GLUCOSE-AMINO ACID METABOLISM IN TUMOUR TISSUES

by

Gyan Chandra Shrivastava M.Sc. (Bom.)

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Department of Biochemistry,
McGill University,
Montreal.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
DPN	Diphosphopyridine nucleotide
TPN	Triphosphopyridine nucleotide
TPNH	Reduced triphosphopyridine nucleotide
HMP	Hexose monophosphate shunt
EMP	Embden-Meyerhoff pathway
Pi	Inorganic orthophosphate

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Abstract

Tumour tissues *in vitro* show an incorporation of glucose carbon into glycine, alanine, glutamate and aspartate. The incorporation into glutamate and aspartate is lower than that observed in normal rat brain cortex slices or chick embryo. This may be due to a lower rate of operation of the citric acid cycle compared with that of glycolysis in tumours.

When normal rat brain cortex slices are incubated with tumour tissues *in vitro*, in a medium containing 5 mM glucose-U-C^{14}, there is a decrease in the amount of glutamine found in the brain cortex slices, with a concomitant increase in the incorporation of glucose carbon into glutamate, aspartate and alanine of the tumour tissues. It is shown that glutamine will leak out of brain slices when there is a lack of glucose. This is subsequently utilized by the tumour tissue. Dialysate obtained from Ehrlich ascites cells also decreases the formation of glutamine by brain cortex slices *in vitro*. This may be due to some factor in the dialysate which can stimulate the activity of glutaminase.

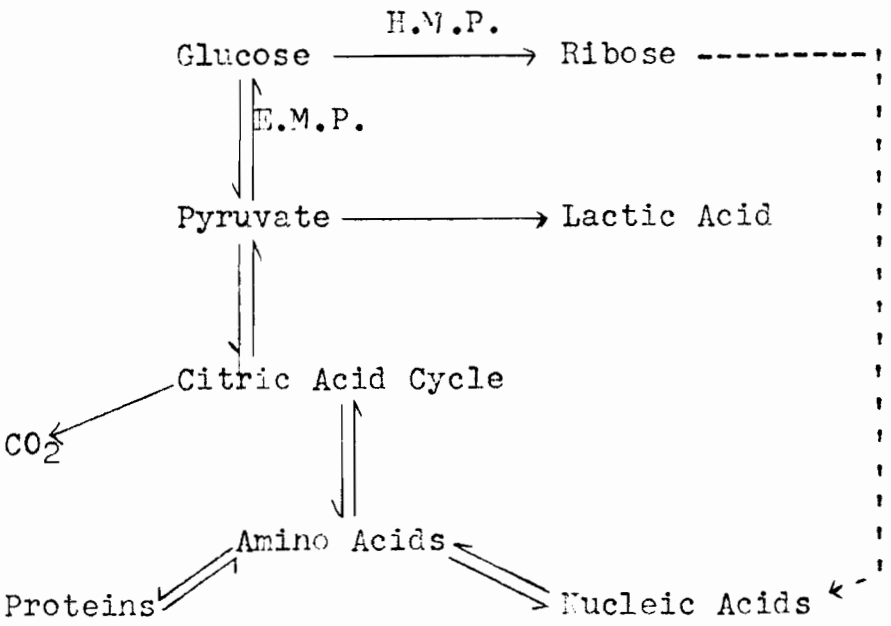
Formate, pyruvate and cysteine increase the oxidation of glucose-1-C^{14} to $C^{14}O_2$ by Ehrlich ascites cells. These effects may be due to a stimulation of TPNH oxidation.

INTRODUCTION

Despite great advances in our knowledge about the metabolism of tumours and about the nature of many carcinogenic factors, the fundamental nature of the neoplastic process remains unknown. However, the biochemical approach to tumour metabolism seems to be the most promising approach for understanding and elucidating the neoplastic process, particularly in view of the high glycolytic rate observed in tumours, which so far, has been found to be the most striking biochemical property of tumour tissues. Qualitatively no special metabolism of tumours has been observed; the biochemical differences are mainly of a quantitative nature especially with reference to the role of glycolysis in tumour metabolism.

Carbohydrates have been known for a long time to play an important role in the metabolism of microorganisms and animal tissues, but it has occupied a place of greater importance in the field of cancer research since, Warburg's discovery (1923, 1924, 1936) that tumour tissues exhibit a higher rate of aerobic and anaerobic glycolysis than do normal tissues. Since then extensive work has been done on the role of carbohydrates in the synthesis of complex molecules, viz. proteins, nucleic acids, etc. Evidence has been compiled to show that neoplastic tissues like normal tissues, synthesise complex molecules from carbohydrates, as described in the pathway in Figure 1.

Figure 1


H.M.P.
Glucose
Ribose
E.M.P.
Pyruvate
Lactic Acid
Citric Acid Cycle
CO_2
Amino Acids
Proteins
Nucleic Acids

ROLE OF GLYCOLYSIS IN TUMOUR TISSUES

Tumour Glycolysis in vivo

Cori and Cori (1925a, b) were the first to compare the differences in the levels of lactate and glucose of blood from normal veins to that from veins draining tumour lesions of a Rouse chicken sarcoma. They reported that the blood in the tumour-draining vein had 23 mg% less glucose and 16.2 mg% more lactate than that found in venous blood draining other tissues. Similar results were obtained from other tumour-bearing animals. Subsequently, Warburg, Wind and Negelein (1926) compared arterio-venous differences of blood glucose and blood lactate for the Jensen rat sarcoma with the jugular renal, ileal and portal areas of the same animal and from this calculated the rate of glycolysis. Their results indicated that the tumour glycolyzed 7% of its dry weight, of glucose per hour at a blood glucose level of 0.2% (200 mg%). As regards the production of lactate, they found that normal tissues lowered the blood lactate level during its passage through them while the sarcoma raised the blood lactate level from 30 to 76 mg%. It has been shown that the presence of intraperitoneal implants of Novikoff hepatoma or Walker 256 carcinosarcoma reduced the blood glucose level of rats with alloxan diabetes (Gorenson and Tilser 1955).

The high rate of glycolysis of the tumour cells *in vivo* was further demonstrated by the studies of Victor and

Potter (1938) who used mice bearing Line I lymphatic leukemia. They observed that in these animals the blood glucose level fell to a value one third of normal during the course of ether anesthesia. More recent studies of Kemp and Mendel (1957), on the rapid rate of diffusion of glucose into the ascitic fluid and of lactate from the ascitic fluid into the blood stream, in the mouse bearing Ehrlich ascites carcinoma, have shown that 14 mg. glucose could diffuse into and 13 mg. lactate, out of 5 ml. of ascitic fluid in one hour. This once again demonstrated the high rate of lactic acid production by tumours in vivo. The above data indicates that in tumours considerable part of the energy must be obtained through glycolysis, and that, unlike normal tissues, neoplastic tissues could survive on the energy supplied by glycolysis.

Measurements of changes in pH of tissues has led investigators to the conclusion that tumour tissues have a higher rate of lactic acid production than do normal tissues. Voegtlin, Kahler and Fitch (1935) have reported that the resting pH of several animal carcinomas and sarcomas, including Jensen rat sarcoma and Flexner-Jobling carcinoma, was about 7.0 but it decreased following injections of glucose, mannose, fructose, xylose or maltose, although unaffected by galactose or lactose. Other workers (Kahler and Robertson 1943) have shown that whereas the normal resting

pH of rat liver is 7.4 and is not changed on the injection of glucose, the rat hepatoma has a resting pH of 7.0 which drops to 6.4 following glucose administration. Eden, Haines and Kahler (1955) by using multiple electrode systems, which allowed them to measure pH changes in a number of areas simultaneously, confirmed earlier results that the pH of tumour tissue drops to around 6.5 on administration of glucose whereas, in the normal tissues there is no drop in the pH.

Tumour Glycolysis in vitro

As in many other fields of biochemistry, the early work on tumour glycolysis in vivo was followed by work on more purified systems, such as tissue extracts and homogenates. Early work in this field had shown that tumour tissues were unable to utilize phosphorylated hexoses at rates comparable to that of glucose utilization and hence they came to the conclusion that glycolysis in tumours was of a non-phosphorylating type. (Tzusuki 1936, Matsuzuki 1933, and Harrison and Mellanby 1930). However, Boyland and Boyland (1938) were able to show rapid glycolysis of phosphorylated intermediates by tumour extracts obtained from Crocker 180 mouse sarcoma, provided ATP and DPN were present. The controversy regarding the type of glycolysis in tumour tissues was finally resolved by Le Page (1948a, b, c, d) who isolated glucose-1-phosphate, glucose-6-phosphate,

fructose-6-phosphate, hexose diphosphate, phosphoglyceric acid, ATP, ADP, etc. from primary mouse carcinoma, rat liver carcinoma, Walker 256 carcinosarcoma, etc. The levels of these phosphorylated derivatives were comparable to the levels found in normal tissues, such as brain, muscle, liver, kidney and heart. In the same year Le Page and Schneider (1948) reported that the soluble fraction of the Flexner-Jobling carcinoma (S_2) was the only fraction which possessed any glycolytic activity by itself, although its activity was greatly enhanced by the addition of nuclei, microsomes or mitochondria. A similar localization of the glycolytic system was reported by these workers for rabbit liver, and confirmed by Kennedy and Lehninger (1949), later for rat liver. Le Page compared the factors affecting the rate of glycolysis in tumours and normal tissues; he (1950) reported that homogenates of Flexner-Jobling carcinoma, Walker 256 carcinosarcoma, Jensen sarcoma, heart, kidney, brain etc. displayed rapid lactate formation from hexose diphosphate; he further reported that both normal and neoplastic tissues could utilize glucose-6-phosphate and fructose-6-phosphate, whereas only liver and tumours could utilize glucose. He concluded that there was a blocking of hexokinase in liver, kidney, diaphragm and skeletal muscle in vitro. Other investigators (Woods et al. 1953, 1955, 1959, ~~1960~~) proposed that the initial phosphorylation

of glucose was of critical importance with regard to the high rate of glycolysis in tumours, and that this initial high rate of glucose phosphorylation was due to a decreased inhibition of mitochondrial hexokinase by anti-insulin hormones. The localization of hexokinase in the mitochondria of Ehrlich ascites cells was observed by Acs, Garzo, Grosz, Molnar, Stephaneck and Straub (1955). More recently Wu and Racker (1959) have reported that in both Ehrlich ascites cells and brain the hexokinase was located in the particulate fraction, although all the other glycolytic enzymes were present in the soluble cytoplasmic fraction. Yushok (1959) made a detailed study of the substrate specificity of the hexokinase of Krebs-2 ascites cells and of brain and reported a great resemblance between the two. Later McComb and Yushok (1959) isolated hexokinase from Krebs-2 ascites carcinoma cells and determined Michaelis constants for 11 sugars as well as the maximum rate of phosphorylation of these compounds and on comparing it with the hexokinase from brain, found that there was no significant difference between the two enzymes.

Thus from the above discussion it could be concluded that, although tumour tissues show a higher rate of aerobic and anaerobic glycolysis as compared to normal tissues, there seems to be no significant difference in either the level of the various glycolytic enzymes or their localization in the cell.

Effect of Tumours on Glycolytic Enzymes in Blood

Levels of enzymes in the blood have been used as indices of many varied pathological conditions. Some work has been done on the effect of cancerous growth in the animal upon the level of glycolytic enzymes with diagnostic intentions.

Aldolase

Of all the glycolytic enzymes, serum aldolase levels have been extensively studied in many clinical disorders, and to a lesser extent in neoplastic diseases. Warburg and Christian (1942) were the first workers to report an elevation of this enzyme in animals with Jensen sarcoma. Later studies of Sibley and Lehninger (1949a, b) and Baker and Govan (1953) have demonstrated increased levels of serum aldolase in patients suffering from neoplastic diseases.

Phosphohexoisomerase

Although increased levels of blood phosphohexoisomerase were reported in tumour-bearing animals by Warburg and Christian in 1942 this enzyme has not attracted much attention. In 1956 Bodansky and Scholler reported an increase in the level of this enzyme during the growth of Walker 256 carcinosarcoma in rats. Israels and Delury (1956) have also found an increase in the level of serum phosphohexoisomerase in patients suffering from chronic myeloctic leukemia although

the same could not be demonstrated in the case of chronic lymphatic leukemia.

Lactic Dehydrogenase

Hill and Levi (1954) reported an elevation in the levels of serum lactic dehydrogenase during neoplastic diseases and pregnancy. Elevation of serum lactic dehydrogenase, following implantation of tumours in mice, has been observed by Hsieh, Suntzeff and Cowdry (1955). Later Hsieh, Mao and Sasananonth (1959) reported that the level of this enzyme declined slowly upon removal of the transplanted tumour; they concluded that the tumour may induce the production of lactic dehydrogenase by the host's normal tissue. There have also been a number of clinical reports showing higher levels of this enzyme in patients suffering from cancer. Lactic dehydrogenase inhibitors have been shown, by Papaconstantinou and Colowick (1960), to inhibit the growth of HeLa cells in tissue culture.

OXIDATIVE METABOLISM

Role of oxidative enzymes in vitro

The presence of enzymes in the tumour tissues for the aerobic oxidation of glucose was suspected long before the complete mechanism of the process was postulated by Krebs and Johnson in 1937. There were, however, at first

many contradictory reports as to the existence and the level of oxidative enzymes in tumour cells.

First reports concerning the presence of cytochrome in neoplastic tissues were given by Bierih and Rosemblohm in 1926 and Yaoi, Tamiya and Nakahara (1928). Both found that the levels of cytochromes in malignant tissues were not appreciably lower than in normal tissues. In 1932 Barron demonstrated the presence of succinic dehydrogenase in slices of Walker 256 carcinosarcoma. However, Elliott, Penoy and Baker (1935) studied lactate and pyruvate metabolism of rat kidney and Philadelphia I sarcoma and found that succinic and malic dehydrogenases and lactic oxidase were not very active in these tissues. They suggested that the initiation of malignancy may well be due to low activities of these enzymes. Oxidation of Kreb's cycle intermediates by tumour-homogenates was studied by Potter, Le Page and Klug (1948) using whole potassium chloride homogenates in medium fortified with ATP, inorganic phosphate, magnesium, cytochrome C and DPN. They found no oxidation of oxalacetate by Walker 256 carcinosarcoma and Flexner-Jobling carcinoma, whereas rat kidney, heart and brain homogenates oxidized this substrate very well. On the other hand, Pardee, Heidelberger and Potter (1950) reported no oxidation of acetate-1-C^{14} by tumour tissues and suggested that tumour tissues lack the oxidative enzymes. A similar suggestion was made by Potter and Busch (1950) because they

did not get accumulation of citrate *in vivo* after fluoroacetate injection into tumour-bearing animals.

In contrast with the early studies cited above, later work has established beyond doubt that the citric acid cycle does operate in tumours. Oxidation (substrate dependent oxygen uptake) with Krebs cycle intermediates was reported in both tumour homogenates and tumour mitochondria by Williams-Ashman, and Lehninger (1951), Williams-Ashman and Kennedy (1952), Siekevitz, Simonsen and Potter (1952), Kielley (1952) and Wenner and Weinhouse (1953). Oxidation of succinate, α-ketoglutarate, glutamate and pyruvate was obtained with various types of tumours such as mouse and rat hepatomas, Jensen sarcoma, Flexner-Jobling carcinoma, Ehrlich ascites tumour, mouse mammary carcinoma, amalanotic melanoma.

