUTE/µg PROTEIN; and PER MINUTE/ µg DNA-P.

µmoles NADP reduced/ min/cell x 10^6		µmoles NADP/ min/µg Protein		µmoles NADP/ min/µg DNA-P	
C13	TC11	C13	TC11	C13	TC11
3.616	2.496	0.0398	0.0336	3.168	3.280
8.416 7.440	6.272 4.320	0.0435 0.0506	0.0296 0.0205	5.536 4.416	3.264 2.256
6.224 6.208 6.400	2.768 3.616 3.584	0.0973 0.1296 0.1048	0.0408 0.0552 0.0656	5.056 – –	3.328 – –
16.000 15.360	6.672 5.184	– –	– –	12.304 11.808	8.560 6.720
2.320 2.320	2.160 2.560	– –	– –	– –	– –
2.5% > P > 1%		2.5% > P > 1%		2.5% > P > 1%	

TABLE 38.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY IN A AND Y CELLS, EXPRESSED AS μmoles NADP REDUCED PER MINUTE/MILLION CELLS; PER MINUTE/μg PROTEIN; and PER MINUTE/μg DNA-P.

μmoles NADP reduced/ min/cell x 10^6		μmoles NADP/ min/μg Protein		μmoles NADP/ min/μg DNA-P	
A	Y	A	Y	A	Y
2.432 2.192	2.352 2.656	-	-	-	-
2.032 2.048	2.416 2.832	0.0397 0.0349	0.0240 0.0291	2.704 1.576	2.112 2.704
1.432 1.272	1.232 1.328	-	-	1.056 1.027	0.651 0.840
2.400	3.280	0.0506	0.0262	1.952	1.872
3.328 2.880	2.752 2.848	0.0536 0.0477	0.0242 0.0245	5.200 3.520	2.864 2.336
7.328 6.848	4.384 4.736	-	-	6.662 6.976	5.280 5.728
7.152 7.136	4.736 5.152	-	-	4.504 8.256	7.104 6.128
7.360 7.200	5.152 4.992	-	-	8.763 7.664	5.392 5.024
7.296 6.144	4.896 5.600	-	-	7.488 7.232	5.696 5.440
2.736 2.880 2.944 2.480	2.256 2.000 2.480 2.640	-	-	1.914 2.144 2.624 2.528	1.888 1.952 2.256 2.192
0.5% > P > 0.1%		0.5% > P > 0.1%		P < 0.1%	

TABLE 39.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY IN C AND Z STRAIN CELLS, EXPRESSED AS µmoles NADP REDUCED PER MINUTE/MILLION CELLS; PER MINUTE/µg PROTEIN; and PER MINUTE/µg DNA-P.

µmoles NADP reduced/min/cell x 10^6		µmoles NADP/min/µg Protein		µmoles NADP/min/µg DNA-P	
C	Z	C	Z	C	Z
7.440	4.800	0.0490	0.0296	7.200	2.368
9.280	5.760	0.0579	0.0301	6.480	2.512
12.720	8.080	0.1568	0.0728	14.400	13.200
9.280	5.600	0.1552	0.0573	10.560	16.800
10.400	7.360	-	-	9.872	4.672
9.200	8.480	-	-	9.760	4.512
9.712	6.624	-	-	12.320	3.408
P < 0.1%		5% > P > 2.5%		P > 10% n.s.	

creased nucleic acid synthesis in the tumour cell should be expected. The activity of glucose-6-phosphate dehydrogenase in the tumour cell must be sufficient for this purpose.

If this is true, to what purpose is the excess activity *via* the hexose-monophosphate shunt put in the normal cell? Possible explanations and the significance of these in carcinogenesis will be presented later.

6-Phosphogluconate Dehydrogenase (Tables 40, 41, 42).

Very little difference in 6-phosphogluconate dehydrogenase activity was found between the 'normal' cells and the 'transformed' cells.

Glock and McLean (1954) found no differences when they compared the activity of this enzyme in normal and tumour tissues of rats and mice. The range of activities in this series was from 63 to 350 units and the large scatter of values for the normal and the tumour groups made comparisons difficult. The present system however eliminated this problem and shows clearly the similarity in 6-phosphogluconate dehydrogenase activity.

TABLE 40.

6-PHOSPHOGLUCONATE DEHYDROGENASE ACTIVITY IN C13 AND TC11 CELLS, EXPRESSED AS µmoles NADP REDUCED PER MINUTE/MILLION CELLS; PER MINUTE/µg PROTEIN; and PER MINUTE/µg DNA-P.

umoles NADP reduced/ min/cell x 10^6		µmoles NADP/ min/µg Protein		µmoles NADP/ min/µg DNA-P	
C13	TC11	C13	TC11	C13	TC11
2.256	1.800	0.0248	0.0242	1.984	2.368
4.704	5.728	0.0243	0.0270	3.088	2.976
4.416	6.256	0.0301	0.0296	2.624	3.328
2.544	3.216	0.0446	0.0483	2.064	3.984
2.656	3.282	0.0554	0.0493	-	-
2.632	2.672	0.0595	0.0490	-	-
4.912	3.088	-	-	-	-
5.200	3.264	-	-	-	-
P > 10% n.s.		P > 10% n.s.		P > 10% n.s.	

TABLE 41.

6-PHOSPHOGLUCONATE DEHYDROGENASE ACTIVITY IN A AND Y CELLS, EXPRESSED AS µmoles NADP REDUCED PER MINUTE/MILLION CELLS; PER MINUTE/µg PROTEIN; and PER MINUTE/µg DNA-P.

µmoles NADP reduced/ min/cell x 10^6		µmoles NADP/ min/µg Protein		µmoles NADP/ min/µg DNA-P	
A	Y	A	Y	A	Y
2.680 2.816	1.920 2.224	- -	- -	1.968 2.256	1.016 1.408
3.488	5.168	0.0736	0.0413	2.832	2.384
2.464 3.088	3.680 3.424	0.0394 0.0509	0.0323 0.0296	3.840 3.792	3.824 3.856
P > 10% n.s.		P > 10% n.s.		P > 10% n.s.	

TABLE 42.

6-PHOSPHOGLUCONATE DEHYDROGENASE ACTIVITY IN C AND Z CELLS, EXPRESSED AS µmoles NADP REDUCED PER MINUTE/MILLION CELLS; PER MINUTE/µg PROTEIN; and PER MINUTE/µg DNA-P.

µmoles NADP reduced/ min/cell x 10^6		µmoles NADP/ min/µg Protein		µmoles NADP/ min/µg DNA-P	
C	Z	C	Z	C	Z
5.760	5.280	0.0379	0.0328	5.584	2.160
5.728	6.480	0.0413	0.0339	4.000	2.816
5.616	4.656	0.0352	0.0240	3.520	1.920
4.160	3.280	0.0509	0.0296	5.184	5.344
3.280	4.576	0.0544	0.0234	3.728	6.800
3.264	6.992	-	-	3.104	3.632
4.160	9.872	-	-	4.400	5.344
3.840	12.240	-	-	4.896	3.152
P > 10% n.s.		P > 10% n.s.		P > 10% n.s.	

D I S C U S S I O N.

1. Carbohydrate Metabolism in Malignant Cells.

(a) Warburg's Theory.

Warburg proposed that irreversible damage to the respiration of cells was the cause of the carcinogenic change. As a result of this lesion most of the cells died but some developed the ability to produce energy by increased glycolysis and so proliferated, as cancer cells (Warburg, 1930, 1956).