Isotopic studies have played a very important role in the elucidation of oxidative pathways in both normal and neoplastic tissues. Olson and Stare (1949) studied the comparative rates of oxidation of C^{14}-labelled pyruvate in slices and homogenates of hepatoma and normal liver, and showed that while slices of hepatoma oxidized pyruvate to CO_2 readily, homogenates of this tumour were inactive. On the other hand, both slices and homogenates of normal liver were able to oxidise pyruvate equally well. Later work of Groth, LePage, Heidelberger and Stoesz (1952) showed that pyruvate-2-C^{14} was oxidized by Flexner-Jobling carcinoma

slices at 25% the rate of oxidation observed in kidney and also proved that the oxidation in tumour takes place through citric acid cycle. Weinhouse, Millington and Wenner (1951) compared the oxidation of C^{14}-labelled glucose, lactate-1-C^{14} and palmitate-1-C^{14} by rat and mouse hepatoma, sarcoma 37, rhabdomyosarcoma, mouse mammary carcinoma and Ehrlich ascites carcinoma with that of rat and mouse liver, kidney, heart, muscle, rat brain and spleen. They found that the tumours could oxidize all these three substrates as rapidly as the normal tissue slices. They used transaconitate, a known inhibitor of aconitase, and obtained an inhibition of glucose and palmitate oxidation, with accumulation of citrate. Later Wenner, Spirtes, and Weinhouse (1952) made a survey of the various enzymes of citric acid cycle in same transplanted tumours as used by Weinhouse et. al. (1951) and provided definite evidence that tumours possessed all the enzymes of the tricarboxylic acid cycle. The enzymes were found to be present at levels comparable to normal tissues, with the exception of aconitase, and α-ketoglutaric dehydrogenase. They suggested that the low activity of these last two enzymes might have been the reason for previous investigators to believe that tumours lacked oxidative enzymes.

In vivo

Busch and his colleagues have studied the operation of the tricarboxylic acid cycle in vivo in tumour-bearing

rats by estimating the radio activity in various Krebs cycle intermediates and by-products such as alanine, aspartate, glutamate, after injecting C^{14}-labelled pyruvate, succinate and glutamate (Busch 1955; Nyhan and Busch 1957; Nyhan and Busch 1958a, b) they observed that normal tissues converted a large percentage of labelled pyruvate to alanine, aspartate and glutamate. On the other hand in tumours, e.g. Flexner-Jobling sarcoma, more than 50% of the labelled pyruvate was converted to lactate. Busch (1955) thus concluded that tricarboxylic acid cycle was not very active in tumours. But subsequent studies of Nyhan and Busch (1957, 1958) with C^{14}-labelled succinate and glutamate in the presence and absence of malonate, a known inhibitor of succinic dehydrogenase, indicated that the citric acid cycle was operative in tumours *in vivo* at rates comparable to those found in normal tissues, and that transformation of pyruvate to amino acids proceeded via the cycle.

Hexose Monophosphate Shunt

There is little doubt that the bulk of carbohydrate oxidation in tumours proceeds via the Embden-Meyerhoff pathway and the citric acid cycle though some oxidation does take place via the hexose monophosphate shunt.

Dickens and Glock (1951) showed that tumour tissues, like liver, kidney and brain could oxidize glucose-6-phosphate, 6-phosphogluconic acid, and ribose-5-phosphate. Later Glock

and McLean (1954) made a detailed survey of the various shunt enzyme systems in ox and rat adrenal gland, rat thymus, spleen, embryo, brain, placenta, and mammary gland and compared them with mouse sarcoma 37, benzpyrene mouse sarcoma, benzpyrene mouse epithilioma, Walker 256 rat carcinosarcoma and found that the enzyme levels in tumour tissues were within the range of those found in normal tissues. Williams-Ashman (1953) has shown the presence of glucose-6-phosphate, and 6-phosphogluconate dehydrogenase activity in acetone powder preparations of Ehrlich ascites carcinoma cells. Villavincencio and Barron (1955) have reported the presence of the enzyme for ribose-5-phosphate metabolism in extracts of Gardner lymphosarcoma. Bosch, van Vals and Emmelot (1956), using cell free extracts, homogenates, and slices of mammary carcinoma, ovarian tumours and a hepatoma, have demonstrated the enzymes, intermediates and reactions of the hexose monophosphate shunt. Studies utilizing glucose-1-C^{14} and glucose-6-C^{14} also have revealed the presence of active shunt mechanism in tumours (Agranoff, Brady and Colodzin 1954). These workers used normal and fasted rat liver, regenerating liver, butter yellow rat hepatoma, and transplanted mouse hepatoma. The presence of the alternate pathway in liver tumours was confirmed by Abraham, Hill and Chaikoff (1955). Emmelot, Bosch and van Vals (1955), Emmelot, Bos and Brombacher (1956) and van Vals, Bosch

and Emmelot (1956), studying induced and spontaneous mammary tumours, hepatoma, ovarian tumours, and a sarcoma, reported the presence of shunt pathway although in some tissues its operation could only be revealed by using malonate. A study of the hexose monophosphate shunt in normal lymphatic tissue and lymphatic tumours was made by Kit (1956), he reported the percentages of CO_2 produced from specifically labelled glucose by various tissues, which were not produced via the Embden-Meyerhoff pathway and therefore, presumably through the shunt. Appendix 11%, thymus 13%, spleen 37%, Gardner lymphosarcoma 32%, Ehrlich ascites cells 23%. He further observed that tumour cells formed 2 to 5 times more pentose from glucose than did normal lymphatic cells. Wenner and Weinhouse (1956) calculated that 2 to 16% of the oxidation of glucose in both normal and tumour tissues proceeded via the hexose monophosphate shunt.

Thus studies of enzyme and coenzyme levels and studies following the oxidation of specifically labelled glucose indicated the presence of an active hexose monophosphate shunt in tumours. Quantitatively, this pathway in tumours appears to be at least as important as in the homologous normal tissues.

AMINO ACID METABOLISM

Free Amino Acid Patterns in Tumours

It has been known for a long time that free amino acids were present in tissues of animals in the post absorptive state at 3 to 9 times their concentration in blood. The patterns are highly characteristic for each tissue of a given organism and are maintained at relatively constant levels despite physiological alterations.

Roberts and Tishkoff (1949) showed that the free amino acid pattern of squamous cell carcinoma was entirely different from that found in the epidermis of normal adult and new born mice. On extending this observation to other tissues, Roberts and Frankel (1949) found that each tissue of normal healthy mouse had a characteristic amino acid pattern, while quite similar patterns of free amino acids were obtained from different types of transplantable and spontaneous tumours.

The possibility that interconversion with amino acids represents a significant metabolic function for intermediates of tricarboxylic acid cycle became apparent during the study of the overall metabolism of acetate-1-C^{14} by Busch, Hurlbert and Potter (1952), Busch (1953) and Busch and Baltrush (1954) in normal and tumour-bearing rats. They showed that a small percentage of the C^{14}-acetate was converted to $C^{14}O_2$ and that the major portion of the radioactivity

was found in amino acids, mainly in glutamic acid. Subsequently Busch and Baltrush (1955) showed that the route of entry of acetate-C^{14} into glutamic acid was through the citric acid cycle. During his study of pyruvate-2-C^{14} metabolism *in vivo*, in rats bearing Flexner-Jobling carcinoma, Walker 256 carcinosarcoma, and Jensen carcinoma, Busch (1955) reported that in non-tumour tissues 30 - 80% of the isotope was transferred to alanine, glutamic acid and aspartic acid after about 1 minute of injection of pyruvate-2-C^{14}, but the incorporation of amino acids in tumour tissues was much slower and lower than in the non-tumour tissues. Kit (1955) reported that on incubation of Gardner lymphosarcoma suspensions with acetate-2-C^{14}, the C^{14} was incorporated into the α-carbon of glycine. Kit and Graham (1956) studied amino acid biosynthesis from C^{14} glucose in the ascitic forms of Mecca and Gardner lymphosarcomas, Ehrlich ascites tumour, lymphatic leukemia (L-5147) and Harding Passey and Cloudman melanomas, *in vitro*, and reported labelling in aspartate, alanine, glutamate, serine and glycine. Later Campbell and Halliday (1957) reported the conversion of C^{14}-glucose into alanine and glutamic acid by rat liver, liver tumours and kidney *in vivo*. Recently Busch, Fujiwara and Keer (1960) studied the conversion of glucose-1-C^{14} in tissues of tumour-bearing rats and found incorporation of C^{14} into lactate, alanine, and glutamate by the Walker 256 carcinosarcoma.

It would seem evident from the above discussion that although the tumour and normal tissues both produce the same amino acids from C^{14} glucose, acetate or pyruvate, there is a quantitative difference between the two types of tissues. The production of glutamate and aspartate from C^{14} glucose both *in vivo* and *in vitro* once again shows that citric acid cycle does operate in tumour tissues.

Amino Acid Requirements in Tissue Culture

Most of our knowledge on amino acid requirements of both normal and tumour tissues has been obtained through studies in tissue culture done mainly in the laboratories of Parker, Morgan and Eagle.

Morgan, Morton and Parker (1950) were the first to prepare complete synthetic media for tissue culture; they studied the nutritional requirements of cultures of leg muscle from 11 days old chick embryo with respect to amino acids, vitamins and other growth factors. Twenty amino acids were found to be essential for the growth of these cells. These included the L-isomers of arginine, histidine, lysine, tyrosine, tryptophane, phenylalanine, cystine, methionine, serine, threonine, leucine, isoleucine, valine, glutamic acid, aspartic, alanine, proline, hydroxyproline, glycine, cysteine and glutamine. However, for the growth of a mouse heart fibroblast strain "L" only 12 amino acids were found to be essential by Eagle (1955a). These were

arginine, histidine, isoleucine, leucine, valine, methionine, phenylalanine, threonine, tryptophane, tyrosine, and glutamine. The amino acid requirements for the growth of HeLa cells, a cell line derived from human epidermoid carcinoma of uterine cervix, were found to be similar to those for strain "L" cells, Eagle (1955b), although the amounts of amino acids required for the HeLa cells were 2 to 4 times higher than those for normal "L" cells. Difference in the numbers of essential amino acids in the medium of Morgan et al. (1950) and Eagle (1955) may be due to the fact that, whereas Eagle's medium is supplemented with 10% serum, there is no serum in Morgan's medium. Hence the lack of added amino acids in the former medium is compensated by the serum. More recently Pasieka, Morton and Morgan (1960) studied the glutamine requirements of both normal and malignant cells and found that this amide was not very readily utilized by cultures of normal cells, but that the tumour cells utilized it more readily.

This requirement for glutamine in the culture of tumour cells was shown by other workers much earlier and is one of the striking differences between normal and cancerous cells in tissue culture.

The results obtained by in vitro studies regarding the glutamine requirements and the growth of tumour tissues seem contrary to the findings of Skipper and Thomson (1958)

on the amino acid requirements of tumour-bearing animals. They reported that growth of tumour was lower when rats bearing sarcoma 180 were fed diets deficient in valine, leucine, isoleucine, histidine and methionine, as compared to animals fed a nutritionally complete diet. Deletion of glutamic acid, glutamine, and aspartic acid had no effect on tumour growth, though omission of these amino acids from the diet resulted in a loss of body weight of the host animal. Hence there was no selective inhibition of tumour growth due to lack of glutamine. This apparent discrepancy between the in vivo and in vitro results regarding the requirement of glutamine for growth of tumours can be explained on the basis of sufficient production of glutamine by the host tissue for tumour growth. Hence making it unnecessary to supply the tumour-bearing animal with extra glutamine.

Glutamine Metabolism in Tumours

Since there seems to be an especially high and specific requirement for glutamine by tumours it would be pertinent to give a short discussion on the various metabolic pathways for glutamine metabolism and its role in tumour tissues.

Glutamine has been a subject of continuous research since its first isolation from beet juice by Schulze and Bosshard (1883). Glutamine was not suspected to occur in mammalian tissue till McIlwain, Fildes, Gladston and Knight

(1939) observed an increase in the growth of Streptococcus haemolyticus by addition of heart muscle extract to the growth medium. The growth promoting factor was identified as glutamine. Since then this amide has been found in several other normal tissues and has been shown to participate in various metabolic reactions.

Glutamine has however never been detected in tumour tissues in the free state. The lack of free glutamine in tumour tissues could be ascribed to a decreased synthesizing ability and to a rapid rate of utilization of this amide. Levintow (1954) assayed a number of tumours for glutamine synthesizing capacity, and in most cases reported a low level of glutamyl transferase activity. Recent studies on the glutamine synthetase and glutamyl transferase activities in hepatomas by Wu and Baur (1960) have revealed a lowering of activity of these enzymes with the progression of the tumours.

Roberts and Borges (1955) studied the levels of free glutamine in developing and regressing tumours and showed synthesis of glutamine in tumours but that the synthetic activity of tumour was exceeded by the rate of utilization. This was shown by using a line of leukemia which grows in two strains of mice, one of which is resistant to leukemia. In this strain the tumour progresses for some time and then starts to regress. No trace of glutamine was

detected in either strains during the tumour progression but as the tumour started to regress, in the resistant strain, free glutamine appeared in the tumour.

Synthetic Reactions of Glutamine

Glutamine has been shown to play an important role in various synthetic processes. Rabinovitz, Olson and Greenberg (1956) showed a stimulation in the incorporation of several amino acids into proteins of Ehrlich ascites carcinoma cells *in vitro*, in the presence of glutamine. In the absence of glutamine, methionine sulfoximine, inhibited the incorporation of amino acids into proteins. The mechanism of inhibition by methionine sulfoximine indicated an interference with the synthesis of glutamine. The role of glutamine in the biosynthesis of proteins in tissue cultures has been investigated by Levintow, Eagle and Piez (1957). They used both C^{14} and N^{15} labelled glutamine and found that this amide was incorporated, without prior degradation, into the proteins of HeLa cells. Their work also showed the incorporation of the carbon chain of glutamine into glutamic acid, aspartic acid, proline and to a smaller extent alanine, serine and glycine.

Levintow (1957) showed the incorporation of the amide group of glutamine into the amide group of asparagine without intermediate formation of free ammonia in HeLa cells grown in tissue culture.

Glutamine has also been shown to play an essential role in the synthesis of purine and pyrimidine constituents of nucleic acids Goldthwait (1956); Levenberg and Buchanan (1957); Hartman and Buchanan (1958); Abrams and Bentley (1959).

Conversion of uridine-5 phosphate to cytidine nucleotides by soluble enzyme systems from rat Novikoff hepatoma has been shown by Hurlbert and Karnmen (1960) to be dependent upon ATP, Mg^{++} and glutamine.

Pathways for glutamine degradation

The degradation of glutamine with formation of ammonia can be brought about in four possible ways; hydrolysis or transfer of γ-glutamyl to an acceptor (γ-glutamyl transferase); phosphate linked hydrolysis (glutaminase I); glutamine transamination, depending upon the presence of α-keto acids (glutaminase II); and by the action of DPN Synthetase on the following reaction:

$$\text{DPN} + \text{Glutamate} \xrightleftharpoons{\text{ATP} + \text{Pi}} \text{Glutamine} + \text{ATP} + H_2O + \text{Desamido DPN}$$

Studies of Kvamme and Svenneby (1961) on the effect of glucose on the utilization of glutamine in Ehrlich ascites carcinoma cells have revealed the presence of glutaminase I with a pH optimum of 9. They also showed that α-keto acids have little effect on the glutamine uptake by cells under aerobic conditions. Glutamine was utilized with the

production of equivalent amounts of ammonia, 50 to 70% appeared as glutamate and only 0.5% as α-ketoglutarate.

Presence of characteristically low levels of glutamine in tumour tissues coupled with the presence of a high rate of glutaminase activity has focused the attention of various workers on the possible disturbance of tumour metabolism by interference with glutamine metabolism as shown by Ayangar and Roberts (1952). γ-L glutamyl hydrazide, an inhibitor of glutamine synthetase, was found to do maximum cytological damage to Yoshida sarcoma cells and also produced changes in the free amino acid patterns, including the appearance of glutamine in both cells and fluid, by Roberts (1960). But the same compound was found to have no effect on Ehrlich ascites carcinoma cells, and had no effect on the tumour growth.

Levels of Glutamine in Tumour-Bearing Animals

White, Ross and McHenry (1954) studied the glutamic acid and glutamine levels in the yolk sacs of chick embryos injected with rat liver carcinoma. They observed that whereas in the control eggs the levels of glutamic acid decreased, the tumour-injected eggs showed a progressive increase in glutamic acid with a concomitant decrease in the level of free glutamine. Later, White, Ozawa, Ross and McHenry (1954) demonstrated a similar change in the glutamic acid and glutamine levels in the plasma of Novikoff

hepatoma and methylcholanthrene-induced tumour-bearing rats. It is of interest to note that in patients suffering from acute myeloblastic, lymphoblastic, and chronic lymphocytic leukemias, the blood plasma levels of glutamine were found to be generally lower than the normal levels (Rouser 1957). Similar lowered plasma glutamine levels were reported by the same author in cases of breast cancer, Hodgkin's disease, lymphosarcoma, and reticulum cell sarcoma.