Numerous studies have been reported on this topic (Introduction, pages 35-38). Most of the evidence suggests that there is an alteration in carbohydrate metabolism in tumours, with a resultant high glycolytic rate. However, his theory of an impaired respiration as the causal factor has not been supported. In many of the studies on this topic tissues grown *in vivo* were used. Since environmental factors had been shown to affect the glycolytic rate of cells grown in culture (Paul, 1959; Zwartouw and Westwood, 1958) it was suggested that the high glycolytic rate found in tumours might be explained by their poor blood supply (Agol, 1960; Paul, 1961). Such a poor blood supply would result in a lowered oxygen tension, a condition under which glycolysis is increased *in vitro*.

In the system used in the present studies it is apparent that the glycolytic rate of the 'transformed',

tumour-producing cells was still much higher than that of the 'normal' cell in aerobic and anaerobic conditions (page 128). It is therefore improbable that the increased glycolysis of tumour tissues is primarily due to conditions imposed in vivo.

(b) System of Study.

Environmental factors have been shown to affect the glycolytic rate of normal and malignant cells in culture in a similar manner (Paul, unpublished). The respiratory rates of a number of cell strains have been studied. With minor deviations the effects of a number of environmental factors on the respiratory rate were found to be similar in normal and malignant cells. However the alterations in respiration rate which were produced were in some instances considerable. It was therefore apparent that any comparison of respiratory and glycolytic rates in normal and malignant cells must be carried out under strictly controlled conditions.

During the actual course of a biochemical experiment conditions usually are controlled, but the results of the above studies showed that the conditions in which the cells were maintained before the experiment were also important. Thus respiration of cells which had been grown for 72 hours in media of different pH's was considerably different, although all measurements of respiration were made at one pH (page 101).

The response of respiratory rate to glucose concentration in the environment differed between various cell strains studied (HeLa, HLM, Y5 and L5178Y, pages 104, 105). This difference could not be attributed to malignant as opposed to non-malignant cells. It was therefore important to study metabolism in malignant cells and comparable normal cells.

Thus the two essential conditions are the use (1) of strictly controlled environmental conditions; and (2) of malignant cells in comparison with their normal cells of origin. The only system available at present, which fulfils both these conditions, is tissue culture.

(c) Tissue Culture.

The use of tissue culture in the study of malignancy has often been criticised; all cultured cells are considered by some people to be malignant to some extent. It is true that cells grown *in vitro* may behave quantitatively differently from the tissues from which they were derived. Cells *in vivo* are normally maintained in a resting state by control mechanisms of the animal body, while still retaining their capacity to grow. *In vitro* the control mechanisms no longer exist and the cells grow to the limit of their capacity in a simple environment.

It is also true that cells grown in culture for

some time do show changes in chromosome content (Hauschka and Levan, 1958). Some cell strains have been shown by transplantation studies to have become malignant after growth *in vitro* (Gey, 1956). Other cell strains, however, seem to have lost their malignant capacities after culture for some time (Hsu and Klatt, 1959).

With regard to carbohydrate metabolism it has been stated that cultured cells always revert to a glycolytic type of carbohydrate metabolism. It is true that within a day or two of explantation the amount of lactic acid produced by tissue cultures increases greatly (Gershanovitch *et al.*, 1958; Paul and Pearson, 1957a,b; Suschny *et al.* 1958; Warburg *et al.* 1958) and that the respiration of some cultures may at the same time diminish.

The cells, of normal origin, which developed the capacity to produce tumours had almost invariably been grown in culture for very long periods of time. It is therefore more likely that the alteration in carbohydrate metabolism, which occurs within a short time after explantation, is due to change in the cell environment than to carcinogenesis, as Warburg proposed (Warburg, 1930).

Thus there are disadvantages in the use of tissue culture, particularly the somewhat artificial nature of the system and the occurence of malignant cells after generations *in vitro*. These must be taken into consider-

ation in assessing the results obtained. On the other hand differences in metabolism between normal and malignant cells in culture cannot be attributed to differences between resting and growing cells but may be characteristic of the cancer process.

Tissue culture offers a system in which cells, judged malignant or non-malignant on a basis of explantation into suitable animals, growing to the limit of their ability, can be studied in a controlled environment. With the possibility of transforming cells judged non-malignant on the above criterion into malignant cells in vitro, tissue culture also supplies a system in which malignant cells can be compared with the non-malignant cells from which they have arison.

2. Environmental Factors in Carbohydrate Metabolism.

(a) Pasteur effect.

The original studies on this phenomenon were carried out by Pasteur on yeasts capable of aerobic and anaerobic existence. He found that under anaerobic conditions much more sugar was consumed by the yeast than under aerobic conditions. The sugar however was utilised much less effectively, in the sense that the ratio of yeast formed to sugar utilised was much lower in anaerobic conditions than in aerobic conditions (Pasteur, 1879).

This respiratory inhibition of glycolysis has been shown to occur in most tissues studied since then. Although the phenomenon can be simply described, the mechanism by which the inhibition is produced remains obscure.

Pasteur effect in yeasts. Meyerhof (1925) demonstrated a Pasteur effect in yeasts with varying respiratory rates - from almost anaerobic brewer's yeast to almost completely aerobic wild yeast - and such yeasts have been used considerably in the study of the Pasteur effect.

Dinitrophenol, which uncouples oxidative phosphorylation, has been shown to release the Pasteur effect, but proprionitrile, which has no effect on respiration or on phosphorylation, also specifically inhibited the Pasteur effect (Strickland, 1956).

Lynen measured the levels of sugar phosphates and

adenine nucleotides during the transition from nitrogen to oxygen and found that, while the adenine nucleotide levels remained essentially unchanged, the level of glucose-6-phosphate rose rapidly during the transition and there was a corresponding fall in hexosediphosphate level (Lynen, 1958).

Lynen believes that the Pasteur effect is due to unavailability of ATP due to compartmentation within the cell. This theory visualises that the ATP produced during respiration is not available for glycolysis since the oxidative enzymes are found in the mitochondria while most of the glycolytic enzymes are located in the soluble fraction of the cytoplasm. Hexokinase, however, which catalyses the production of glucose-6-phosphate, is situated in the mitochondria and therefore the increased glucose-6-phosphate found by Lynen can be correlated with the theory of compartmentation (Lynen et al., 1959).

Pasteur effect in ascites cells. Racker and Wu studied the distribution of glycolytic enzymes in the mitochondrial and soluble cytoplasmic fractions of ascites cells. They found only one enzyme of glycolysis, hexokinase, present in the mitochondrial fraction (60 per cent. of the total hexokinase was in the mitochondria). The rate of glycolysis of extracts and homogenates supplemented with co-factors greatly exceeded that of the intact cell and

they therefore concluded that glycolysis in the intact cell was not limited by enzyme levels but by co-factors or intermediates. The addition of inorganic phosphate most markedly stimulated glycolysis in the intact cell. The intracellular level of ATP in those cells could be depleted by preincubation with dinitrophenol but under those conditions glycolysis continued at the maximal rate. They concluded therefore that glycolytically generated ATP was more effective in phosphorylating glucose than ATP formed by oxidative phosphorylation due to some type of compartmentation (Wu and Racker, 1959).

Particulate systems. A Pasteur effect has been demonstrated in a system in which glycolysis in a supernatant fraction of brain or tumour was inhibited by the addition of varying amounts of liver mitochondria. Glucose uptake and also lactate formation were inhibited. The inhibited glycolytic system had higher levels of glucose-6-phosphate and fructose-6-phosphate but lower levels of hexose diphosphate and glyceraldehyde-3-phosphate than did the uninhibited system, while the levels of adenine nucleotide were similar in both (Aisenberg, 1959; Aisenberg and Potter, 1957; Aisenberg et al., 1957). The results with this system agree with Lynen's findings.