Simonsen and Roberts (1960) studied changes in the free amino acid patterns during the course of tumour growth in animals and the effect of tumour growth on the free amino acids of host tissues. In studies ranging from 48 hours after injection of Ehrlich tumour cells until 11 days, the tumour showed constant levels of free amino acids. Free glutamine could not be detected in either cells or fluid at any time. Although there was no change in the free amino acid levels of the host tissues for a week after tumour implantation, after 8 days, however there appeared to be a decrease in the glutamine levels of both muscle and liver. The same authors performed similar studies on rats bearing Murphy lymphosarcoma with the same results, except that in this case there was a lowering in the level of glutamine in the kidney after 8 days of tumour implantation. Similar results were also obtained with rats bearing Walker 256 carcinosarcoma by Simonsen and Roberts (1960).

It could be concluded from the above discussion that the free amino acid levels of the host tissues of tumour-bearing animals are generally quite constant and that only in the terminal stages there appears to be alterations; the most frequently observed change being a decrease in free glutamine levels.

Use of Transplantable Tumours

The study of cancer in man is, for obvious reasons, very limited hence most of the experimental study of the disease has been mainly done with laboratory animals. Although clinical observations on cancer were recorded as long ago as 1500 B.C. it was not until the beginning of this century that a systematic and extensive study of cancer was undertaken (Greenstein 1954). The early studies were limited to a few spontaneous tumours, but a great step forward in the understanding of tumour growth came at the end of last century when Jensen succeeded in transplanting a tumour from one rat to another. This opened up a new avenue of approach in the experimental study of cancer and the ready availability of tumour material resulted in extensive use of tumour slices in the biochemical study of cancer (Crabtree 1929; Warburg 1930; Dodds and Greville 1934; Victor and Potter 1935).

Lowenthal and Jahn (1932) innoculated mice intraperitoneally with a suspension of Ehrlich carcinoma cells and obtained an ascites tumour containing a large number of

cells. They found that this ascites tumour was readily transmissible and had a seemingly characteristic invariant growth pattern. An ascites type of tumour has many advantages over a solid tumour, the cells are easily obtained in a relatively pure state, and there is no complicating connective stroma as in solid tumours.

Aim of Present Study

It has been observed by Rusch and his colleagues (c.f. amino acid metabolism) that tumour tissues were not as active as normal tissues in the production of amino acids *in vivo*. Hence it was of interest to compare the activities of normal and tumour tissues in so far as the incorporation of glucose carbon into amino acids is concerned *in vitro*.

In vivo tumour-host relationship with reference to the level of free glutamine has been shown quite clearly in both, animals bearing transplanted tumours as well as in patients suffering from malignant diseases. *In vitro* studies were performed to throw some light on the effect of tumour tissues on the incorporation of glucose into free amino acids of host tissues, and also to find possible reasons for the low levels of glutamine in normal tissues of tumour-bearing animals.

An attempt has also been made to evaluate the role of hexose monophosphate shunt in tumours and the effect of some substances on that pathway.

MATERIALS AND METHODS

Materials

Tumours

1. Ehrlich Ascites Carcinoma Cells

Ehrlich Ascites carcinoma cells have been grown in this laboratory for the past five years by intraperitoneal injection of 0.4 ml. of cell suspension into CF_1, Swiss white mice, the transplantations being done every seven days.

Animals were killed by cervical dislocation, six to seven days after transplantation, and the cells removed with a Pasteur pipette. The peritoneal cavity was washed with ice-cold Kreb-Ringer salt solution, pH 7.4. The suspension was centrifuged at 800 g for twenty seconds; this sedimented the cells. The supernatant was discarded and the cells were resuspended in cold salt solution and recentrifuged. The process was repeated till the cell preparation was free of any perceptible blood contamination. The cells were then centrifuged at 800 g for two minutes to pack them thoroughly; and appropriate dilution of the packed cell was made so as to give 10 mg. dry weight of cells in 0.2 ml. of suspension, which was the quantity used per Warburg vessel for all the experiments.

2. Ehrlich Solid Tumour

Solid tumour was obtained by injecting the ascitic

form subcutaneously into CF_1, Swiss white mice; and they were generally used twelve days after transplantation. The animals were killed by cervical dislocation and the tumour was removed and chilled in crushed ice. Slices weighing approximately 50 to 60 mg. wet weight and of about 0.3 to 0.4 mm. thickness were cut with a chilled Stadie-Riggs tissue slicer (1944). As far as possible the slices from one single tumour were always used for one experiment. If this was not possible then the slices from several tumours were randomly distributed.

3. Melanoma

Melanoma S91 was obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbour, Maine, U.S.A. It was transplanted subcutaneously every four weeks into DBA/1 mice weighing about 15 grams. Tumours used in the experiments were about four weeks old and the slices were prepared in the same manner as described for the Ehrlich Solid tumours.

4. Novikoff Hepatoma

Both solid and liquid types of Novikoff hepatoma were obtained through the courtesy of Dr. V.N. Nigam of the Montreal Cancer Institute, Montreal, Quebec, Canada.

Solid hepatoma was transplanted by injecting a tumour suspension intraperitoneally into hooded rats.

Slices of 7 day old tumours were utilized for experimental purposes.

Liquid hepatoma was obtained by intraperitoneal injection of tumour cells into hooded rats. The suspension of the cells was prepared the same way as described for Ehrlich Ascites cells.

5. Walker 256 Rat Carcinosarcoma

Walker 256 Rat carcinosarcoma was obtained originally from the laboratories of Dr. H. Selye, University of Montreal, Montreal, Quebec, Canada.

Tumour was injected intramuscularly into the hind leg of hooded rats and used ten to twelve days after implantation.

Tumour transplantations were done by Miss I. Cyr; and the author is thankful for it.

Chemicals

All chemicals were of reagent grade and were used without any further purification.

Radioactive Materials

1. Radioactive Glucose

Glucose-1-C^{14}, Glucose-6-C^{14} and Glucose-U-C^{14} were obtained from Radiochemical Centre, Amersham, Buckinghamshire, England.

2. Formate-C^{14}

This was supplied by Merck and Co., Montreal, Quebec, Canada.

3. L-Glutamine-U-C^{14}

This was supplied by Merck and Co., Montreal, Quebec, Canada.

4. L-Glutamic Acid-U-C^{14}

This was obtained from Radiochemical Centre, Amersham, Buckinghamshire, England. L-Glutamic acid-U-C^{14} was in the form of ammonium salt.

Preparation of Solutions

Solutions of radioactive materials were prepared in distilled water so as to give an approximate desired activity. Exact counts per unit volume was determined by counting an aliquot after proper dilution. All radioactive solutions were stored at -20°C.

In the preparation of corresponding carrier solutions, due allowance was made for the contributions from the radioactive material. The carrier solutions were also kept frozen at -20°C.

Incubation Media

A Krebs-Ringer medium of the following composition was used for all incubations: 145 mM NaCl; 1.5 mM KH_2PO_4;

1.5 mM $MgSO_4$; 5.8 mM KCl; and 3.6 mM $CaCl_2$; Phosphate buffer pH 7.4, to give a final concentration of 10 mM per vessel, incubation volume being 1 ml. Final glucose concentration per vessel was 5 mM. The concentrations of all other metabolites used are described in the text.

Methods

Incubation Procedure

All incubations were done in a conventional Warburg manometric apparatus. All solutions were placed in the main chamber of the Warburg vessel. 0.1 ml 20% KOH was put in the centre well which contained a small roll of filter paper. The vessels were gassed for 5 minutes with pure (therapy) oxygen and then placed into the bath at 37°C. After 5 minutes of equilibration the manometers were closed and the initial reading taken. Incubation was carried on for 60 minutes.

Chromatography of Amino Acids

The method for the chromatography of free amino acids, obtained through the oxidation of radioactive substrates, was carried out as described by Kini and Quastel (1959).

After the incubation period the cells were tipped into ice cold Krebs-Ringers solution and centrifuged for two

minutes at 800 g. The sediment was washed once again with cold Krebs-Ringer solution and the walls of the tubes were dried with the help of a roll of Kleenex Tissue. The cells were then suspended in 5 ml. of 95% ethanol and allowed to stand for two hours at 4°C for complete extraction of the amino acids.

The tumour slices, after incubation, were taken out, washed in ice cold water and put in tubes containing 2 ml. of 95% ethanol. The slices were homogenized; and the homogenizer was rinsed with 2 ml. and 1 ml. of 95% ethanol to give a final volume of 5 ml. in each tube. The tubes were kept at 4°C for two hours for the extraction of free amino acids.

After two hours of extraction, the contents of the tubes were centrifuged and the supernatant collected in fresh tubes. The sediment was extracted twice with 1 ml. of 95% ethanol and once with 1 ml. of distilled water and all the washings were pooled. The ethanol extract was dried at 30°C in a current of air. The dried residue was dissolved in 0.5 ml. of 95% ethanol; the sides of the tubes were scratched thoroughly to bring everything into solution. 0.1 ml. (100 µl) was plated on aluminium planchets and counted in a flow counter to determine the total activity of the extract. The remaining ethanolic extract was spotted on 9½" x 9½" sheet of Whatman No. 1 filter paper for two dimensional ascending chromatography.

Phase I

Sec. Butanol: 95% Formic Acid: Water (70:11:17/v/v/v).

Phase II

Liquid Phenol: Water: Ammonia (946 ml.: 106 ml.: 1.7 ml. NH_4OH Sp. Gr. 0.88).

The papers were kept in each phase for twelve hours. After removal from each phase the papers were thoroughly dried.

Chromatograms were then kept in contact with Kodak X-ray film (no Screen), size 10" x 12", in the dark room. The time of exposure depended upon the activity at the origin of the paper. Films were then developed and fixed, using Kodak X-Ray film developer and fixer. After drying, the spots on the film were marked with a wax pencil and the corresponding positions on the chromatogram were traced and counted, using a closed window Tracer lab Geiger-Mueller probe with a mica window 28 mm. in diameter and a thickness of 1.5 to 1.8 mg/cm^2.

All results are expressed as mμ atoms of substrate carbon incorporated per 100 mg. wet weight tissue.

Calculation of mμ atoms of Metabolite Carbon Incorporated

The calculations were done in the following manner. An example of the calculation is as follows:

$$\text{Factor} = \frac{\text{M}\mu \text{ moles glucose carbon} \times 6}{\text{Total activity of glucose u-C}^{14}\text{ (cpm)}}$$

∴ For a 5 mM Glucose in medium

$$\text{Factor} = \frac{6 \times 5000}{200{,}000} = 0.15$$

∴ mμ atoms of glucose carbon incorporated / 100 mg. wet weight tissue

$$= \frac{\text{cpm of individual amino acid spot} \times \text{total activity of alcohol extract} \times 0.15 \times 100}{\text{Total activity on paper} \times \text{tissue weight}}$$

$C^{14}O_2$ Determination

Incubation with tumour slices and ascites cells was carried out as previously described. At the end of the incubation period 0.2 ml. of 30% Trichloroacetic acid from the side arm was tipped into the main chamber, to stop the reaction as well as to liberate bound $C^{14}O_2$. The incubation was continued for another 30 minutes to complete the evolution of $C^{14}O_2$. The alkali soaked filter paper was removed from the centre well and transferred to centrifuge tubes containing 0.4 ml., 1.3% sodium carbonate solution (1 ml. ≡ 25 mg. $BaCO_3$), added as carrier to give a total of approximately 11 mg. barium carbonate. The centre well was washed three times with distilled water and the washings transferred to the centrifuge tubes. The volume in the tubes was made up to about 5 ml. with distilled water, and tightly stoppered and left overnight to elute $K_2C^{14}O_3$ from the filter paper. On the following day the filter papers

were removed from each tube and rinsed with distilled water. 0.2 ml. of 2M Ammonium chloride and 0.5 ml. saturated barium chloride solutions were added to precipitate barium carbonate. The precipitate was washed twice with distilled water and once with acetone and finally resuspended in about 0.2 ml. acetone. The suspension was quantitatively transferred to weighed aluminium planchets (4.3cm^2), dried and counted in a flow end window counter. All activities were corrected for background and for self-absorption of $BaCO_3$.

Ammonia Estimations

Ammonia estimations were done by the method of Fraganca, Quastel and Schucher (1954). After initial incubation period of 1 hour at 37°C the vessels were placed in crushed ice. The brain cortex slices were taken out. The centre well of the Warburg vessel contained a small roll of filter paper soaked with 0.2 ml. of $\frac{1}{3}H_2SO_4$, and 0.3 ml. of saturated K_2CO_3 was added to the sidearm. The vessels were reattached to the manometers and the stoppers closed. The K_2CO_3 solution was then tipped into the main compartment. This raises the pH of the medium to about 10.5 and thus arrests the course of enzyme reaction. Under these conditions the ammonia present in the medium is liberated and is absorbed by H_2SO_4 soaked filter paper. The vessels were kept in the bath for 3 hours, this period of time was found to be

adequate for complete diffusion and absorption of ammonia. The vessels were then removed and filter paper transferred to a test tube containing 2 ml. water. The centre well was washed thrice with distilled water and the washings transferred to the tube. The tubes were stoppered and kept overnight. The filter papers were then taken out and washed with distilled water and the washings added to the tubes. The contents of the tubes were transferred into Folin-Wu tubes for Nesslerization.

Colour was developed by the addition of 1.0 ml. of Nessler solution and 2.0 ml. of 2N NaOH. The intensity of colour was estimated at 425 mμ with a Unicam colourimeter and the ammonia content of the samples was determined from the standard curve which was done concurrently with all experiments. The results are expressed as μgs of ammonia/ 100 mg. wet weight tissue.

CHAPTER I

AMINO ACID FORMATION FROM GLUCOSE BY NEOPLASTIC AND NORMAL TISSUES

INTRODUCTION

There have been many reports on the production of amino acids from C^{14}-labelled glucose, acetate and pyruvate both *in vivo* and *in vitro* by tumour tissues. Busch and his coworkers (1955, 1956) studied the production of amino acids *in vivo* from acetate-1-C^{14} and pyruvate-2-C^{14} by tumour-bearing animals and reported that the major portion of C^{14} gets incorporated into glutamate, alanine and aspartate in normal and tumour tissues, but that the level of C^{14} incorporation in the latter was significantly lower than the former. Later Busch, Fujiwara and Keer (1960) studied the metabolic patterns of animals bearing Walker 256 carcino-sarcoma, after injecting glucose-1-C^{14}, and observed maximum labelling in lactate, followed by alanine and glutamate. Kit (1955) reported the incorporation of acetate-2-C^{14} into the α-carbon of glycine by suspensions of Gardner lympho-sarcoma *in vitro*. Later Kit and Graham (1956) studied the *in vitro* formation of amino acids from labelled glucose in various tumours and reported labelling in aspartate, alanine, glutamate, serine and glycine.

It was therefore thought to be interesting to

compare the amino acid patterns, from glucose, of various tumours with some normal fast-growing as well as slow-growing tissues.

RESULTS

Amino Acid Formation by Tumours

Both liquid and solid types of tumours were incubated for 1 hour in a normal Krebs-Ringer medium in the presence of 5 mM glucose-U-C^{14} and incorporation of glucose carbon into amino acids were determined (Table 1). All results in Table 1 are expressed mμ atoms of glucose carbon incorporated per 100 mg. wet weight tissue.

Ehrlich ascites cells seem to be more active than either their solid form or the other solid tumours used, in incorporating glucose carbon into amino acids. Another interesting point of difference between the Ehrlich ascites carcinoma cells and other solid tumours is their higher incorporation of glucose into alanine.

Amino Acid Formation by Normal Tissues

The amino acid formation by 4-day old chick embryos, rat brain cortex slices, spleen, kidney cortex, liver and 24 hour regenerating liver slices were determined and results are presented in Table II.

Four-day old chick embryos were taken and put into Warburg vessels, after weighing, with the incubation

TABLE I

AMINO ACID FORMATION FROM GLUCOSE BY TUMOUR TISSUES

Amino Acids	Ehrlich Ascites Cells	Ehrlich Solid	Walker 256	Novikoff Hepatoma	Melanoma
Glycine	41 ± 5	55 ± 5	78 ± 8	77 ± 9	29 ± 3
Glutamate	211 ± 30	101 ± 6	250 ± 15	217 ± 20	347 ± 35
Glutamine	Nil	Nil	Nil	Nil	Nil
Aspartate	56 ± 8	24 ± 2	66 ± 4	100 ± 15	121 ± 12
Alanine	495 ± 50	44 ± 4	49 ± 3	169 ± 18	126 ± 10
QO_2	7.8	4.0	5.6	3.0	4.0

All results are expressed as mμ atoms glucose carbon incorporated / 100 mg. wet weight tissue.