In another particulate system purified glycolytic enzymes were added in correct amounts to mitochondria.

By limiting inorganic phosphate in the presence of added ATP (to maintain respiration and glycolysis) a Pasteur effect could be demonstrated. However the limiting level of inorganic phosphate was much lower than the levels found in the intact cell and it was therefore unlikely that such a mechanism operated in the intact cell (Gatt et al., 1956; Gatt and Racker, 1959). Chance has pointed out that, by manipulating levels of enzymes, nucleotides, and inorganic phosphates, it is possible that Pasteur effects are obtained which may bear no relationship to the mechanism within the cell (Chance and Hess, 1959).

Theories of the mechanism of the Pasteur effect. Engelhardt and Sakov (1943) suggested that the mechanism of the Pasteur effect was non-stoichiometric, i.e. that the presence of oxygen or respiration, rather than the amount of respiration was responsible for the inhibition of glycolysis. They proposed that, in the presence of oxygen, phosphohexokinase reaction was inhibited, tissue respiration occurring through the hexose monophosphate shunt.

The group of theories which are more widely held imply competition between respiration and glycolysis for a common metabolic intermediate. Ball et al. (1942) suggested that the common intermediate was NAD. This mechanism, which he proposed on theoretical grounds, has little experimental confirmation and the most favoured

intermediate is inorganic phosphate or phosphate acceptor (Lynen, 1941; Johnson, 1941).

However many of the results obtained were not consistent with the theory of a low availability of intracellular inorganic phosphate under aerobic conditions, e.g. Stickland showed that the level of inorganic phosphate was only 20 per cent. lower in aerobic conditions than in anaerobic conditions.

Racker, Lynen and Chance had suggested from their investigations that oxidatively generated ATP was somehow unavailable for glucose phosphorylation. The theory of compartmentation has thus evolved.

This theory supposes that there is some interaction between respiration and glycolysis involving the adenine nucleotide - inorganic phosphate system. Under aerobic conditions inorganic phosphate and/or phosphate acceptor is utilised for oxidative phosphorylation in the mitochondria and is therefore unavailable for the glyceraldehyde-3-phosphate dehydrogenase reaction in the soluble fraction of the cytoplasm. This leads to a lack of glycolytically generated ATP in the cytoplasm thereby limiting glucose phosphorylation.

Wu and Racker suggested that this compartmentation (either structural or functional) might be less rigid in anaerobic conditions and also in tumour cells as compared

to normal cells, thus explaining the high aerobic glycolysis of tumour cells.

The Pasteur effect in tumours. When it was shown that the range in respiratory rates in normal and cancer tissues was similar, Warburg rephrased his hypothesis; that the respiration of tumour tissues is inadequate since glycolysis is maintained in aerobic conditions, i.e. the Pasteur effect is incomplete. It is true that many normal tissues studied show no appreciable aerobic glycolysis. However measurements of the Pasteur effect and the percentage Pasteur effect (expressed as a function of oxygen uptake) of large numbers of normal and of cancer tissues have shown that the Pasteur effect is functioning in tumours in the same qualitative and quantitative manner as in normal tissue. The high aerobic glycolysis of tumours is a manifestation of the high anaerobic glycolysis in the presence of a normal respiratory rate.

The Pasteur and Crabtree effects. The Pasteur effect differs from the Crabtree effect (the inhibition of respiration by glycolysis) in one significant aspect. The Crabtree effect is a tightly coupled phenomenon, that is, the energy lost by glucose-suppressed respiration is exactly replaced by glycolytically formed high energy phosphate (Quastel and Wickis, 1959). On the other hand, much more energy is available in aerobic conditions in

both normal and cancer tissues than in anaerobic conditions, since 1 mole of oxygen respired inhibits only 1 to 2 moles of lactate formation.

(b) Crabtree Effect.

Crabtree (1929) observed that the addition of glucose, but not of xylose, to slices of several sarcomas (Jensen rat sarcoma, Crocker mouse sarcoma and a mouse tar sarcoma) inhibited the oxygen uptake of the slice. The inhibition was not large (10 - 20 per cent.) but was consistent and normal tissues similarly treated were not affected.

The term 'Crabtree effect' (or 'reversed Pasteur effect') is now used to describe the inhibition of respiration observed after the addition of any hexose or hexose analogue capable of inhibiting respiration. Such an inhibition has been shown in many tumour tissues, and also in normal tissues which exhibit aerobic glycolysis.

The majority of the studies on the mechanism of the Crabtree effect have been made with Ehrlich ascites tumour cells following reports of Kun et al. (1951) which showed a very considerable Crabtree effect (50 per cent.) in these cells.

Not only glucose but also fructose, mannose (Brin and McKee, 1956; Clowes and Ketch, 1954; Kun et al., 1951; Racker, 1956) and 2-deoxyglucose (Ibsen et al.,

1958; Packer and Golder, 1960) can induce respiratory inhibition.

Two patterns of inhibition have been demonstrated. In the present studies, and other manometric investigations (McKee et al., 1953; Quastel and Dickis, 1959), the addition of low concentrations of glucose stimulates respiration but as the glucose concentration is further increased the respiratory rate falls. Manometric techniques necessitate the equilibration of the cells in the apparatus for some time (at least an hour in the Cartesian diver technique). However respiration can be measured constantly before and after glucose addition by the use of the oxygen electrode method. The use of this technique has shown that the addition of minute quantities of glucose doesinhibit respiration (Chance and Hess, 1959; Ibsen, 1960). This inhibition by small quantities of glucose would not be observed manometrically, since this small quantity would be fully utilised during the equilibration period, thereby releasing the depression of respiration. The actual stimulation of respiration shown manometrically by low concentrations of glucose is thought to be due to availability of pyruvate, formed from the glucose (Ibsen, 1960).

Therefore the oxygen electrode technique is more suitable for following the time-course of the Crabtree

effect with small quantities of glucose. This time-course of inhibition has been shown to consist of an initial stimulatory phase, lasting 20 to 120 seconds (Chance and Hess, 1956; Packer and Golder, 1960) and probably again due to pyruvate. This is followed by an inhibitory phase which lasts until all the glucose has been utilised and thereafter the respiratory rate can return to normal (Ibsen et al., 1958).

Inhibitors of the Crabtree effect. Inhibition of respiration by glucose can be released by substances which uncouple oxidative phosphorylation, for example 2-4 dinitrophenol (Chance and Hess, 1959; Kun et al., 1951; Racker, 1956; Wenner and Weinhouse, 1955) and by dicoumarol (Chance and Hess, 1959).

Mechanism of action of Crabtree effect. Several mechanisms have been proposed to explain the Crabtree effect.

1. pH: In cells which produce lactate aerobically the increased hydrogen ion concentration in the medium might cause the respiratory inhibition. That this is not the primary cause of the Crabtree effect has been shown by the fact that inhibition by glucose is removed immediately the glucose is utilised. While dinitrophenol releases the respiratory inhibition it does not decrease the change in pH found in the presence of glucose alone

(Ibsen et al. 1958). Bloch-Frankenthal and Weinhouse (1957) suggest that the production of H^+ at specific sites within the cell may cause the inhibition and it has been shown that the change in pH may be greater within the cell than in the medium (Dewey and Green, 1959). The mechanism by which dinitrophenol could protect such sites and thereby release the respiratory inhibition is more difficult to understand.

2. Competition for substrates: As early as 1936 Belitzer (1936) suggested that the Crabtree effect was due to competition between respiration and glycolysis for common intermediates. Dinitrophenol which is known to uncouple phosphorylation from oxidation (Loomis and Lipmann, 1948) releases inhibition of respiration by glycolysis. It was therefore suggested that the common intermediate in question was either inorganic phosphate or adenine nucleotide. In the presence of the uncoupling agent the inhibition would be released since the rate-limiting mitochondrial phosphorylation would be uncoupled from oxidation and therefore substrates of phosphorylation would no longer limit respiration.

Further evidence for a competition for inorganic phosphate was that the level of inorganic phosphate was lowered during glycolysis (Hess and Chance, 1959; Ibsen et al., 1958; Racker, 1956; Wu and Racker, 1959), while

raising the inorganic phosphate level of the medium reduced the Crabtree effect (Brin and McKee, 1956; Wu and Racker, 1959).

Ibsen (1960) has shown however that much of the stimulation that an increased inorganic phosphate concentration exerts on respiration in the presence of glucose is due to a direct effect on glycolysis and not on respiration, while Bloch-Frankenthal and Ram (1959) found that increase in inorganic phosphate level may increase the Crabtree effect. Furthermore, the inorganic phosphate level, although it initially drops, returns to the endogenous level within three minutes, while the Crabtree effect continues much longer.

There is more evidence that ADP may be the limiting factor. This evidence includes the observations that an equal quantity of phosphorylation occurs in the presence or absence of glucose under aerobic conditions (Creaser et al., 1959; El'tsina, 1960), that in isolated mitochondria ADP controls respiration more effectively than inorganic phosphate (Hess and Chance, 1961; Ibsen et al., 1958), and that respiration in reconstructed systems can be inhibited by limiting ADP (Gatt and Racker, 1959).

2-deoxyglucose, which also causes a Crabtree effect in Ehrlich ascites tumour cells, is phosphorylated but metabolised no further while iodoacetic acid inhibits

aerobic glycolysis at the triose phosphate dehydrogenase step but does not release the Crabtree effect (Chance and Hess, 1959; Hess and Chance, 1958; Racker, 1956; Wu and Racker, 1959). There is some controversy on this latter point. It has been reported that iodoacetic acid releases the Crabtree effect only in the presence of phosphate (Seelich and Letnansky, 1957) but other workers who found no effect of iodoacetic acid on the Crabtree effect employed phosphate buffers (Ibsen et al., 1958; Racker, 1956).

A direct competitive mechanism for ADP does not explain a Crabtree effect induced by 2-deoxyglucose or glucose plus iodoacetic acid and so mechanisms of indirect competition have been suggested. Two different theories have been evolved, both based on the concept of intracellular barriers.

Ibsen et al. (1958) suggested that hexokinase, located in the mitochondria (Wu and Racker, 1959) utilised a portion of the normal mitochondrial ATP, making it unavailable for the transport of cytoplasmic ADP through the membrane to the sites of oxidative phosphorylation within the mitochondria. This theory is based on Siekevitz and Potter's theory (1955) of nucleotide transport through the mitochondrial membrane by the adenylate kinase reaction.

Chance and Hess (1956) and Racker (1956) suggested that ATP becomes trapped within the mitochondria because of the initial stimulation of oxidation and an associated change in mitochondrial permeability. In support of this theory Hess and Chance (1961) found more ATP within the mitochondria of cells exposed to glucose while Packer and Golder (1960) found that the early stage of stimulated oxidation and high cytoplasmic ADP seemed to be associated with structural changes in the mitochondria.

The Crabtree effect in other tissues. Many observations have been made on the Crabtree effect in various normal and neoplastic tissues. The results are varied and many of the normal tissues require special conditions before any effect is shown. With leukocytes in particular (normal and leukaemic) the range of the Crabtree effect found is very great (from no effect at all to 30 per cent. inhibition).

It is possible that in the tissues studied factors such as pH, hormones mask the Crabtree effect or that too high a cell to glucose ratio was used so that glucose was fully utilised before the inhibition was measured. In the present studies, all cell strains, of normal or malignant origin, demonstrated a marked Crabtree effect under standard conditions at high glucose concentrations. At lower concentrations however variations in pattern were

obtained. Most cell strains showed an initial rise in respiration when glucose concentration was very low. As has been explained, this rise may be due to increase in citric acid cycle substrate levels and to the delay in measuring respiration inherent in the manometric technique used. The small concentration of glucose added may therefore have been utilised before respiration was measured. (Fig.9 and page 105).

In two cell strains however a subsequent fall in respiration with increased glucose concentration was followed by a sharp rise before a classic Crabtree effect was shown. The reason for this truncated pattern is not obvious. In one case, that of the L strain cells, it was suggested that the reason was the 'leaky' nature of the cells, since the respiration of these cells had been shown to be affected by the concentration of citric acid cycle intermediates in the medium (Danes and Paul, 1961). However, the other cell strain showing this truncated pattern (Y5 strain) did not 'leak' citric acid cycle intermediates into the medium, as judged by respiration measurements in fresh medium with and without the addition of citric acid cycle intermediates (page 228).

(c) Other Environmental Factors.

Other factors which have been found to affect carbohydrate metabolism have not been studied in such detail.

pH. There is a progressive transition from a more glycolytic to a more aerobic pattern of metabolism as the pH is lowered in a number of cell strains studied, but the actual amount of glycolysis at any given pH varies considerably from cell strain to cell strain (Paul et al., 1962). This phenomonon of decreased glycolysis with decreased pH does not seem to be directly related to respiration, since in most cell strains the highest respiratory rate is found at pH 7.4 (page 101).

Carbon dioxide. Carbon dioxide is essential for cellular growth (Geyer and Chang, 1958; Geyer and Neimark, 1958; Harris, 1954; Swim and Parker, 1958; Whitfield and Rixon, 1961). It has been suggested that this requirement is due to decarboxylation of oxaloacetic acid which occurs readily in the absence of carbon dioxide. Such an affect would explain the decrease in respiratory rate found in the absence of carbon dioxide (Biez, 1960; Danes and Kieler, 1958; Kieler, 1960a,b; present work, page 106). This theory is supported by the observation that cells will grow in a carbon dioxide-free medium if oxaloacetic acid is added (Gwatkin and Siminovitch, 1960).

Non-specific effects of serum and other components

on carbohydrate metabolism have been reported (Kuwabara, 1959; Phillips and Andrews, 1960; Wu, 1959) but no detailed study has yet been made of them. It is difficult to separate these factors from some of the effects which have already been described.

3. Glycolysis and Carcinogenesis.

(a) Carbohydrate metabolism in BHK21 cells.

Once the factors that affect the respiration and glycolysis of cultured cells had been determined and it was evident that their effect on normal and malignant cells was similar, it became possible to compare carbohydrate metabolism in the two cell types.

In the system of baby hamster kidney fibroblasts, two types of cell were used. One, specified 'normal', did not produce tumours on inoculation into hamster brain. The other, designated 'transformed', was derived from the same parent cell but had been 'transformed' *in vitro* by polyoma virus. This 'transformed' cell did produce tumours in hamster cheek pouch or brain.

The respiratory rates of 'normal' and 'transformed' BHK21 cells in a controlled environment were not significantly different (pages 121-123). The glycolytic rates of the two cell types were however markedly different under all the conditions used (pages 129-131) glycolysis

of the 'transformed' cell being substantially greater than that of the 'normal' cell in anaerobic and aerobic environments.