Incubation medium as described in "Materials and Methods". All incubations carried out in an atmosphere of O_2.

Values given are the average of at least four experiments.

mixture containing 5 mM glucose-1-C^{14} and incubated for 1 hour in an oxygen atmosphere and the incorporation of glucose carbon into amino acids were determined. All other tissues were sliced with the help of a Stadie-Riggs slicer and slices weighing between 60 - 70 mg. wet weight used.

The results of Table II could be divided into three groups, with respect to their activity in the incorporation of glucose carbon into amino acids. The first group comprising the actively metabolising tissues like chick embryos and rat brain cortex slices. The second consisting of tissues having medium metabolic activity like than in spleen and kidney cortex slices. Lastly liver and regenerating liver slices which have a very low activity.

Both chick embryos and rat brain cortex slices can incorporate glucose carbon into glutamate, glutamine, aspartate and γ-aminobutyric acid, but neither of these tissues show any formation of glycine from glucose. It is however, surprising that there is no incorporation of glucose carbon into alanine by chick embryo.

Spleen and kidney cortex slices incorporate glucose carbon into glutamate, aspartate and alanine but not into glycine or glutamine, or γ-aminobutyric acid. Lack of incorporation into the last mentioned amino acid is not surprising as it is only found in nervous tissues

TABLE II

AMINO ACID FORMATION FROM GLUCOSE BY NORMAL TISSUES

Amino Acids	Chick Embryo	Rat Brain Cortex Slices	Spleen	Kidney Cortex	Rat Liver	Regenerating Rat Liver
Glycine	Nil	Nil	Nil	Nil	Nil	Nil
Glutamate	240 ± 25	1056 ± 44	259 ± 13	205 ± 20	30 ± 2	40 ± 2
Glutamine	300 ± 20	440 ± 21	Nil	Nil	Nil	Nil
Aspartate	100 ± 12	122 ± 10	109 ± 8	52 ± 7	Nil	Nil
Alanine	Nil	190 ± 11	82 ± 10	66 ± 4	Nil	Nil
γ -Amino butyric acid	80 ± 6	300 ± 5	Nil	Nil	Nil	Nil
QO_2	3.2	10.0	6.2	13.5	4.0	6.0

All results expressed as mμ atoms glucose carbon incorporated/100 mg. wet weight tissue.

Incubation procedure same as indicated in Table 1.

Liver and regenerating liver slices show incorporation into glutamate only, although it is very much lower than other tissues. This could be due to the reason that most of the glucose in liver tissue goes to form glycogen.

EFFECTS OF VARIOUS COMPOUNDS ON AMINO ACID FORMATION BY TUMOUR TISSUES

It is seen from the results presented in Tables I and II that tumour tissues in general have a lower capacity for incorporating glucose carbon into amino acids, than the two actively metabolising tissues namely chick embryos and rat brain cortex slices. Hence an attempt was made to see whether providing amino group donors or pyridoxal, which is implicated in transamination reactions, could increase the levels of incorporation into amino acids by tumour tissues.

Effects of Glutamate

5mM glutamate was added to the incubation medium in addition to 5mM glucose and results obtained with Ehrlich ascites carcinoma cells and Ehrlich solid tumour slices are presented in Tables III and IV. It may be observed that there is no effect whatsoever of added glutamate on the amino acid formation by either tumours. This may be due to the possibility that enough extra cellular glutamate cannot enter the tumour tissues as seen from the results obtained with glutamate-1-C^{14} and presented in Table III. There is

TABLE III

EFFECT OF GLUTAMATE ON AMINO ACID FORMATION BY EHRLICH ASCITES CARCINOMA CELLS

Amino Acids	5mM Glucose-U-C^{14}	5mM Glucose-U-C^{14} + 5mM Glutamate	5mM Glutamate-U-C^{14}	5mM Glutamate-U-C^{14} + 5mM Glucose
Glycine	38 ± 2	49 ± 5	Nil	Nil
Glutamate	215 ± 30	225 ± 40	547 ± 67 (65%)	1397 ± 19 (87%)
Glutamine	Nil	Nil	Nil	Nil
Aspartate	57 ± 6	51 ± 4	287 ± 39	128 ± 12
Alanine	500 ± 49	600 ± 65	Nil	Nil

All results expressed as mμ atoms glucose carbon incorporated/loo mg. wet weight tissue. Incubation procedure same as before.

Figures in parenthesis represent percent of total glutamate-U-C^{14} into the cells remaining as glutamate.

TABLE IV

EFFEDT OF GLUTAMATE ON AMINO ACID FORMATION BY EHRLICH SOLID CARCINOMA TISSUE

Amino Acids	5mM Glucose-U-C^{14}	5mM Glucose-U-C^{14} + 2mM Glutamate	5mM Glucose-U-C^{14} + 5mM Glutamate
Glycine	55 ± 5	51 ± 4	60 ± 7
Glutamate	101 ± 4	98 ± 9	109 ± 10
Glutamine	Nil	Nil	Nil
Aspartate	24 ± 2	22 ± 4	24 ± 3
Alanine	44 ± 4	60 ± 7	39 ± 4

Results expressed as mμ atoms glucose carbon incorporated/100 mg. wet weight tissue.

Incubation procedure same as before.

higher transport of glutamate in the presence of glucose than in its absence, but there is more breakdown of glutamate without glucose, to give aspartate, than in its presence.

Effects of Ammonium Chloride

Effects of various concentrations of ammonium chloride on the incorporation of glucose carbon into amino acids are presented in Table V. 1 mM NH_4Cl gives a stimulation of 89% in glycine formation whereas the incorporation into other amino acids remain about the same as in the controls. But when the concentration of NH_4Cl is increased to 2.5 or 5.0 mM, glycine formation is completely inhibited and so is the incorporation of glucose carbon into aspartate. Although the formation of glutamate remains about the same as in the controls, in the presence of 2.5 mM NH_4Cl, it decreases by about 48% at a NH_4Cl concentration of 5.0 mM.

Effects of Pyridoxal

Since melanoma and Ehrlich solid tumours show a lower transaminating activity than Ehrlich ascites cells, it was thought to be of interest to see whether supplying pyridoxal might bring up the levels of amino acids in these tumours. 2 mM pyridoxal was used in the presence of 5 mM glucose-^{1}-C^{14} as a substrate. It can be seen from Table VI that in the case of melanoma there is about 37% increase in glycine formation accompanied with a decrease of 33%

TABLE V

EFFECT OF AMMONIUM CHLORIDE ON AMINO ACID FORMATION BY EHRLICH ASCITES CARCINOMA CELLS

Amino Acids	5 mM Glucose-U-C^{14}	5mM Glucose-U-C^{14} + 1mM NH_4Cl	5mM Glucose-U-C^{14} + 2.5mM NH_4Cl	5mM Glucose-U-C^{14} + 5mM NH_4Cl
Glycine	41 ± 5	78 ± 10	Nil	Nil
Glutamate	211 ± 30	232 ± 31	245 ± 20	109 ± 9
Glutamine	Nil	Nil	Nil	Nil
Aspartate	56 ± 8	48 ± 6	Nil	Nil
Alanine	495 ± 50	534 ± 29	547 ± 50	Nil

All results expressed as mμ atoms of glucose carbon incorporated/100 mg. wet weight tissue.

Incubation media same as before.

TABLE VI

EFFECT OF PYRIDOXAL ON AMINO ACID FORMATION BY TUMOURS

Amino Acids	Melanoma		Ehrlich Solid Tumour	
	5mM Glucose-U-C^{14}	5mM Glucose-U-C^{14} + 2mM Pyridoxal	5mM Glucose-U-C^{14}	5mM Glucose-U-C^{14} + 2mM Pyridoxal
Glycine	29 ± 3	40 ± 5	55 ± 5	17 ± 2
Glutamate	347 ± 35	150 ± 11	101 ± 4	70 ± 6
Glutamine	Nil	Nil	Nil	Nil
Aspartate	121 ± 12	80 ± 6	24 ± 2	30 ± 5
Alanine	126 ± 10	180 ± 20	44 ± 4	20 ± 2

All results are expressed as mμ atoms of glucose carbon incorporated/100 mg. wet weight tissue.

Incubation procedure same as before.

and 56% in the incorporation of glucose carbon into aspartate and glutamate respectively. Alanine increases by about 42% in the presence of 2 mM pyridoxal. The results obtained with Ehrlich solid tumour slices are quite different from the ones obtained with melanoma. There is lower incorporation of glucose carbon in all the amino acids in the presence of 2mM pyridoxal.

Effects of Sodium Bicarbonate

The low incorporation of glucose carbon into amino acids by tumours could possibly be due to a shift in intracellular pH owing to a high amount of lactic acid production, which may not be taken care of by the phosphate buffer. Hence sodium bicarbonate was tried at various concentrations in the presence of phosphate buffer and the results are presented in Table VII.

It is seen from the data that the formation of glycine and alanine are completely inhibited by all of the three concentrations of $NaHCO_3$. Aspartate remains unaffected in the presence of 0.5mM concentration of $NaHCO_3$ but it is inhibited by 1.0 and 2.0mM concentrations. Maximum increase in the incorporation of glucose into glutamate is obtained in the presence of 0.5mM $NaHCO_3$. If we examine the amount of incorporation of glucose into lactate, which of course is not very reliable as lactate leaks out into the medium quite readily, it would seem that there is an increase in the level

TABLE VII

EFFECT OF Na-BICARBONATE ON AMINO ACID FORMATION BY EHRLICH ASCITES CARCINOMA CELLS

Amino Acids	5mM Glucose-U-C^{14}	5mM Glucose-U-C^{14} + 0.5mM $NaHCO_3$	5mM Glucose-U-C^{14} + 1mM $NaHCO_3$	5mM Glucose-U-C^{14} + 2mM $NaHCO_3$
Glycine	41 ± 5	Nil	Nil	Nil
Glutamate	211 ± 30	400 ± 50	340 ± 25	260 ± 30
Glutamine	Nil	Nil	Nil	Nil
Aspartate	56 ± 8	60 ± 6	Nil	Nil
Alanine	495 ± 50	Nil	Nil	Nil
Lactate	950 ± 80	1440 ± 150	1700 ± 125	1860 ± 185

All results are expressed as mμ atoms glucose carbon incorporated/100 mg. wet weight tissue.

Incubation procedure same as before.

of glucose carbon incorporation into lactate with increase in bicarbonate concentration.

Discussion

It is evident from the results presented above, that tumour tissues, irrespective of their site of origin, incorporate glucose carbon into the same amino acids, although there may be quantitative difference between tumours. Although neoplastic tissues are fast growing, their efficiency of amino acid formation from glucose is much lower than slow growing tissues like brain cortex or even a fast growing tissue like chick embryo. This observation regarding lower capability of tumour tissues to synthesise amino acids is in keeping with results obtained by Busch (1955) in his studies *in vivo* with C^{14} labelled acetate, pyruvate and succinate, where he reported a lower level of incorporation of C^{14} into glutamate, aspartate, and alanine by tumour tissues than by their normal counterpart. The low *in vitro* activity reported above does not seem to be due to lack of endogenous amino acids for transamination with α-keto acids, viz. pyruvate, α-keto glutarate or oxalacetate. This is quite evident from the results obtained in the presence of added glutamate in the medium. Although the amount of glutamate transported into the tissues is not very high, yet if there was a deficiency of amino group donor for transaminating system this low amount of glutamate should have given higher

incorporation into aspartate, through glutamate - oxalacetate transaminase and also there should have been higher levels of alanine, through glutamate - pyruvate transaminase. But there was no difference observed between the control and glutamate system, thereby showing that low activity of transamination is not due to lack of glutamate or the amino group donor. It could be argued that probably the tumours cannot utilize bound group of amino acids and that they may need free amino group for transaminase activity. This possibility could also be ruled out from the results obtained with ammonium chloride. With increasing concentration of ammonium chloride, the amino acid formation seems to get lower, except in the case of glycine which shows an increase of 89% in the presence of 1.0mM NH_4Cl but with an increase in concentration of NH_4Cl to 2.5mM ot 5.0mM, glycine is completely inhibited. The inhibitory action of higher concentrations of NH_4Cl might be due to a toxic effect of NH_4Cl on the tissues. The effect of pyridoxal on the two tumours used indicates that in melanoma, this cofactor has some effect on the glycine and alanine production but not on glutamate or aspartate, but the same is not true for Ehrlich solid tumour. This increase in glycine and alanine formation in melanoma may be due to some effect on the glycolytic pathway. But the citric acid cycle does not seem to be affected. In conclusion it may be said that the lower

capability for incorporating glucose into amino acids may not be due to lack of either amino group donator or cofactors but it might well be due to a slow operation of the citric acid cycle.

The effect of sodium bicarbonate on the amino acid formation is quite interesting. Although bicarbonate apparently increases glycolysis, as seen from the increase in lactate production, it suppresses glucose incorporation into amino acids. Nissen (1960) has shown that increased CO_2 tension lowers the formation of oxalacetate in Ehrlich ascites cells and thus interferes with the operation of the Tricarboxylic acid cycle. Hence it may be possibly due to the above-mentioned phenomenon, that there is a complete inhibition of amino acid formation at higher concentrations of $NaHCO_3$.

There are two well-marked differences between the tumour tissues used and chick embryos or rat brain cortex slices; whereas both the normal tissues have quite a high incorporation of glucose into glutamine, there is no indication of the same in the neoplastic tissues. This result can be substantiated with the findings of Simonsen and Roberts (1960) and of Kit and Graham (1956) who were not able to detect any glutamine in tumours either <u>in vivo</u> or <u>in vitro</u>. This lack of glutamine could be due to the presence of high glutaminase activity reported by Rabinovitz,

Olson and Greenberg (1956), or it may even be due to a lower synthesising capacity of tumours reported by Wu and Baur (1960). Results presented later in the thesis may tend to indicate that there may be higher breakdown, rather than low synthesis of glutamine.

The other point of difference between the two principal normal tissues used, namely chick embryo and brain cortex slices and the tumour tissues, is the glycine formation by ~~by~~ neoplastic tissues and its absence in normal tissues under our conditions. It is quite surprising that although Kit (1955) reported incorporation of acetate-2-C^{14} into both serine and glycine, we in our experiments failed to detect any serine. This might be because the spots of glycine and serine come very near to each other in the solvents used.

Only one aspect of difference between normal and neoplastic tissues, namely lack of glutamine formation by tumour tissues is studied to some extent and is reported in the subsequent chapter. The implication of this phenomenon on normal tissues (brain cortex slices) in vitro is also reported.

SUMMARY

1. Ehrlich ascites carcinoma cells, Ehrlich solid tumour, melanoma, Novikoff hepatoma and Walker 256 carcinosarcoma have been shown to form glycine, glutamate, alanine and aspartate, from glucose.
2. Rat brain cortex slices and 4-days old chick embryo have been shown to produce glutamate, aspartate, glutamine and γ-aminobutyric acid from glucose. Whereas the rat brain cortex slices show incorporation of glucose carbon into alanine the chick embryo does not.
3. Spleen and kidney cortex slices show incorporation of glucose carbon into glutamate, aspartate and alanine. Rat liver and regenerating rat liver slices do not incorporate glucose carbon into any other amino acid other than glutamate.
4. Addition of L-glutamate, ammonium chloride or pyridoxal to the medium does not increase the incorporation of glucose carbon into various amino acids by tumour tissues.
5. Addition of sodium bicarbonate to the incubation medium increases the glycolysis, but decreases the formation of amino acids from glucose by tumour tissues.