(b) Importance of glycolysis.

The results obtained refute Warburg's theory of carcinogenesis quite clearly, as far as this system is concerned. The increased glycolysis reported in cancer cells is however substantiated and the question is raised as to the importance of the increase in glycolysis in carcinogenesis. It is possible that this phenomenon is purely incidental in tumour induction. If it is not, is the effect primary in the carcinogenic progress of the cell or a secondary mechanism of adaptation of malignant cells to their environment?

An alteration in carbohydrate metabolism is the most constantly found difference in metabolism between normal and malignant cells. While most of the studies have been performed on tissue slices of animals and therefore environmental factors are difficult to assess, increased glycolysis has been shown in other reasonably controlled systems too; in chorioallantoic membrane and Rous infected chorioallantoic membrane (Weber et al., 1961a,b) and in tissue culture systems.

With all the evidence that has been accumulated on this aspect of metabolism it would be surprising if the

alteration in glycolytic metabolism in tumours was purely incidental. If on the other hand it is a secondary effect of carcinogenesis, as is proposed by many investigators (Potter, 1964), what advantages do an increased glycolytic metabolism confer on the cancer cell?

(c) Advantages.

It has been suggested that aerobic pathways are essential for specialised functions in differentiated cells whereas the energy for fundamental synthetic reactions involved in undifferentiated growth can be derived directly from glycolysis. In support of this theory Allsopp and Fell (1950) found that heart explants ceased to beat when they were treated with inhibitors of the citric acid cycle, such as fluoroacetate, although they continued to survive under these conditions. Such a tissue would require an exceptionally high glycolytic rate to produce enough energy for continuous contraction. However, by a respiratory metabolism 15 to 16 times as much ATP would be obtained from a given amount of glucose as by glycolysis.

Moreover, certain processes demand intact electron transfer pathways with a hydrogen acceptor, almost invariably oxygen. The synthesis of many mucopolysaccharides for example requires the formation of glucuronic acid from glucose. This process requires an oxidative metabolism since the reduction of pyruvate to lactate can only

account for the electrons made available in the formation of pyruvate from glucose by glycolysis. Therefore the normal functioning of specialised cells producing acid mucopolysaccharides must as a rule require oxygen. A similar requirement governs synthesis of many other special molecules.

Thus a glycolytic metabolism would be less favourable for differentiation. The increased glycolysis of tumours grown *in vivo* may be correlated with growth in conditions with poor blood supply and resultant low oxygen tension, but tumour cells grown *in vitro* still display a high glycolytic rate. It is difficult to envisage in what manner increased glycolysis, in the presence of a normal respiratory rate, is advantageous to the cancer cell.

(d) Amino acid incorporation.

It has been suggested that tumour tissues in general are able to incorporate amino acids anaerobically. Quastel and Bickis (1959) measured the efficiency quotient of incorporation of glycine, defined as the ratio of amino acid incorporated into protein to ATP, theoretically made available by respiration or by glycolysis (assuming a P/O ratio of 3.0). They found that in the five tumours studied, the anaerobic efficiency was as great as, and often greater than, the aerobic efficiency. Embryo tissue also showed anaerobic incorporation but the efficiency in

aerobic conditions was greater.

In the series of normal tissues studied, including regenerating liver, the anaerobic efficiency was extremely low. They suggest the cause of diminished efficiency in these tissues may be the development in adult tissues of new enzyme systems, dependent on ATP which are needed for special functions of these tissues. There is less ATP available therefore for amino acid incorporation systems.

(e) Glycolysis and Growth.

Is glycolysis essential for growth? Oxamic acid competitively inhibits lactic dehydrogenase but does not interfere with metabolism of carbohydrate *via* pyruvic acid and the citric acid cycle. In the presence of this inhibitor, aerobic and anaerobic glycolysis of Ehrlich ascites cells were equally inhibited but the inhibition of anaerobic glycolysis was temporary, due to accumulation of pyruvic acid (Papaconstantinou and Colowick, 1961). When this inhibitor was present in HeLa cell cultures growth was completely inhibited but the inhibition could be prevented by the addition of pyruvate (Papaconstantinou and Colowick, 1961).

Earlier work had shown however that HeLa cells could grow well with galactose as the carbohydrate source. Under these conditions little or no lactic acid was produced (Eagle *et al.*, 1958; Rueckert and Mueller, 1958), since

galactose is phosphorylated in HeLa cells at an extremely low rate (Eagle et al., 1958). HeLa cells are now known to be sensitive to oxamate only under conditions where they are producing lactic acid rapidly from glucose (Goldberg, 1961).

The inhibition of lactic dehydrogenase by oxamate in HeLa cells is thought to lead to accumulation of pyruvate and $NADH_2$, and this in turn to lead to failure of triose phosphate oxidation, which requires NAD. There is therefore a corresponding failure in ATP synthesis so that the ATP level falls because of its rapid use for the phosphorylation of glucose.

Thus the inhibition by oxamate in HeLa cells may be due to a diminished system for regeneration of NAD, as suggested by Boxer and Devlin (1961) and discussed in a later section.

(f) Potter's Theory and Glycolysis.

If carcinogenesis is considered to be due to a defect in control of the formation of an enzyme system, the question then arises as to which activities of the cell metabolism can, when repressed or derepressed, result in tumour cells. Can the primary effect be in carbohydrate metabolism? Potter and his colleagues picture the limiting type of their minimal deviation hepatomas as one in which only the group of enzymes responsible for DNA production

and mitosis are derepressed, while all other enzymes are present in normal amounts and with normal activities (Potter, 1964).

The activities of the enzymes involved in these processes have not been measured in the present system, but growth rates of the two cell types (as measured by increase in population) are similar. It is therefore less likely that a pattern such as Potter described is present.

Potter (1964) considers that the increased glycolysis shown in so many tumours is not related to the carcinogenic reaction but to a later stage of adaptation. Minimal deviation hepatomas have been shown to possess glycolytic rates similar to, higher or lower than, normal liver (Aisenberg and Morris, 1963; Elwood et al., 1963; Weber et al., 1961). More recently Burk et al. (1965) have found that rates of anaerobic and aerobic glycolysis of minimal deviation hepatomas were several folds higher than those of liver tissue from the host animal or from hepatoma-free animals, if an adequate $NAD/NADH_2$ ratio was maintained.

Potter also refers to the evidence of Homburg et al. (1961). However this study of respiration and glycolysis of malignant and non-malignant lymphoblasts in culture did show an increased (50 per cent.) anaerobic glycolysis

in the malignant cell though little difference was found in aerobic conditions. Respiration in the malignant lymphoblast was not impaired.

With the BHK21 cell system it is easily apparent that the 'transformed' cell has a much higher glycolytic rate than the 'normal' cell by the change in pH of the culture medium with growth. Since the culture medium contains methyl red as a pH indicator, a fall in pH is marked by a change in colour from orange-red to yellow. Stoker and Macpherson (1961) found in the initial studies on BHK21 cell transformation by polyoma virus that cultures of cells which had been exposed to virus could when examined after 7 days, be distinguished from control cultures of unexposed cells by the acid pH of the culture as well as by morphology.

It is therefore not impossible that an alteration in carbohydrate metabolism may be inherent in carcinogenesis, rather than a secondary adaptation of cancer cells.

4. Possible Mechanisms for Increased Glycolysis.

The extent to which the glycolytic pathway functions may be altered by a number of factors, for example the activity of the enzymes involved, in particular of the rate-limiting enzymes, the availability of substrate or of co-factors.

(a) Enzymes.

Hexokinase has been claimed by several people to be the rate-limiting enzyme in glycolysis (Beck, 1958; Di-Pietro et al. 1962). In the present study the level of activity of hexokinase was in most cases considerably higher in the 'transformed' cells than in the 'normal' cells in correlation with the glycolytic rates in these cells. Other investigators have reported a similar correlation, that the rate of glucose utilisation and the glycolytic capacity of any given cell are directly related to hexokinase activity for that cell (Warburg et al. 1958; Wu, 1959).

It thus seems a reasonable theory that in most instances the primary factor controlling the catabolism of glucose is the hexokinase system permitting entry of glucose to the appropriate metabolic pathways.

(b) Availability of substrate (glucose).

It has been known for many years that in tissue cultures glucose is the main source of energy (Astrup et.

al. 1947; Gemmill et al. 1940; Lewis, 1922; Villmer, 1942; Wilson et al. 1942). While glycolysis could be controlled by the level of activity of hexokinase it could also be controlled by the availability of intracellular glucose.

Glucose appears to be actively transported across the cell membrane, i.e. transported by a process requiring metabolic energy. One theory regarding this transport is that hexose molecules may associate in pairs by the action of a 'dimerizer' in the membrane. The paired molecules could then cross the membrane because of their increased solubility (Stein, 1961).

Another theory envisages a carrier molecule for transport across the cell membrane and subsequent dissociation on the inner side. The evidence suggests that the carrier molecule is enzymic; the reaction is specific e.g. D-galactose enters the red blood cell while L-galactose is excluded (Park et al., 1959) and the kinetics of the transfer seem to follow Michaelis-Menton kinetics for an enzyme-catalysed reaction (Morgan et al., 1961).

Transport across the membrane antecedes and is distinct from phosphorylation and may be regarded as the first step in glucose metabolism. It could be a rate-limiting step in cases where the intracellular free glucose was so low that phosphorylation by hexokinase was below capacity.

It has been suggested recently that aerobic glycolysis is a defence mechanism against a high glucose influx. Graff et al. (1965) have found that L929 mouse fibroblasts and Ehrlich ascites cells grew well in very low glucose concentrations (5 mg./100 ml. or less). These cells did not produce lactic acid until the glucose concentration was higher than 5 mg./100 ml. Media used in tissue culture normally contain 100 to 200 mg. glucose per 100 ml., a concentration at which Graff et al. found prolonged generation rates and inhibition of DNA and RNA synthesis. Thus the increase in aerobic glycolysis found on explantation of tissues may be due to a glucose concentration effect.

Graff et al. suggest that in vitro the defence mechanism of aerobic glycolysis is manifest because the hormonal action on glucose transport across the cell membrane is absent or not operating. In vivo only those cells which are refractory to glucose flux regulation would exhibit aerobic glycolysis (cancer cells).

The cell membrane of normal and tumour cells will be considered in a later section; it may however be mentioned at this point that alterations in the cell membrane might affect the uptake of glucose and hence be involved in the increased glycolysis found in tumour cells.

(c) Co-factors.

Glock and McLean (1955, 1957) found that NAD and NADP were in general lower in concentration in tumour tissues than in normal tissues, particularly NADP. In both tissues the form of NAD and NADP was similar, i.e. the bulk of NAD was in oxidised state and of NADP was in the reduced state.

Boxer and Devlin (1961) consider that the absence of enzymatic hydrogen-carrying systems is a factor in the aerobic glycolysis of malignant tissue. Their theory is based on the assumption that normal tissues do not exhibit aerobic glycolysis - an assumption which is not true for all normal tissues and particularly in cultured cells, but the theory can be applied to such systems if the difference between normal and malignant is quantitative.

The enzymes and co-factors involved in glycolysis are primarily located in the cell sap while the terminal oxidative reactions of carbohydrate metabolism occur in the mitochondria. Since NAD and $NADH_2$ are present only in catalytic quantities in both normal and malignant cells (Carruthers and Suntzeff, 1954) and the mitochondrial membrane is impermeable to $NADH_2$ (Lehninger, 1951), there must be a 'shuttle' system of some kind operating across the mitochondrial membrane to regenerate

NAD from $NADH_2$. One such 'shuttle' system is the glycerophosphate shuttle in which α-glycerophosphate, produced from dihydroxyacetone phosphate with the regeneration of NAD from $NADH_2$, is oxidised by an intramitochondrial enzyme. The enzymic equipment for such a shuttle has been shown in normal mammalian tissues, but is very low or lacking in the tumours studied. In the glycolytic pathway NAD can only be regenerated by the action of lactic dehydrogenase and formation of lactic acid which is not further metabolised.

Boxer and Shonk (1960) measured the activities of lactic and glycerophosphate dehydrogenases and found the ratio of lactic dehydrogenase to glycerophosphate dehydrogenase varied between 0.5 and 7 to 1, while in tumours the ratio ranged from 10 to 1 to several hundreds to 1.

Other similar 'shuttle' systems have been described, for example acetoacetate-β-hydroxybutyrate shuttle and again the tumour tissue appeared to be deficient in this system.

While such a theory gives a lucid explanation for the increase in aerobic glycolysis found in malignant tissues, it does not explain the increased glycolysis of malignant tissues over normal tissues found in anaerobic conditions. It would thus seem more probable

that a higher availability of glucose and/or an increase in the rate-limiting enzyme of glycolysis is the reason for the increased glycolysis of malignant cells.

In the system of 'normal' and 'transformed' cells used in the present work the quantities of lactic and of pyruvic acid produced were estimated as well as of glucose used. It is interesting to note that in the A strain cells ('normal') more lactic acid was produced than pyruvic acid while in the Y strain ('transformed') the opposite was found. No difference in activity of lactic dehydrogenase was found between the two strains. It would appear that there was some difference in content of $NADH_2$ available for reduction of pyruvic to lactic acid but, opposed to the theory of Boxer and his colleagues, less $NADH_2$ was available for this reduction in the 'transformed' cell than in the 'normal' cell.

5. Other Aspects of Carbohydrate Metabolism.

(a) The Hexose Monophosphate Shunt in Tumours.

There can be little doubt that the major function of the hexose monophosphate shunt in many tissues is the synthesis of pentose (Horecker and Hiatt, 1958). Most of the pentose formed is incorporated into nucleic acid. It has therefore been difficult to compare differences in the extent of this pathway or the activities of the enzymes since in very few systems of normal and malignant cells are the growth rates similar. The existence of the pathway in tumours has however been clearly established (Dickens and Glock, 1951; Glock and McLean, 1954; Bosch et al., 1956).

In the present system the 'normal' and 'transformed' cells are growing at similar rates. The activities of two of the enzymes of the pathway were measured. One, 6-P-gluconate dehydrogenase, was present at similar levels of activity in both cell types. The other, glucose-6-phosphate dehydrogenase, occurred at a lower level of activity in the 'transformed' cell than in the 'normal' cell. As these cell types are growing at similar rates the activity of glucose-6-P dehydrogenase in the 'transformed' cell must be sufficient for the production of nucleic acids during growth. Therefore some other aspect of metabolism, involving this enzyme,

must be altered during 'transformation' into tumour-producing cells.

(b) Lipogenesis.

It has been suggested that there is a strong relationship between the hexose monophosphate shunt and lipogenesis. Mammary gland slices from lactating rats, which exhibit a high degree of glucose catabolism through the hexose monophosphate shunt, convert a major proportion of the utilised glucose to fatty acids (Abraham and Chaikoff, 1959). On the other hand tissues such as adult rat brain and muscle, which do not form fatty acids from glucose to any extent, appear to utilise this glucose glycolytically, (Bloom and Stetten, 1953; Tower, 1958).