CHAPTER II

EFFECTS OF TUMOUR TISSUE ON GLUTAMINE FORMATION FROM GLUCOSE BY RAT BRAIN CORTEX SLICES IN VITRO

INTRODUCTION

The effects of tumour tissues on the free glutamine levels of different tissues of tumour-bearing animals have been a subject of some study. White et al. (1954) reported a change in the levels of glutamate and glutamine in the plasma of rats bearing Novikoff hepatoma and methylcholanthrene induced tumours. They found an increase in the amount of plasma glutamate with a concomitant decrease in the level of plasma glutamine. Clinical reports have also shown a greatly reduced concentration of glutamine in blood plasma of patients suffering from lymphoblastic and chronic lymphocytic leukemias (Rowser 1957). Recent studies of Simonsen and Roberts (1960) have indicated a decrease in free glutamine levels in both muscle and liver of mouse bearing Ehrlich ascites carcinoma. Similar results were also obtained, by the same authors, in cases of rats bearing Murphy lymphosarcoma or Walker 256 carcinosarcoma. Wu and Baur (1960) have observed a progressive decrease in the concentrations of free glutamine in plasma, liver and muscle, with increase in the size of Walker 256 carcinosarcoma in

rats. They have ascribed this decrease to a reduced synthesis of glutamine. Therefore, it appears that the presence of tumours in the animals depletes the host tissue of glutamine. It was therefore of interest to see if the same effect could be obtained when rat brain cortex slices, a potent producer of glutamine (c.f. Table II), are incubated in the presence of tumour tissues *in vitro*.

The results presented in this chapter confirm the findings *in vivo* and a possible mechanism for this phenomenon is discussed.

RESULTS

Amino Acid Formation from Glucose by Tissues from Normal and Tumour-bearing Rats

Brain cortex, spleen and kidney cortex slices, from normal and rats bearing liquid hepatoma, were examined for amino acid formation from glucose-U-C^{14} (5mM) *in vitro*.

It is seen in Table VIII that the presence of hepatoma increases the formation of glutamate and aspartate from glucose by brain cortex slices, by 39% and 73% respectively, and decreases the incorporation of glucose carbon into glutamine by 25%, when compared to the values obtained from the brain cortex slices from normal rats. No significant difference in the formation of alanine or γ-aminobutyric acid, from glucose, was found in the brain cortex slices from tumour-bearing rats.

TABLE VIII

AMINO ACID FORMATION FROM GLUCOSE BY TISSUES FROM NORMAL AND RATS BEARING LIQUID NOVIKOFF HEPATOMA

Amino Acids	Brain cortex slices		Spleen slices		Kidney cortex slices	
	Normal	Hepatoma	Normal	Hepatoma	Normal	Hepatoma
Glycine	Nil	Nil	Nil	Nil	Nil	Nil
Glutamate	1056 ± 44	1475 ± 24	256 ± 8	258 ± 6	200 ± 5	92 ± 3
Glutamine	440 ± 21	333 ± 6	Nil	Nil	Nil	Nil
Aspartate	122 ± 10	212 ± 12	114 ± 5	127 ± 2	50 ± 2	33 ± 4
Alanine	190 ± 11	185 ± 10	82 ± 10	117 ± 3	68 ± 2	76 ± 5
γ-amino butyric acid	300 ± 15	300 ± 12	Nil	Nil	Nil	Nil
QO_2	10.0	10.0	5.0	7.0	13.5	12.0

All results are expressed as mμ atoms of glucose carbon incorporated/100 mg. wet weight tissue.

Incubation medium same as given in "Materials and Methods", with 5mM glucose-1-C^{14}. Incubations done in presence of pure oxygen phase.

The incorporation of glucose carbon into glutamate and aspartate in spleen slices is not affected by the presence of tumour *in vivo* but the formation of alanine is increased by 40% in the spleen slices of tumour-bearing animals.

With the kidney cortex slices the results are opposite to those obtained with spleen. Kidney cortex slices from tumour-bearing rats show increased incorporation of glucose carbon into glutamate and aspartate by 52% and 34% respectively. There is, however, no difference in the formation of alanine between normal kidney cortex slices and those from rats bearing hepatoma.

Effect of Ehrlich Ascites Carcinoma Cells on Amino Acid Formation from Glucose by Normal Rat Brain Cortex Slices *in vitro*.

The data obtained with rat brain cortex slices indicate that the presence of tumour lowers the incorporation of glucose carbon into glutamine, hence an effort was made to find out whether there would be any effect of tumour tissues on the glutamine formation from glucose by brain cortex slices *in vitro*.

Brain cortex slices from normal rats were incubated for 1 hour in the presence of Ehrlich ascites carcinoma cell suspensions with 5mM glucose-U-C^{14}. At the end of the incubation period the brain cortex slices were removed, rinsed in chilled distilled water and the amino acids formed

were determined. The Ehrlich ascites cells were analysed separately for their amino acid patterns from glucose. In all subsequent discussion of results the increase or decrease is in relation to the values obtained from the experiments where the two tissues were incubated separately.

It is seen from Table IX that the level of incorporation of glucose carbon into glutamate and aspartate in the brain cortex slices is unaffected by the presence of Ehrlich ascites cells. However, the presence of carcinoma cells decreases the formation of glutamine, from glucose by brain cortex slices, by nearly 50%, and increases the incorporation into alanine by 155%.

On the other hand, the presence of brain slices produces an increased incorporation of glucose into glutamate, aspartate and alanine of tumour cells by 272%, 28% and 63% respectively. There is, however, a decreased incorporation into glycine.

The inhibitory effect on glutamine formation from glucose by the rat brain cortex slices is lost when Ehrlich ascites cells are heated for 10 minutes at 100°C before incubation.

In another set of experiments ascites cells were disrupted by repeated freezing and thawing and the supernatant fluid was incubated with rat brain cortex slices. The results obtained are reported in Table IX. Brain

TABLE IX

EFFECT OF EHRLICH ASCITES CARCINOMA CELLS ON THE AMINO ACID FORMATION BY RAT BRAIN CORTEX SLICES

Amino Acids	Ehrlich Ascites Cells	Brain Cortex Slices	Brain Slices + Ascites Cells		Heated Ascites Cells + Brain Slices	Soluble fraction + Brain Slices
			Brain	Ascites Cells	Brain	Brain
Glycine	41 ± 5	Nil	Nil	26 ± 4	Nil	Nil
Glutamate	211 ± 30	1056 ± 44	1065 ± 25	785 ± 25	1190 ± 35	1383 ± 58
Glutamine	Nil	440 ± 21	220 ± 5	Nil	427 ± 22	298 ± 25
Aspartate	56 ± 8	157 ± 12	172 ± 21	70 ± 5	245 ± 20	207 ± 22
Alanine	495 ± 50	190 ± 11	485 ± 10	805 ± 60	265 ± 15	267 ± 9
γ-amino butyric acid	Nil	300 ± 15	410 ± 10	Nil	270 ± 15	228 ± 19
QO_2	7.8	10.0	10.2		9.5	9.0

All results are expressed as mμ atoms of glucose carbon incorporated/100 mg. wet weight tissue.
Incubation mixture as before. Brain Cortex slices were incubated in the presence of Ehrlich Ascites cell suspension for 1 hour then cortex slices and Ascites cells were separately analysed for amino acids.

cortex slices show an increase in the incorporation of glucose into glutamate, aspartate and alanine by 30%, 31% and 40% respectively. But the formation of glutamine from glucose is decreased by 48%.

Effect of Solid Ehrlich Carcinoma

In view of the results obtained with Ehrlich ascites carcinoma cells it was thought of interest to see if the reduction in the formation of glutamine from glucose by brain cortex slices could also be brought about in the presence of solid tumours.

It is seen from the results in Table X that the incubation of brain cortex slices with solid Ehrlich tumour slices causes a decreased formation of glutamine, from glucose, by 66% in the brain slices. The glucose incorporation into glutamate and aspartate of brain cortex slices remains unchanged but the incorporation into alanine increases by 37%.

Just as the Ehrlich ascites cells, the solid Ehrlich tumour slices show an increase in the formation of glutamate, from glucose, by 221% and of aspartate and alanine by 25% and 38% respectively. Glycine formation is inhibited by 43% in the tumour slices.

Effect of Walker 256 carcinosarcoma

The effect of Walker 256 carcinosarcoma slices on

TABLE X

EFFECT OF EHRLICH SOLID CARCINOMA SLICES ON AMINO ACID FORMATION BY RAT BRAIN CORTEX SLICES

Amino Acids	Tumour Slices	Brain Cortex Slices	Tumour + Brain Cortex Slices	
			Brain	Tumour
Glycine	55 ± 5	Nil	Nil	31 ± 1
Glutamate	101 ± 3	1174 ± 70	1037 ± 24	325 ± 20
Glutamine	Nil	389 ± 9	234 ± 6	Nil
Aspartate	24 ± 2	149 ± 10	156 ± 4	30 ± 3
Alanine	44 ± 4	200 ± 15	275 ± 12	60 ± 2
γ-amino butyric acid	Nil	300 ± 15	360 ± 15	Nil
QO_2	4.0	11.0	6.0	

All results are expressed as mμ atoms glucose carbon incorporated/100 mg. wet weight tissue.
Incubation technique same as in Table IX.

brain cortex slices are similar to the ones obtained with the two types of neoplastic tissues reported above.

Brain cortex slices show a decreased incorporation of glucose carbon, into glutamine of 47% (Table XI) in the presence of tumour tissue slices. There is, however, no effect on the formation of glutamate or aspartate from glucose, but alanine was increased by 25%.

Tumour slices on the other hand show an increase in the incorporation of glucose into glutamate, aspartate and alanine by 212%, 139% and 89% respectively. Glycine formation from glucose is completely inhibited due to the presence of brain cortex slices.

Effect of Normal Tissues

Having observed the reduction of formation of glutamine in the presence of tumour tissues, it deemed interesting to find out if this phenomenon was characteristic of only neoplastic tissues or any other normal tissue would give a similar picture with rat brain cortex slices. In Chapter I (c.f. Table II) it was observed that like tumour tissues, both spleen and kidney cortex slices failed to show any incorporation of glucose carbon into glutamine. Hence these tissues were chosen to study effects of normal tissues on glutamine formation from glucose on rat brain cortex slices *in vitro*.

TABLE XI

EFFECT OF WALKER 256 CARCINOSARCOMA SLICES ON AMINO ACID FORMATION BY RAT BRAIN CORTEX SLICES

Amino Acids	Tumour Slices	Brain Cortex Slices	Tumour + Brain Cortex Slices	
			Brain Slices	Tumour Slices
Glycine	75 ± 8	Nil	Nil	Nil
Glutamate	250 ± 15	1056 ± 44	931 ± 10	780 ± 28
Glutamine	Nil	440 ± 21	231 ± 4	Nil
Aspartate	66 ± 4	157 ± 12	148 ± 5	158 ± 3
Alanine	49 ± 3	190 ± 11	239 ± 6	90 ± 12
γ-amino butyric acid	Nil	300 ± 15	265 ± 4	Nil
QO_2	5.6	10.0	9.5	

All results are expressed as mμ atoms glucose carbon incorporated/100 mg. wet weight tissue.

Incubation procedure same as in Table IX.

Effect of Rat Kidney Cortex Slices

The effects of kidney cortex slices on rat brain cortex slices are reported in Table XII.

It is seen from the table that the presence of the kidney cortex slices have a stimulatory effect on the incorporation of glucose carbon into the amino acids of the rat brain cortex slices, except in the case of γ-amino-butyric acid where a decrease of 29% is obtained. Formation of glutamine from glucose under such conditions, unlike the results observed with neoplastic tissues, is increased by 10%.

Effect of Rat Spleen Slices

It is seen from results presented in Table XIII that the presence of spleen slices had no significant effect on the formation of glutamine, from glucose, by rat brain cortex slices.

From the results obtained on the effects of various tissues on the glucose carbon incorporation into glutamine by rat brain cortex slices *in vitro*, it appears that the decreased level of incorporation of glucose into glutamine by brain tissue is brought about by tumours only and is a characteristic of tumour tissues.

Various factors were investigated to find by what mechanism tumour cells brought about this decrease in the

TABLE XII

EFFECT OF RAT KIDNEY CORTEX SLICES ON AMINO ACID FORMATION BY RAT BRAIN CORTEX SLICES

Amino Acids	Kidney Cortex Slices	Brain Cortex Slices	Kidney Cortex + Brain Cortex	
			Brain	Kidney
Glycine	Nil	Nil	Nil	Nil
Glutamate	205 ± 10	992 ± 61	1265 ± 65	430 ± 15
Glutamine	Nil	400 ± 22	440 ± 5	Nil
Aspartate	52 ± 8	140 ± 6	212 ± 12	80 ± 4
Alanine	34 ± 2	164 ± 14	436 ± 18	70 ± 6
γ-amino butyric acid	Nil	256 ± 8	180 ± 20	Nil
QO_2	14.5	10.0	12.0	

All results are expressed as mμ atoms glucose carbon incorporated/100 mg wet weight tissue.

Incubation procedure same as in Table IX.

TABLE XIII

EFFECT OF RAT SPLEEN SLICES ON AMINO ACID FORMATION BY RAT BRAIN CORTEX SLICES

Amino Acids	Spleen Slices	Brain Cortex Slices	Spleen + Brain Cortex Slices	
			Brain	Spleen
Glycine	Nil	Nil	Nil	Nil
Glutamate	259 ± 13	1174 ± 70	1081 ± 109	400 ± 40
Glutamine	Nil	389 ± 9	360 ± 10	Nil
Aspartate	109 ± 8	117 ± 10	187 ± 10	121 ± 3
Alanine	82 ± 10	255 ± 35	376 ± 20	60 ± 4
γ-amino butyric acid	Nil	262 ± 11	216 ± 16	Nil
QO_2	6.0	11.0	9.0	

All results expressed as mμ atoms glucose carbon incorporated/100 mg. wet weight tissue.

Incubation techniques as described in Table IX.

incorporation of glucose carbon into glutamine by rat brain cortex slices *in vitro*. It is possible that this apparent decreased formation of glutamine from glucose, may be caused by a leakage of this amide from the brain tissue into the surrounding medium rather than a partial inhibition of synthesis. This is substantiated by the observations that with a decreased formation of glutamine in brain slices there was a concomitant increase in tumour glutamate and aspartate.

From the experiments with effects of boiled ascites cells on brain slices it is evident that the glutamine lowering phenomenon is linked with viability of the cells or in other words, linked with some enzymatic process. It has also been shown above the activity is located in the supernatant of the disrupted ascites cells. Warburg and other workers have shown that tumour has a very high rate of aerobic glycolysis and that tumour tissues can utilise high amounts of glucose. Hence the first possibility tried was to see whether the glutamine leakage from the brain cortex tissue in the presence of neoplastic tissue might be due to a lack of glucose for proper metabolism of brain tissue slices.

Effect of various concentrations of Glucose

The effects of various concentrations of glucose on amino acid formation are reported in Tables XIV, XV and XVI.

In the presence of 1mM glucose-U-C^{14} there is a decreased incorporation of glucose carbon into all the amino acids in both tumour tissue as well as the brain cortex slices. The presence of Ehrlich ascites cells decreases the formation of glutamine from glucose by 81% and increases the alanine by 76%, but the incorporation of glucose into glutamate and aspartate are unaffected (Table XIV).

On the other hand, under the same conditions, Ehrlich ascites cells show an increased incorporation into glutamate and aspartate by 98% and 30% respectively. However, alanine formation from glucose goes down by 36%.

In the presence of tumour with 3mM glucose-U-C^{14}, the incorporation of glucose carbon into glutamine goes down by 44%, whereas the formation of alanine goes up by 144% in the brain cortex slices. There is, however, no change in the formation of glutamate and aspartate from glucose, by the brain tissue.

Tumour cells under similar conditions show an increased formation of glutamate, aspartate and alanine by nearly 190%, 50% and 51% respectively. However, the formation of glycine in tumour cells remains unaffected by the presence of brain tissue.

Results obtained with 5mM glucose are similar to the ones described before (Table XV).