NADP is suggested as being a regulator in lipogenesis (Abraham and Chaikoff, 1959; Horecker and Hiatt 1958; Langdon, 1957). This coenzyme is required for the activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase and in fatty acid synthesis. It is present in tissues mainly in the reduced form (Glock and McLean, 1955). The opposite hypothesis has therefore been proposed; that NADP, derived from the utilisation of $NADPH_2$, in reductive steps of fatty acid synthesis, stimulates the hexose monophosphate shunt (Marks, 1956; Siperstein,1959; Siperstein and

Fagan, 1958).

However, in whichever direction the regulation occurs, it would appear that a lowered activity of glucose-6-phosphate dehydrogenase with a concurrent low level of $NADPH_2$ would parallel a decrease in fatty acid synthesis and other pathways using $NADPH_2$.

A growing awareness of the importance of lipid, especially phospholipid, in cell membranes and sub-cellular particles such as mitochondria, along with the advent of suitable analytical techniques, has stimulated studies of phospholipid composition of tumour tissues. Kasawaki, et al. (1958) isolated a characteristic phospholipid from several human tumours, which they could not detect in normal tissues.

Gray (1963) has measured the lipid composition of Landschutz ascites carcinoma cells and BP8/C3H ascites sarcoma cells in comparison with normal tissues, with special reference to phospholipids. He found a preponderance of C_{18} saturated acid as compared with C_{16} saturated acid in tumours, while in normal tissues the opposite emphasis occurred.

In analysis of the lipids of normal rat liver and a primary hepatoma VeerKamp et al. (1961) found a considerable change in the ratio of the saturated C_{17} fatty acid stearic acid to the unsaturated oleic acid. This

ratio was approximately 1.5 : 1 for normal liver and 1 : 2 for the hepatoma.

6. The Cell Membrane.

(a) Permeability.

De Grier and Van Deenen (1961) have shown a direct correlation between permeability and phospholipid composition when erythrocytes of various species were compared. A correlation was found between permeability to glycol and percentage of lecithin in the membrane.

A change in permeability in tumour cell membranes has been reported. The result of carcinogenesis seems to be increased permeability or leakiness of the cells. Thus proteolytic enzymes have been shown to be released from ascites tumour cells (Malmgrén *et al.*, 1955) while Dosch (1958) found ribose-5-phosphate, glucose, and fructose were metabolised by enzymes liberated into the extra-cellular medium by ascites tumour cells.

In the present work some cell strains were shown to 'leak' citric acid cycle intermediates into the medium. Respiration of these cells was decreased after inoculation into fresh medium and the decrease could be prevented by the addition of citric acid cycle intermediates to the medium (page 99). The BHK21 cells, 'normal' and 'transformed' did not show this leakiness with citric acid cycle intermediates (page 116).

This however does not detract from an hypothesis that the decrease in glucose-6-phosphate dehydrogenase is either the cause or result of altered and possibly decreased lipid synthesis. The alteration in phospholipid concentration in the cell membrane may in turn cause an alteration in permeability of the cell membrane to glucose, facilitating its entry. The increased intracellular concentration of glucose might then result in induction of a higher activity of hexokinase, thus producing an increased rate of glycolysis.

(b) Theories of carcinogenesis involving membranes.

Several theories have been advanced that carcinogenesis may be related to changes in the cell surface. Pardee (1964) suggests that the loss of the mechanism of regulation of growth in the cancer cell may be related to a loss of intercell control of cell division. Since various normal adult tissues will divide under certain conditions, for example in regenerating liver, and also in wound healing, it has been suggested that the loss of cell division depends on the relation between the cells and not on some property within the cells (Abercrombie, 1957; Paschkis, 1958). Pardee suggests that growth-regulating substances are unable to penetrate the tumour cell due to altered permeability of the cell surface.

Green's immune theory of cancer (page 22) can also

be correlated with changes in surface of the malignant cell, by virtue of the positioning of antigens on the cell surface.

Kalckar (1964) found that four types of mammalian cells with malignant potentialities showed a loss of epimerase activity in converting glucose to galactose. He suggests that this enzyme inhibition could cause a lack of galactosyl compounds in the cell membrane, which may be of extreme importance in carcinogenesis.

(c) Membrane contacts between cells.

It has been shown that when normal cells, moving in culture in liquid medium on a glass surface, make contact with each other, movement is stopped. This phenomenon has been termed 'contact inhibition' (Abercrombie and Heaysman, 1954; Abercrombie et al. 1957). The region of contact between two cells is as a rule a firm but not indissoluble adhesion between the two plasma membranes (Abercrombie and Ambrose, 1958; Weiss, 1958).

This 'contact inhibition' is much less in evidence in malignant cells (Abercrombie and Ambrose, 1958). Electrophoresis of cells has shown that kidney tumour cells and hepatoma cells in culture have much greater electrophoretic mobility than the normal cells from which they are derived.. This increased mobility can be explained by an increase in the net negative charge per

unit area carried by the tumour membrane.

There thus seems evidence that the cell membrane in tumour cells is different from that in normal cells.

7. Glucose-6-phosphate and Isocitric Dehydrogenases.

It is possible however that the decrease in glucose-6-phosphate dehydrogenase found in the 'transformed' cell has a simpler explanation. The third enzyme which was found to be present in different amounts in 'normal' and 'transformed' cells was isocitric dehydrogenase. These two enzymes and also 6-phosphogluconate dehydrogenase require NADP whereas the other adenine nucleotide-requiring enzymes in carbohydrate metabolism require NAD. 6-Phosphogluconate dehydrogenase was present at similar levels in the 'normal' and 'transformed' cells while glucose-6-phosphate dehydrogenase was lower and isocitric dehydrogenase higher in activity in the 'transformed' cell than in the 'normal' cell. If the ratios of these enzymes are compared within the sets of strains of cells, it can be seen that there is no significant difference (Table 43). It is possible therefore that an equilibrium exists between glucose-6-phosphate dehydrogenase and isocitric dehydrogenase, governed in some way by the availability of NADP for each enzyme at a given time.

T A B L E 43

COMPARISON OF THE RATIO OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY IN 'NORMAL';'TRANSFORMED' CELLS WITH THE RATIO OF ISOCITRIC DEHYDROGENASE ACTIVITY IN 'TRANSFORMED':'NORMAL'.

Cell Strains	Glucose-6-phosphate Dehydrogenase Ratio:- Normal : Transformed	Isocitric Dehydrogenase Ratio:- Transformed:Normal	%P
	MEAN ± St.E.	MEAN ± St.E.	
C13 & TC11	1.76 ± 0.60	1.70 ± 0.44	n.s. P > 10
A & Y	1.17 ± 0.27	1.27 ± 0.33	n.s. P > 10
*C & Z	1.48 ± 0.18	2.37 ± 0.69	n.s. P > 10

Both enzyme activities are expressed as μmoles NADP reduced/minute/10^6 cells.

*Z-test showed standard deviations of C and Z cells significant at the 5% level.

8. Conclusion.

The results reported for this system of 'normal' and 'transformed' BHK21 cells in culture showed that respiration in standard conditions was not inhibited in the 'transformed' cell. Glycolysis however was greatly stimulated and it has been suggested that a change in the constitution and permeability of the cell membrane might be responsible. It would be interesting therefore to characterise the cell membranes of the two types of cell, qualitatively and quantitatively. Studies of glucose uptake, using radioactive glucose, might show whether in fact there was an increased availability of glucose in the 'transformed' cell.