TABLE XIV

EFFECT OF VARIOUS CONCENTRATIONS OF GLUCOSE ON AMINO ACID FORMATION OF EHRLICH ASCITES CELLS AND RAT BRAIN CORTEX SLICES

	1.0mM Glucose				3.0mM Glucose			
			Ehrlich Cells + Brain Slices				Ehrlich Cells + Brain Slices	
Amino Acids	Ehrlich Cells	Brain Slices	Brain	Ehrlich Cells	Ehrlich Cells	Brain Slices	Brain	Ehrlich Cells
Glycine	13 ± 2	Nil	Nil	11 ± 1	12 ± 1	Nil	Nil	11 ± 1
Glutamate	71 ± 2	406±11	367±14	141± 3	51 ± 4	495±15	481±12	148± 8
Glutamine	Nil	75 ± 5	14 ± 2	Nil	Nil	139± 5	77 ± 4	Nil
Aspartate	13 ± 3	112± 7	111± 8	17 ± 2	20 ± 3	83 ± 4	88 ± 9	30 ± 1
Alanine	152±20	47 ± 5	83 ± 4	111± 8	136±10	45 ± 2	110± 3	206± 3
γ-amino butyric acid	Nil	77 ± 6	65 ± 4	Nil	Nil	61 ± 8	76 ± 6	Nil

All results expressed as mμ atoms glucose carbon incorporated/100 mg. wet weight tissue.

Incubation procedure same as in Table IX.

TABLE XV

EFFECT OF VARIOUS CONCENTRATIONS OF GLUCOSE ON THE AMINO ACID FORMATION BY EHRLICH ASCITES CELLS AND RAT BRAIN CORTEX SLICES

	5mM Glucose				10mM Glucose			
			Ehrlich + Brain				Ehrlich + Brain	
Amino Acids	Ehrlich Cells	Brain Slices	Brain	Ehrlich Cells	Ehrlich Cells	Brain Slices	Brain	Ehrlich Cells
Glycine	50 ± 6	Nil	Nil	26 ± 3	1120±56	Nil	Nil	720±37
Glutamate	189±12	1141±29	1129±99	600±15	186± 8	1267±97	1291±101	272±15
Glutamine	Nil	425±30	173±6	Nil	Nil	468±30	554±28	Nil
Aspartate	42 ± 2	138±13	120±3	65 ± 4	35 ± 4	166± 9	144 ± 3	48 ± 0
Alanine	450±27	152±12	520±32	730±19	494±31	290±15	755± 0	344±24
γ-amino butyric acid	Nil	275±20	383±16	Nil	Nil	302±4	574±36	Nil

All results expressed as mμ atoms glucose carbon incorporated/100 mg. wet weight tissue.

Incubation procedure same as in Table IX.

When the results of 10mM and 20mM concentrations are considered (Tables XV and XVI) it is seen that the incorporation into glutamine by brain cortex slices increases, even in the presence of Ehrlich carcinoma cells, the increase being 18% and 25% respectively, but glutamate and aspartate values remain the same as in the controls.

Tumour cells under the same conditions show some increase in the incorporation of glucose carbon into glutamate and aspartate, although it is not as high an increase as is observed in the lower concentrations of glucose. Alanine formation from glucose, however, shows some decrease. Glucose incorporation into glycine, in both the control experiments as well as in the experiments where Ehrlich ascites cells were incubated together, show an increase over the values obtained with lower concentrations of glucose.

Effect of Glutamine on Amino Acid Formation from Glucose by Ehrlich Ascites Cells

Since an increase in the incorporation of glucose was observed in glutamate, aspartate and alanine of Ehrlich ascites cells in the presence of brain cortex slices, and this had been thought to be due to a leakage from brain tissue and its subsequent hydrolysis by tumour tissue, it was of interest to see if glutamine in the presence of glucose-U-C^{14} and Ehrlich ascites cells would also give a similar picture.

TABLE XVI

EFFECTS OF 20mM GLUCOSE-U-C^{14} ON AMINO ACID FORMATION BY EHRLICH ASCITES CELLS AND RAT BRAIN CORTEX SLICES

Amino Acids	Ehrlich Cells	Brain Slices	Ehrlich Cells + Brain Slices	
			Brain	Ehrlich Cells
Glycine	639 ± 45	Nil	Nil	350 ± 30
Glutamate	245 ± 10	1466 ± 86	1441 ± 79	331 ± 65
Glutamine	Nil	594 ± 21	746 ± 0	Nil
Aspartate	35 ± 2	194 ± 14	147 ± 14	44 ± 9
Alanine	532 ± 15	340 ± 20	778 ± 71	314 ± 60
γ-amino butyric acid	Nil	393 ± 59	589 ± 61	Nil

All results expressed as mμ atoms glucose carbon incorporated/100 mg. wet weight tissue.

Incubation procedure as described in Table IX.

In one set of experiments (Table XVII) glucose-U-C^{14} (5mM) was added together with 1mM glutamine-U-C^{14} to the incubation medium. This was done so as to imitate the conditions of previous experiments where brain cortex slices were incubated with neoplastic tissues. The glutamine leaking out in such cases would be tagged with C^{14} from glucose-U-C^{14} hence, its breakdown products would be detectable by our detection techniques.

Results obtained in such experiments where both glucose and glutamine are labelled with C^{14} show an increase in the incorporation of glutamate, aspartate and alanine of 682%, 500% and 161% respectively, when compared to the values obtained in the presence of 5mM glucose-U-C^{14} only. However, there is no apparent increase in the formation of glutamate, and aspartate where cold glutamine is added; this is due to the fact that glutamine does give rise to glutamate and aspartate but the increase is not observable as these amino acids do not have C^{14} and hence cannot be detected. The only amino acid that does show an increase throughout is alanine as this may be due to the fact that there is a faster transamination taking place, between either glutamine directly or its product glutamate, with pyruvate.

Values given for glutamine, in the table, denote the mu atoms of glutamine remaining in the cell after 1 hour of incubation and are not indicative of any formation of this amide by tumour cells.

TABLE XVII

EFFECT OF GLUTAMINE ON AMINO ACID FORMATION BY EHRLICH ASCITES CELLS

Experimental Conditions / Amino Acids	1mM Glutamine-U-C^{14}	5mM Glucose-U-C^{14}	5mM Glucose-U-C^{14} + 1mM Glutamine-U-C^{14} *	5mM Glucose-U-C^{14} + 2mM Glutamine	5mM Glucose-U-C^{14} + 5mM Glutamine
Glycine	Nil	41 ± 5	30 ± 2	30 ± 4	32 ± 3
Glutamate	500 ± 9	250 ± 15	1957 ± 102	255 ± 14	240 ± 10
Glutamine	1089 ± 115	Nil	2357 ± 93	Nil	Nil
Aspartate	566 ± 16	59 ± 4	359 ± 14	141 ± 1	99 ± 3
Alanine	49 ± 2	525 ± 35	1324 ± 30	1710 ± 28	1364 ± 36
QO_2	9.5	7.0	7.6	7.8	7.8

Results expressed as mu atoms of glucose or glutamine carbon incorporated/100 mg. wet weight tissue.

Incubation carried out in normal Krebs-Ringer Phosphate media pH 7.4 for 1 hour at 37°C in O_2 atmosphere.

* Both glucose-U-C^{14} and glutamine-U-C^{14} have the same specific activities.

Effect of ∝-Alanine on Ehrlich Ascites Cells

In the experiments described before the presence of brain cortex slices caused significant increase in glutamate, aspartate and alanine formation from glucose by the Ehrlich ascites cells. Hence the effect of alanine on amino acid formation from glucose was studied to see if added alanine would give rise to a high incorporation of glucose carbon into glutamate.

The results obtained from such experiments are described in Table XVIII. It is seen from the table that the presence of alanine (1mM) increases the incorporation of glucose carbon into glutamate, glycine and alanine by 35%, 200% and 77%, but the highest increase in the formation of glycine and alanine is obtained in the presence of 2mM alanine; glutamate formation is, however, maximum in the presence of 1mM alanine.

Thus it may be concluded that an increase in glutamate and aspartate is not mainly due to increased formation of alanine, but it is due to a breakdown of glutamine by tumour cells.

Effect of Dialysis of Ehrlich Ascites Cells.

Effects of dialysis were seen on the formation of amino acids from glucose by brain cortex tissues. Suspensions of Ehrlich ascites cells were dialysed against Krebs-Ringer phosphate medium pH 7.4 for 6 hours at 0°C. Both the

TABLE XVIII

EFFECTS OF 1, 2 AND 5mM ALANINE ON AMINO ACID FORMATION FROM GLUCOSE BY EHRLICH ASCITES CARCINOMA CELLS

Amino Acids	5mM Glucose-U-C^{14}	5mM Glucose-U-C^{14} + 1mM Alanine	5mM Glucose-U-C^{14} + 2mM Alanine	5mM Glucose-U-C^{14} + 5mM Alanine
Glycine	18 ± 1	54 ± 2	63 ± 3	39 ± 0
Glutamate	143 ± 3	194 ± 2	167 ± 2	173 ± 1
Glutamine	Nil	Nil	Nil	Nil
Aspartate	34 ± 1	50 ± 4	54 ± 4	62 ± 0
Alanine	381 ± 38	676 ± 28	1134 ± 128	1082 ± 86

All results expressed as mμ atoms glucose carbon incorporated/100 mg. wet weight tissue.

dialysed cells and the dialysate were incubated with rat brain cortex slices in the presence of glucose.

The results indicate that both the dialysate and the dialysed cells lower the incorporation of glucose carbon into glutamine by 80% and 72% respectively (Table XIX). The dialysed cells maintain all their original activity, as far as the incorporation of glucose carbon into amino acids are concerned.

Ascitic fluid from tumour-bearing mice was used to determine its effect on the formation of glutamine by the brain cortex slices. It seems to have no effect on the glucose incorporation into amino acids by brain cortex slices.

Effect of Dialysate on the Production of Ammonia

Since the dialysate did not show either oxygen consumption or any anaerobic glycolysis, the possibility of leakage of glutamine from brain cortex slices due to a rapid utilisation of glucose was ruled out. An attempt was made to see if dialysate has properties of hydrolyzing glutamine by estimating the production of ammonia in the presence of brain cortex slices.

Both the dialysed Ehrlich ascites cells and dialysate were incubated with and without brain cortex slices in the presence of 5mM glucose for 1 hour and the ammonia produced estimated as described in "Materials and Methods".

TABLE XIX

EFFECT OF DIALYSED EHRLICH ASCITES CELLS AND ASCITIC FLUID ON THE AMINO ACID FORMATION BY RAT BRAIN CORTEX SLICES

Amino Acids	Dialysed Cells	Brain Slices	Dialysed Cells + Brain Slices		Dialysate + Brain Slices Brain	Ascitic Fluid + Brain slices Brain
			Brain	Cells		
Glycine	36 ± 2	Nil	Nil	19 ± 1	Nil	Nil
Glutamate	275 ± 17	1056 ± 44	1107 ± 42	680 ± 16	1237 ± 87	985 ± 50
Glutamine	Nil	465 ± 20	127 ± 3	Nil	93 ± 6	500 ± 25
Aspartate	34 ± 2	120 ± 5	135 ± 9	48 ± 2	135 ± 5	121 ± 8
Alanine	421 ± 7	210 ± 15	369 ± 19	652 ±	174 ± 6	219 ± 22
γ-amino butyric acid	Nil	325 ± 18	429 ± 35	Nil	355 ± 5	317 ± 20

All results expressed as mμ atoms glucose carbon incorporated/100 mg. wet weight tissue.

3.0 ml. of 1:1 dilution of Ehrlich Ascites cells were dialysed for 6 hours against 30 ml. Krebs-Ringer Phosphate medium, pH 7.4, for 6 hours at 0°C. After dialysis cell suspension containing 10 mg. dry weight was added per Warburg vessel.

0.5 ml. of dialysate was used in the presence of brain cortex slices.

It is seen from Table XX that the presence of freshly prepared cell suspension with brain cortex slices increases the ammonia level by 287% as compared to the sets where ascites cells were incubated alone. On the other hand the dialysed cells increase the ammonia level by 435% and the dialysate produces 960% more ammonia , when incubated in the presence of brain cortex slices.

Effect of Hyaluronidase and Hyaluronic acid on Amino Acid Formation from Glucose by Brain Cortex Slices

Invasiveness of tumour tissues has been linked to the presence of hyaluronidase or some enzymes related to it. An attempt was therefore made to see if hyaluronidase has any effect on the formation of amino acids from glucose by brain cortex slices in vitro.

The results presented in Table XXI indicate that there is no effect of either hyaluronic acid or hyaluronidase on the incorporation of glucose carbon into amino acids by brain cortex slices.

TABLE XX

EFFECT OF DIALYSATE AND EHRLICH ASCITES CELLS ON THE PRODUCTION OF AMMONIA IN THE PRESENCE OF 5mM GLUCOSE AND BRAIN CORTEX SLICES

Tissues Used	µg NH_3 produced/100 mg. wet weight tissue	Net increase in NH_3 production
Brain cortex slices	4.3	
Ehrlich Ascites cells	5.6	
Ehrlich cells + brain cortex slices	26.0	16.1 (287%)
Dialysed cells	5.1	
Dialysed cells + brain cortex slices	31.6	22.2 (435%)
Dialysate	0.0	
Dialysate + brain cortex slices	13.9	9.6 (960%)

Ammonia estimations were carried out as described in "Materials and Methods".

TABLE XXI

EFFECT OF HYALURONIDASE AND HYALURONIC ACID ON THE AMINO ACID FORMATION FROM GLUCOSE BY RAT BRAIN CORTEX SLICES

Amino Acids	Brain Cortex Slices	Brain Cortex Slices + Hyaluronidase	Brain Cortex Slices + Hyaluronic Acid + Hyaluronidase	Brain Cortex Slices + Hyaluronic Acid
Glycine	Nil	Nil	Nil	Nil
Glutamate	1056 ± 24	1006 ± 56	956 ± 44	967 ± 58
Glutamine	410 ± 15	395 ± 25	425 ± 17	415 ± 8
Aspartate	118 ± 10	102 ± 3	100 ± 6	122 ± 9
Alanine	190 ± 9	160 ± 2	179 ± 10	177 ± 9
γ-Amino butyric Acid	285 ± 11	300 ± 12	325 ± 15	310 ± 10
QO_2	10.0	10.0	11.0	10.0

All results expressed as mμ atoms glucose carbon incorporated/100 mg. wet weight tissue.

Hyaluronidase and Hyaluronic acid - 100 γ/flask.

Discussion

Studies on the production of amino acids, from glucose, by rat brain cortex slices obtained from normal rats and rats bearing liquid Novikoff hepatoma, presented in this chapter, indicate that the brain cortex slices from the tumour-bearing rats have a lower incorporation of glucose carbon into glutamine than do its counterpart from normal rats. This might indicate that the presence of tumour in the rat decreases the capability of the brain cortex slices to form glutamine from glucose. These findings are in conformity with the findings reported by Wu and Baur (1960). They observed that with the increase in size of transplanted Walker 256 carcinosarcoma in rats, there was a decrease in the level of free glutamine in various tissues of the host rats. According to these authors this phenomenon is due to a progressive decrease in the level of glutamine synthetase activity in the liver of the tumour-bearing rats. This seems to be a paradoxical situation, as these authors have no direct evidence, neither have we in our experiments, that the synthesis of glutamine *in vivo* paralleled the synthetase activity *in vitro*, as it may be possible that the remaining enzyme activity would be commensurate with the normal demand for glutamine *in vivo*. If the conclusions drawn by Wu and Baur (1960), coupled with our findings are true, then it is hard to understand how the

tumour tissue, which has been shown to have a high demand of glutamine for protein synthesis, can survive. Hence there may be a possibility that the glutamine synthesis *in vivo* might be proceeding at a normal rate thereby keeping up with the demands for tumour growth.

Some idea regarding the mechanism through which intracellular glutamine from brain cortex slices is made available to the tumour tissues can be obtained from the results presented in this chapter.