The possibility that an equilibrium exists between glucose-6-phosphate dehydrogenase and isocitric dehydrogenase activities should be investigated. If ratios of these enzymes in the two cell types are equal under different conditions it would be possible to speculate on their relative significance in 'normal' and 'transformed' cells.

These studies however only apply to one isolated system of 'normal' and 'transformed' cells in culture, 'transformation' being induced by a carcinogenic virus. Before conclusions can be reliably drawn as to the relationships previously discussed with carcinogenesis, other similar systems of cells in culture, transformed *in vitro* by viral or chemical means, to cells capable of inducing tumours *in vivo*, must be studied.

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APPENDIX A.

HANKS BALANCED SALT SOLUTION (1949).

	Concentration (g/litre)
NaCl	8.00
KCl	0.40
$CaCl_2$	0.14
$MgSO_4 \cdot 7H_2O$	0.10
$MgCl_2 \cdot 6.H_2O$	0.10
$Na_2HPO_4 \cdot 2H_2O$	0.06
KH_2PO_4	0.06
Phenol red	0.02
$NaHCO_3$	0.35

TRIS CITRATE-BUFFERED BALANCED SALT SOLUTION

(Paul 1962, unpublished).

x 10 BSS	65 ml.
0.2 M Tris	80 ml.
0.1 M Citric Acid	50 ml.
25% $MgCl_2$	10 ml.
Water to	1 litre

Used in same proportion as balanced salt solution (BSS) (Hanks and Wallace, 1949).

WAYMOUTH'S MEDIUM MB 752/1 (1959).

	Concentration mg/litre	mM
NaCl	6000	103.0
KCl	150	2.0
$CaCl_2.2H_2O$	120	0.82
$MgCl_2.6.H_2O$	240	1.18
$MgSO_4.7H_2O$	200	0.81
Na_2HPO_4	300	2.11
KH_2PO_4	80	0.59
$NaHCO_3$	2240	26.7
Glucose	5000	27.8
Ascorbic acid	17.5	0.1
Choline HCl	250	1.8
Cysteine HCl	90	0.57
Glutathione	15	0.05
Hypoxanthine	25	0.18
Glutamine	350	2.38
Thiamine HCl	10	0.03
Ca Pantothenate	1.0	0.003
Riboflavin	1.0	0.003
Pyridoxin HCl	1.0	0.003
Folic acid	0.4	0.0008
Biotin	0.02	0.00008
m-Inositol.$2H_2O$	1.0	0.005

Weymouth's Medium (contd.)

	Concentration mg/litre	mM
Nicotinamide	1.0	0.008
Vitamin B_{12}	0.2	0.00015
L-cystine	15	0.06
Glycine	50	0.66
L-phenylalanine	50	0.30
L-glutamic acid	150	1.02
L-aspartic acid	60	0.46
L-tyrosine	40	0.22
L-lysine HCl	240	1.42
L-proline	50	0.44
L-methionine	50	0.34
L-threonine	75	0.64
L-valine	65	0.55
L-isoleucine	25	0.19
L-leucine	50	0.38
L-tryptophan	40	0.20
L-arginine HCl	75	0.36
L-histidine HCl	150	0.80
NaOH	to pH 7.4	2.5

EAGLES MEDIUM (1955).

	Concentration mg/litre	mM
L-arginine	17.4	0.1
L-cystine	6.0	0.05
L-histidine	3.2	0.02
L-isoleucine	26.2	0.2
L-leucine	13.1	0.1
L-lysine	18.2	0.1
L-methionine	7.5	0.05
L-phenylalanine	8.3	0.05
L-threonine	11.9	0.1
L-tryptophan	2.0	0.01
L-tyrosine	18.0	0.1
L-valine	11.7	0.1
L-glutamine	146.0	1.0
Choline	1.0	
Nicotinic acid	1.0	
Pantothenic acid	1.0	
Pyridoxal	1.0	
Riboflavine	0.1	
Thiamine	1.0	
Biotin	1.0	
Folic acid	1.0	
Glucose	2000.0	

Eagles Medium (contd.)

	Concentration mg/litre	mM
NaCl	8000	
KCl	400	
$CaCl_2$	140	
$MgSO_4 \cdot 7H_2O$	100	
$MgCl_2 \cdot 6H_2O$	100	
$Na_2HPO_4 \cdot 2H_2O$	60	
KH_2PO_4	60	
$NaHCO_3$	350	
Phenol red	20	
Penicillin		0.5

FISCHER'S MEDIUM (1958).

		g/litre.
A.	NaCl	8.0
	KCl	0.4
	$MgCl_2.6H_2O$	0.1
	Na_2HPO_4	0.06
	$NaH_2PO_4.H_2O$	0.067
B.	Glucose	1.0
C.	$NaHCO_3$	1.0
D.	5% acid hydrolysed casein + tryptophan (0.5 mg/ml)	5 ml/litre
		mg/litre
E.	Glycine	20.0
	Cystine	7.5
	Histidine	20.0
F.	Glutamine	200.0
G.	Vitamins:	1.0
	Thiamin HCl	0.5
	Nicotinamide	0.5
	Ca-Pantothenate	0.5
	Pyridoxal HCl	0.5
	D-ribose	0.5
	Riboflavin	0.5
	Choline chloride	1.5
	i-Inositol	1.5
H.	Biotin	0.01
I.	Ascorbic acid	1.6
	Glutathione (reduced)	1.5
J.	Serum	2 - 10%
K.	Peptone	0.06%
L.	Folic acid	10 mg/litre
M.	Penicillin	100 units/ml.
	Streptomycin	0.050 mg/ml.
O.	Phenol red	10 mg/litre.
P.	$CaCl_2.H_2O$	0.182 g/litre.

TRYPSIN-CITRATE SOLUTION AS USED IN REMOVAL OF CELLS FROM GLASS.

Difco trypsin (1:250)	1.25 g.
Sodium citrate	1.48 g.
Sodium chloride	3.00 g.
Water, to	500 ml.

Adjust to pH 7.8

Sterilised by filtration.

SUSPENSION FLUID FOR COUNTING CELLS

Sodium chloride	7 g.
Citric acid	10.5 g.
Formalin (40%)	1 ml.
Water, to	1 litre.

Filter through sintered glass filter to remove particulate impurities.

HEART I MEDIUM.

L-Arginine HCl	-	0.36 mM.
L-Histidine HCl	-	0.80
L-Isoleucine	-	0.76
L-Leucine	-	0.09
L-Lysine HCl	-	1.42
L-Methionine	-	0.10
L-Phenylalanine	-	0.30
L-Proline	-	0.22
L-Serine	-	0.47
L-Threonine	-	0.64
L-Tryptophan	-	0.20
L-Valine	-	0.21
Glutamine	-	2.38
L-Cystine	-	0.06
L-Tyrosine	-	0.22

Concentrations of vitamins and glucose used are as in Waymouth's Medium MB 752/1 (page 221).

SOLUTION A.

(Citric acid cycle intermediates).

Na pyruvate	0.330 g.
Citric acid	0.630 g.
α-Ketoglutarate	0.448 g.
Na succinate	0.311 g.
Na fumarate	0.480 g.
Malic acid	0.402 g.
Oxaloacetic acid	0.390 g.

Dissolved in 30 ml. BSS and pH adjusted to 7.4. The solution was sterilised by filtration.

1 ml. of this solution was used in 100 ml. culture medium, giving a final concentration of 1×10^{-3}M of each constituent.