When tumour tissues and brain cortex slices from normal rats are incubated together in the presence of 5mM glucose-U-C^{14}, an apparent decrease of 50% is observed in the formation of glutamine from glucose in the brain tissue. This effect on cerebral tissue is characteristic of tumour tissues only, and it also seems to be specific for glutamine, as other amino acids are not affected. This apparent decrease in the incorporation into glutamine could be the result of either a decreased synthesis of this amide by the brain slices, or a leakage of glutamine from the brain slices into the medium and its subsequent metabolism by the tumours. The correctness of the latter hypothesis seems to be substantiated by the observations that with a decrease in glutamine from brain cortex slices, there is a concomitant increase in the incorporation of glucose carbon into glutamate and aspartate, of tumour tissues, by 300% and 30% respectively. Results

obtained from the incubation of brain cortex slices and tumour tissues, with various concentrations of glucose in the medium, further prove the validity of the leakage hypothesis. It is seen that with the increase in the concentration of glucose, the effect of tumour tissues on the apparent glutamine formation from glucose is decreased. This effect is completely abolished at a glucose concentration of 10mM. If a comparison is made of the total numbers of mu atoms of glucose carbon incorporated into amino acids, for tumour tissues and brain cortex slices incubated together and when they are incubated separately, it appears that at the lower concentrations of glucose (1, 3 and 5mM) nearly the same number of glucose carbons are utilised under both conditions, but at higher levels of glucose there is a significant increase in the total glucose incorporation when tumour tissues were incubated in the presence of brain cortex slices. This may further indicate that at lower concentrations of glucose, loss in the incorporation in brain slices is compensated by the increase in other amino acids of the tumours. This evidence may further prove our hypothesis that there is a leakage of glutamine from brain tissue at lower glucose concentrations in the presence of tumours. The reason for this phenomenon may be due to a lack of sufficient glucose in the medium for the efficient metabolism of brain tissue. Tumour tissues have been shown

to have a very high rate of aerobic glycolysis, Warburg (1926). It has also been reported that tumour tissues can utilise up to about 10% of their dry weight of glucose per hour *in vitro* (Aisenberg 1961). Hence when neoplastic tissues are incubated with brain cortex slices *in vitro*, tumour tissues by virtue of their high rate of aerobic glycolysis may be using up most of the glucose from the medium, thereby starving the brain slices of glucose. Thus the lack of glucose in the medium, coupled with a high glutaminase activity of tumour tissues may be responsible for an increased leakage of glutamine from the brain cortex slices into the medium.

The results discussed above may also explain to a certain extent the findings of previous workers (Rouser, 1957; Simonsen and Roberts, 1960; Wu and Baur, 1960), that patients suffering from cancer or tumour-bearing animals have a low glutamine content in various tissues and in the blood. The presence of tumours is reported to decrease the blood sugar concentration in the tumour-bearing animals (Warburg *et al*, 1926; Gorenson and Tilser, 1955). This low glucose concentration may cause the host's tissue to lose glutamine, which is taken up readily by tumour, thereby causing a decreased level of glutamine.

This hypothesis may also explain the observations of Skipper and Thomson (1959) that transplanted tumours could

grow even if glutamine is absent in the diet of tumour-bearing animals. This amide could be synthesised by host tissues, from other dietary components, and made available to the tumour through the process described above.

It seems surprising that the dialysate, from the Ehrlich ascites carcinoma cells, can also bring about a diminution in the apparent formation of glutamine, from glucose, by rat brain cortex slices *in vitro*. As the dialysate does not show any aerobic or anaerobic glycolysis, the leakage of glutamine from brain slices is not due to a lack of glucose in the medium. However, there is an increase in the production of ammonia (960%), when brain cortex slices are incubated in the presence of the dialysate and glucose (5m1), indicating thereby an increase in glutamine breakdown. Hence it is possible that the dialysate contains some factor capable of increasing the production of ammonia as well as decreasing the incorporation of glucose carbon into glutamine, by brain cortex slices. The fact that this unknown factor is heat labile may indicate that this may be somewhat enzymic in nature. This idea is further strengthened by the fact that the dialysate produces a precipitate on the addition of 50% trichloroacetic acid indicating thereby the presence of a protein-like substance in the dialysate. The observation of acid precipitable material in the dialysate is substantiated by the findings of Carter and Greenstein

(1946), who reported that on dialysing a mixture of nucleates and fresh tissue against a solution containing sodium chloride and magnesium chloride, particles having 12,000 to 18,000 molecular weight could be detected in the dialysate. Thus it is possible that the dialysate, obtained from Ehrlich ascites cells, also contains small protein molecules which can simulate the action of glutaminase. The only conceivable explanation for the loss of glutamine from brain cortex slices, in the presence of the dialysate is that there may be some leakage of glutamine from the brain tissue, into the medium, and the subsequent rapid breakdown by the factor present in the dialysate causes increased efflux of glutamine. However, further work is necessary to elucidate this unusual, yet interesting, finding.

When tumour tissues are incubated with brain cortex slices, an increased incorporation of glucose carbon into glutamate, aspartate and alanine is observed; this increase is much greater than the loss observed in the glutamine formation, from glucose, by the brain cortex slices, but in the experiments on tumour tissues incubated with glucose-U-C^{14} and glutamine-U-C^{14}, it is seen that the latter at a concentration of 1/5 that of glucose brings about incorporation into glutamate, aspartate and alanine, of the same order as observed with brain slices present in a medium containing 5ml glucose-U-C^{14}. It is known that the breakdown products

of glutamine are glutamate and aspartate (Coles, 1961). The glutamine formed from glucose-U-C^{14}, by brain cortex slices, will be uniformly labelled, hence its breakdown product will also be tagged with C^{14}, thereby allowing a proper detection of the increase in the by-products by the method used in our experiments. Increased breakdown of glutamine by the tumour tissue, in the presence of brain cortex slices, is also indicated by the increase in total ammonia values obtained under similar conditions. With an increased hydrolysis of glutamine, by tumour tissues, there is an accumulation of glutamate, which is then available for transamination with the formation of α-ketoglutarate-C^{14}. This in turn gives rise to labelled oxalacetate, leading to an increased formation of aspartate. There is also the possibility that glutamine may be transaminating independently with pyruvate, to give rise to alanine. This probability is indicated by the results obtained from the experiments where non-radioactive glutamine was used in conjunction with glucose-U-C^{14}. A marked increase in the formation of alanine is observed. However, it must be mentioned that it was not possible to determine whether the increase in the alanine formation is due to a direct transamination of glutamine or is a product of glutamate transamination.

Previous investigators have often linked the invasiveness of the tumours with some kind of "spreading

factor", similar to hyaluronidase. An increase in the blood hyaluronidase activity of patients suffering from cancer has been reported by Büchner and Schöne (1956) and Pavlyushcik (1960). However, it is evident from the results of the experiments with the effects of hyaluronidase on the amino acid formation from glucose, by rat brain cortex slices in vitro, that this enzyme may not be the so-called "Invasive factor" of tumour tissues.

In conclusion it can be said that tumour tissues interfere with the apparent glutamine formation from glucose, by rat brain cortex slices in vitro. This phenomenon is characteristic of the neoplastic tissues only, and is due to the high rate of glucose utilisation by the tumour tissues, coupled with its high glutaminase activity.

Summary

1. Brain cortex slices from rats bearing liquid Novikoff hepatoma have been shown to have a decreased incorporation of glucose carbon into glutamine.
2. Brain cortex slices, incubated in the presence of tumour tissues, exhibit a decreased formation of glutamine from glucose. At the same time there is an increased incorporation of glucose carbon into glutamate, aspartate and alanine, in the tumour tissues.
3. The lowering of apparent glutamine formation from glucose, in brain slices, is due to a leakage of glutamine from brain cortex slices. This phenomenon is due to a lack of glucose in the medium, because of a high rate of glycolysis of the tumour tissues.
4. The ability of Ehrlich ascites cells to bring about the decrease in the incorporation of glucose carbon into glutamine, of brain cortex slices is increased upon dialysis of the cells.
5. The dialysate from the Ehrlich ascites cells has been shown to possess some protein-like component which can simulate the activity of glutaminase, and lower the formation of glutamine from glucose, by rat brain cortex slices *in vitro*.

CHAPTER III

EFFECTS OF FORMATE, PYRUVATE AND CYSTEINE ON THE HEXOSE MONOPHOSPHATE SHUNT IN TUMOUR TISSUES

Introduction

In neoplastic tissues there are at least two major pathways for glucose catabolism - the Embden-Meyerhoff glycolytic pathway, which is quantitatively the more important, and the hexose monophosphate shunt. Dickens and Glock (1951) showed that tumour tissues oxidize glucose-6-phosphate, 6-phosphogluconate and ribose-5-phosphate. Later Kit (1956) determined that in Ehrlich ascites cells, 23% of the total oxidation of glucose goes through the hexose monophosphate shunt. He further observed that lymphatic tumour tissues formed 2 to 5 times more pentose from glucose, than do the normal lymphatic cells. Wenner and Weinhouse (1956) reported that 2 to 16% of the oxidation of glucose, in both normal and neoplastic tissues, proceeded via the shunt mechanism. Thus it is clear that tumour tissues have an active hexose monophosphate shunt. In view of these findings, it was decided to see if this mechanism for glucose oxidation could be accelerated by the use of certain compounds. Formate, pyruvate and cysteine, were found to stimulate the oxidation of glucose-1-C^{14} but not of glucose-6-C^{14}; a possible explanation for this increase is discussed in this chapter.

Results

$C^{14}O_2$ production from Glucose-1-C^{14} and Glucose-6-C^{14} by Tumour Tissues

It is seen from Table XXII, that Ehrlich ascites cells and melanoma slices produce 94% and 49% more $C^{14}O_2$ from glucose-1-C^{14} than from glucose-6-C^{14}. However, in the case of leukemia tissue there is a higher production of $C^{14}O_2$ from glucose-6-C^{14} than from glucose-1-C^{14}.

Effects of Sodium Formate

Islam (1961) reported that the addition of glucose to a medium containing formate, increases the oxidation of formate by leucocytes. Hence it was decided to see the effect of formate on the oxidation of glucose-1-C^{14} and glucose-6-C^{14} by tumour tissues.

Results presented in Table XXIII show that, whereas the presence of 2mM sodium formate stimulates the production of $C^{14}O_2$ from glucose-1-C^{14} in all the neoplastic tissues used, it has an inhibitory effect on glucose-6-C^{14} oxidation. The increase in $C^{14}O_2$ production from glucose-1-C^{14} being, Ehrlich ascites cells, 42%; melanoma, 51%; and leukemia, 156%.

Effects of Malonate on the production of $C^{14}O_2$ in presence of Formate

The effects of malonate, a known inhibitor of

TABLE XXII

$C^{14}O_2$ PRODUCTION FROM GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14} BY TUMOURS

Tumours	QO_2	5mM glucose-1-C^{14}	5mM glucose-6-C^{14}
Ehrlich Ascites Cells	7.0	396 ± 13	204 ± 12
Melanoma	2.4	552 ± 15	372 ± 25
Leukemia	2.0	69 ± 4	277 ± 13

All results expressed as mμ atoms glucose 1 or 6 carbon incorporated per 100 mg wet weight tissue.

TABLE XXIII

EFFECT OF 2mM Na-FORMATE ON $C^{14}O_2$ PRODUCTION FROM GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14} BY TUMOUR TISSUES

Tumours	5mM Glucose-1-C^{14}	5mM Glucose-1-C^{14} + 2mM Na-Formate	5mM Glucose-6-C^{14}	5mM Glucose-6-C^{14} + 2mM Na-Formate
Ehrlich Ascites Cells	404 ± 12	574 ± 30	216 ± 12	190 ± 5
Melanoma	568 ± 20	856 ± 14	402 ± 19	276 ± 15
Leukemia	76 ± 8	195 ± 12	279 ± 20	226 ± 18

All values expressed as mμ atoms glucose 1 ~~and~~ or 6 carbon incorporated/100 mg. wet weight tissue.

succinic dehydrogenase, was studied, to eliminate the possibility of oxidation of glucose-1-C^{14} via the citric acid cycle and also to see if by inhibiting the tricarboxylic acid cycle, more glucose-1-C^{14} could be oxidized through the hexose monophosphate shunt.

The results presented in Table XXIV, indicate that 5mM malonate has no effect on the formation of $C^{14}O_2$ from glucose-1-C^{14}, although it markedly inhibits the oxidation of glucose-6-C^{14}. These results are the same whether formate is present or not.

Effects of Formate on amino acid formation from Glucose-1-C^{14} and Glucose-6-C^{14}.

Formate has been shown to stimulate the oxidation of glucose-1-C^{14}, hence it was thought to be of interest to see whether the increase in the hexose monophosphate shunt, would have any effect on the formation of amino acids, from the glucose-1-C^{14} and glucose-6-C^{14}, by Ehrlich ascites carcinoma cells.

It is seen from Table XXV that the presence of formate decreases the incorporation of glucose-1-carbon into all the amino acids, viz. glycine (83%), glutamate (21%), aspartate (52%) and alanine (16%). The presence of formate has no effect on the glucose-6-carbon into glutamate, aspartate and alanine, although it causes a decrease in the glycine formation.

TABLE XXIV

EFFECTS OF MALONATE ON THE $C^{14}O_2$ PRODUCTION FROM GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14} IN THE PRESENCE AND ABSENCE OF 2mM Na-FORMATE BY EHRLICH ASCITES CELLS

5mM Glucose - 1 - C^{14}

	Glucose-1-C^{14}	+ 2mM Formate	+ 2mM Formate + 5mM Malonate	+ 5mM Malonate
$C^{14}O_2$	390 ± 10	600 ± 30	585 ± 35	402 ± 25
QO_2	6.6	6.9	4.1	4.3

5mM Glucose - 6 - C^{14}

	Glucose-6-C^{14}	+ 2mM Formate	+ 2mM Formate + 5mM Malonate	+ 5mM Malonate
$C^{14}O_2$	196 ± 12	176 ± 11	12 ± 0	14 ± 1
QO_2	6.7	6.8	3.9	4.4

All values expressed as mμ atoms glucose 1 ~~and~~ or 6 carbon incorporated/ 100 mg. wet weight tissue.

TABLE XXV

EFFECT OF 2mM Na-FORMATE ON AMINO ACID FORMATION FROM GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14} BY EHRLICH ASCITES CARCINOMA CELLS

Amino Acids	5mM Glucose-1-C^{14}	5mM Glucose-1-C^{14} + 2mM Formate	5mM Glucose-6-C^{14}	5mM Glucose-6-C^{14} + 2mM Formate
Glycine	12 ± 1	2 ± 0	13 ± 2	6 ± 1
Glutamate	105 ± 8	82 ± 4	119 ± 10	101 ± 3
Glutamine	Nil	Nil	Nil	Nil
Aspartate	17 ± 3	8 ± 1	24 ± 3	22 ± 2
Alanine	117 ± 4	98 ± 6	156 ± 13	138 ± 2

All results expressed as mμ atoms of glucose 1 ~~and~~ or 6 carbon incorporated/ 100 mg. wet weight tissue.

Effect of Pyruvate on $C^{14}O_2$ production from Glucose-1-C^{14} and Glucose-6-C^{14}

Pyruvate has been shown by Wenner, Hackney and Moliterno (1958) to stimulate the oxidation of glucose-1-C^{14} by Ehrlich ascites carcinoma cells. Hence it was decided to see whether by inhibiting the alternate pathway, i.e. the citric acid cycle, there would be a higher stimulation of hexose monophosphate shunt.

Results presented in Table XXVI show that the presence of 2mM pyruvate stimulates the $C^{14}O_2$ production from glucose-1-C^{14}, by about 92%. Malonate at a concentration of 5mM, however, has no effect on the incorporation of glucose-1-carbon into CO_2, under these conditions.

Oxidation of glucose-6-C^{14} is only slightly increased in the presence of pyruvate, but it is greatly reduced, about 75%, with malonate present in the medium.

Effect of Pyruvate on the Amino Acid Formation from Glucose-1-C^{14} and Glucose-6-C^{14}

The presence of 2mM pyruvate decreases the incorporation of glucose-1-carbon into glycine and glutamate by 35%, but has no effect on the formation of alanine. Incorporation of glucose-1-carbon into aspartate is completely inhibited by the presence of pyruvate (Table XXVII).

The addition of pyruvate completely inhibits the incorporation of glucose-6-carbon into glycine without affecting the formation of the other amino acids.

TABLE XXVI

EFFECT OF Na PYRUVATE ON $C^{14}O_2$ PRODUCTION FROM GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14} IN THE PRESENCE AND ABSENCE OF MALONATE BY EHRLICH ASCITES CELLS

5mM Glucose - 1 - C^{14}

	5mM Glucose-1-C^{14}	+ 2mM Pyruvate	+ 2mM Pyruvate + 5mM Malonate	5mM Malonate
$C^{14}O_2$	384 ± 25	740 ± 35	698 ± 30	375 ± 15
QO_2	7.0	6.8	3.6	3.9

5mM Glucose - 6 - C^{14}

	5mM Glucose-6-C^{14}	+ 2mM Pyruvate	+ 2mM Pyruvate + 5mM Malonate	+ 5mM Malonate
$C^{14}O_2$	168 ± 12	208 ± 17	54 ± 6	20 ± 0
QO_2	6.8	6.8	3.7	3.8

All values expressed as mμ atoms glucose 1 or 6 carbon incorporated/ 100 mg. wet weight tissue.

TABLE XXVII

EFFECT OF 2mM Na-PYRUVATE ON AMINO ACID FORMATION FROM GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14} BY EHRLICH ASCITES CARCINOMA CELLS

Amino Acids	5mM Glucose-1-C^{14}	5mM Glucose-1-C^{14} + 2mM Na Pyruvate	5mM Glucose-6-C^{14}	5mM Glucose-6-C^{14} + 2mM Na Pyruvate
Glycine	14 ± 2	9 ± 2	12 ± 0	Nil
Glutamate	100 ± 9	65 ± 4	125 ± 12	130 ± 8
Glutamine	Nil	Nil	Nil	Nil
Aspartate	20 ± 2	Nil	26 ± 3	24 ± 4
Alanine	113 ' 12	110 ' 10	160 ' 11	175 ' 14

All results are expressed as mμ ators glucose 1 or 6 carbon incorporated/100 mg. wet weight tissue.

Effects of L-cysteine on the oxidation of Glucose-1-C^{14} and Glucose-6-C^{14}

Etinhoff and Gersanovitch (1953) have reported that the addition of cysteine, to the medium containing glucose, reversed the "Crabtree effect" or as they call it "Reversed Pasteur Effect" in Ehrlich ascites cells. Therefore it was decided to confirm their report as well as to see whether cysteine has any effect on the glycolysis or on the hexose monophosphate shunt.

The reversal of "Crabtree effect" is seen from the QO_2 values presented in Table XXVIII. Ehrlich ascites carcinoma cells with no added substrate give a QO_2 value of 9.5, which is lowered in the presence of both glucose-1-C^{14} and glucose-6-C^{14} to 6.5, a decrease of nearly 37%. On the addition of 2mM cysteine, in the presence of glucose, the QO_2 value increases to 8.5, an increase of 30% over the value obtained in the presence of glucose alone.

The addition of cysteine, to a medium containing glucose-1-C^{14}, stimulates the incorporation of glucose-1-carbon into CO_2. The increase in $C^{14}O_2$ production being 112%, and is not affected by malonate.

On the other hand, glucose-6-C^{14} is not affected by the presence of cysteine, but its oxidation is markedly inhibited by malonate.

TABLE XXVIII

EFFECT OF L-CYSTEINE ON THE $C^{14}O_2$ PRODUCTION FROM GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14} BY EHRLICH ASCITES CARCINOMA CELLS

		5mM Glucose - 1 - C^{14}				5mM Glucose - 6 - C^{14}			
	Endo-genous	Glucose-1-C^{14}	+ 2mM Cysteine	+ 2mM Cysteine + 5mM Malonate	+ 5mM Malonate	Glucose-6-C^{14}	+ 2mM Cysteine	+ 2mM Cysteine + 5mM Malonate	+ 5mM Malonate
$C^{14}O_2$	-	425±33	902±16	976±25	400±29	215±10	220±15	10±0	14±2
QO_2	9.5	6.5	8.5	4.2	4.3	6.5	8.7	4.0	4.0

All results expressed as mu atoms glucose 1 or 6 carbon incorporated/100 mg. wet weight tissue.

Effect of L-cysteine on Amino Acid Formation

As cysteine gave the highest stimulation of glucose-1-C^{14}, among the three compounds tried, it was of interest to see its effect on the amino acid formation from glucose-1-C^{14} and glucose-6-C^{14}.

It is seen from Table XXIX, that cysteine brings about a marked inhibition in the incorporation of glucose-1-carbon into all the amino acids. Glycine formation is completely inhibited, whereas, the incorporation into glutamate and aspartate is reduced by nearly 50%, in both the cases. The formation of alanine, from glucose-1-C^{14} is inhibited by 75%.

Although the presence of cysteine has a slight stimulatory effect on the incorporation of glucose-6-carbon into glycine, glutamate and aspartate, there is however, a marked inhibition in the formation of alanine.

Discussion

It has been common practice to calculate the ratio between the $C^{14}O_2$ produced from glucose-1-C^{14} and that produced from glucose-6-C^{14}, to evaluate the operation of the hexose monophosphate shunt in various tissues. If the ratio turns out to be 1 or less, it shows that the $C^{14}O_2$ formed from both glucose-1-C^{14} and glucose-6-C^{14} is obtained through the same pathway, namely, the citric acid cycle, and that the shunt is not operating. If this ratio is greater than 1,

TABLE XXIX

EFFECT OF 2mM L-CYSTEINE ON THE AMINO ACID FORMATION FROM GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14} BY EHRLICH ASCITES CELLS

Amino Acids	5mM Glucose-1-C^{14}	5mM Glucose-1-C^{14} + 2mM Cysteine	5mM Glucose-6-C^{14}	5mM Glucose-6-C^{14} + 2mM Cysteine
Glycine	13 ± 1	Nil	15 ± 0	20 ± 3
Glutamate	109 ± 9	50 ± 3	125 ± 10	140 ± 6
Glutamine	Nil	Nil	Nil	Nil
Aspartate	13 ± 3	6 ± 1	29 ± 3	31 ± 2
Alanine	125 ± 5	31 ± 1	164 ± 2	37 ± 2

All results expressed as mu atoms glucose 1 or 6 carbon incorporated/100 mg. wet weight tissue.

it shows that glucose-1-C^{14} is being oxidised faster than glucose-6-C^{14}, and this can happen only in the event of glucose-1-C^{14} going through the shunt, where the first carbon of glucose is decarboxylated during the formation of ribulose-5-phosphate from 6-phosphogluconic acid.

It is evident from the results presented in this chapter that tumour tissues, in general, have an active hexose monophosphate shunt. The ratio between the $C^{14}O_2$ produced from glucose-1-C^{14} and that produced from glucose-6-C^{14}, for Ehrlich ascites cells and S_{91} melanoma tissue, turns out to be 2. The only exception is leukemia which exhibits a ratio of 0.25. Addition of 5 mM malonate does not result in any inhibition of the formation of $C^{14}O_2$ from glucose-1-C^{14} by Ehrlich ascites cells whereas it inhibits that from glucose-6-C^{14} by 80%. These results are similar to those obtained by van Vals et al. (1956), who reported that addition of malonate had no effect on the $C^{14}O_2$ production from glucose-1-C^{14} but markedly inhibited the oxidation of glucose-6-C^{14} by various tumour tissues. It is difficult to explain these results, as a major portion of glucose is metabolised via the citric acid cycle and hence a block in the tricarboxylic acid cycle should bring about at least some reduction in the $C^{14}O_2$ production from glucose-1-C^{14} also. However, further work is necessary to elucidate this phenomenon.

Formate, pyruvate and L-cysteine stimulate the $C^{14}O_2$ production from glucose-1-C^{14}, but have no effect on the production of $C^{14}O_2$ from glucose-6-C^{14}. The specific stimulation on CO_2 formation from glucose-1-C^{14} is an indication that these compounds stimulate the operation of the hexose monophosphate shunt. This assumption is supported by the fact that the presence of malonate, with these three compounds, does not affect the $C^{14}O_2$ production from glucose-1-C^{14}, but markedly inhibits the $C^{14}O_2$ formation from glucose-6-C^{14}. The increase in the $C^{14}O_2$ production from glucose-1-C^{14} results in a decreased incorporation of glucose-1-carbon into amino acids. In all these cases, there is no such effect on the formation of amino acids from glucose-6-C^{14}. It is reasonable to assume that if more glucose-1-carbon is being oxidised to CO_2, via the hexose monophosphate shunt, a lesser amount will be available to go through the glycolytic pathway and the citric acid cycle, to give rise to amino acids. The formation of amino acids, from glucose-6-C^{14}, is unaffected just as the $C^{14}O_2$ production. There is, however, one exception; in the presence of cysteine, suppression of alanine formation from both types of glucose-C^{14} occurs. The reason for this behaviour is not understood.

It has been shown, by the early works of Warburg, Dickens, Lipmann and others, that the initial steps of the

hexose monophosphate shunt involve TPN as a coenzyme for the oxidation of glucose-6-phosphate and of the resulting 6-phosphogluconic acid. Glock and McLean (1955, 1957) have shown that in the neoplastic tissues DPN is generally lower than in most normal tissues, while the level of TPN is negligible, and that the bulk of DPN is in oxidised form and the TPN is in reduced form. Hence it is possible that the stimulatory action of formate, pyruvate and L-cysteine, on the hexose monophosphate shunt in Ehrlich ascites carcinoma cells, is due to their capabilities of oxidizing TPNH, and thereby making more TPN available for the efficient operation of the shunt.

Formate has been shown, by Osborn and Huennekens (1957), to combine with glycine to give rise to serine via the following steps:

$$\text{Formate} + \underset{(\text{THFA})}{\text{Tetrahydrofolic acid}} \xrightarrow{\text{TPNH} + \text{H}^{+}\ \text{TPN}} \text{FormylTHF} \longrightarrow \text{MethyleneTHF} \xrightarrow{+\ \text{glycine}} \text{Serine.}$$

It can be seen that the reduction of FormylTHF is mediated by TPNH. Hence this reaction can be used by the tissues, as a means of oxidation of TPNH. It has also been shown by Herscovics, from our laboratories, that there is a 30 fold increase in the incorporation of formate into serine, in the presence of glucose, by Ehrlich ascites cells. Thus the

effect of formate on glucose-1-carbon oxidation may be attributed to its utilization in the synthesis of serine, which is accompanied by rapid oxidation of TPNH. The effects of glucose and formate, on their oxidation seem to be synergestic. Experiments, on the effect of glucose on the production of $C^{14}O_2$ from formate-C^{14}, show a marked increase in the oxidation of formate-C^{14} in the presence of glucose.

The results obtained in the presence of pyruvate are in agreement with those reported by Wenner et al. (1958). They observed a stimulatory effect of pyruvate on the oxidation of glucose-1-C^{14} by various tumours. It has been suggested by these authors that pyruvate acts as an electron acceptor for TPNH, in the presence of glucose, and gets reduced to lactate, and that in Ehrlich ascites cells lactic dehydrogenase may be TPNH dependent. Thus it seems clear how and why there is an increase in the formation of $C^{14}O_2$ from glucose-1-C^{14}, in the presence of pyruvate.

L-cysteine seems to have the highest stimulatory effect, amongst all the compounds tried, on the oxidation of glucose-1-C^{14} by Ehrlich ascites carcinoma cells. The ratio between the $C^{14}O_2$ produced from glucose-1-C^{14} to that produced from glucose-6-C^{14}, increases from 2, in the absence of cysteine, to 4 in its presence. This increase in the

hexose monophosphate shunt can also be ascribed to the effects on the TPN/TPNH ratio in the system. Szeinberg and Marks (1961) have shown that the addition of TPNH to a solution of cysteine, buffered at pH 7.4, brings about a non-enzymatic oxidation of TPNH. The rate of oxidation increases (but not by a linear relationship) with rising concentration of cysteine to 1.2×10^{-2}M. Further increase in the concentration of cysteine is associated with a fall in the rate of TPNH oxidation. The observations of these workers coupled with the results reported in this chapter explain the stimulatory effect of cysteine on the hexose monophosphate shunt, in Ehrlich ascites carcinoma cells in vitro.

In conclusion it can be said that tumour tissues lack endogenous electron acceptors, and if these are supplied there is a stimulation of the hexose monophosphate shunt. It has been shown by Reynafarje and Potter (1957) that Novikoff hepatoma lacks TPN-cytochrome C reductase as well as transhydrogenase. It is possible that these enzymes are also missing in Ehrlich ascites cells, and that these cells are unable to oxidise TPNH through the usual electron transport pathway, hence an exogenous electron acceptor is required for an efficient oxidation of TPNH, to give TPN, which is an essential cofactor for the operation of the hexose monophosphate shunt.

Summary

1. Ehrlich ascites carcinoma cells and S91 Melanoma have been shown to have an active hexose monophosphate shunt. In AKR leukemia this mechanism seems to be absent.

2. Sodium formate, at a concentration of 2mM stimulates the production of $C^{14}O_2$ from glucose-1-C^{14} by Ehrlich ascites cells and melanoma tissue. The presence of formate has no effect on the oxidation of glucose-6-C^{14}. The increase in the production of $C^{14}O_2$ from glucose-1-C^{14} results in a decreased incorporation of glucose-1-carbon into amino acids.

3. The presence of sodium pyruvate has been shown to increase the oxidation of glucose-1-C^{14}, with no effect on the glucose-6-C^{14} oxidation. The addition of pyruvate also diminishes the incorporation of glucose-1-carbon into amino acids.

4. Among all the compounds tested, L-cysteine shows the highest stimulation of glucose-1-C^{14} oxidation, with a marked decrease in the amino acid formation. The oxidation of glucose-6-C^{14} is not affected by the presence of cysteine.

GENERAL DISCUSSION

"Tumour-host relations usually are regarded as those changes produced in the tissue of the host remote from the tumour and in which no evidence of metastatic malignant cell is found."

Begg (1955).

A tumour can manifest its malignant properties only in the host. It can kill an animal without destroying vital organs; such a cancer death must be a "metabolic death". The ultimate objective of studies of tumour-host relations should be to define the effects, or summation of effects, that lead to the death of the host. A tumour may produce its effects at a distance both by the production of a chemical substance ("toxin") and by concentration of a material necessary for continued normal metabolism in the host.

There have been reports on work, both *in vitro* and *in vivo*, regarding the high requirement of tumour tissues for glutamine. Experiments of Eagle (1955b) and Pasieka *et al.* (1960) have shown that tumour cells growing in tissue culture require high amounts of glutamine and that they utilise glutamine much faster than normal tissues. Reports from Roberts and his group (cf Introduction) have indicated that the tumour growth in animal reduces the glutamine content

of various host tissues. They have also shown that tumour tissue synthesises glutamine, but that its utilisation is more rapid. On the other hand Wu and Baur (1960) have also reported a similar finding, but they ascribe this to a decrease in the glutamine synthesis by host tissue. These authors estimated the glutamine synthesis by liver of the hepatoma bearing animal *in vitro*. It is possible that the conditions *in vivo* may be entirely different and this low synthesising capacity, of liver, may be compensated by the synthesis of this amide by other host tissues and glutamine may then be made available for tumour growth.

This hypothesis is substantiated by the *in vitro* studies, reported in this thesis. It is seen that the presence of tumour tissue, irrespective of the site of its origin, reduces the formation of glutamine from glucose. This is not due to an effect of tumour tissue on the glutamine synthesising system in the brain cortex slices *in vitro*, but it seems to be caused by a lack of glucose in the medium, because of the greater utilisation of glucose by the tumours. This lack of glucose results in a leakage of glutamine which is rapidly broken down by the tumours. Support for this explanation exists in the following observations. First, the decrease in glutamine formation in the brain cortex slices, is accompanied by an increase in the incorporation of glucose carbon into glutamate, aspartate and alanine in

the tumour tissues. Secondly, if a concentration of glucose of 10mM is added to the medium, the brain cortex slices are capable of synthesising as much glutamine, from glucose, as when no tumour is present. These observations do not support the conclusions of Wu and Baur (1960).

It could be argued that the experiments described in this thesis were performed *in vitro* and hence are not applicable to the situation *in vivo*. However, it is known from the observations of Gorenson and Tilser (1955), that a relatively low blood sugar concentration is found in tumour bearing animals. Hence it is conceivable that the low levels of glutamine found in the tissues of tumour-bearing animals may be due to an efflux of glutamine from the tissues in the absence of an adequate concentration of glucose in the blood, and its subsequent utilization by tumour tissue. The situation is then analogous to our experimental conditions when 5mM glucose is used.

It is seen from the results presented in this thesis that there is a lower formation of amino acids from glucose, by tumour tissues, as compared to either a slow growing tissue (brain cortex slices) or a rapidly growing tissue (chick embryo). The decreased incorporation of glucose into glutamate and aspartate, in neoplastic tissues can be so for two reasons; either a slow operation of the citric acid cycle or a lack of amino group donor for transamination.

The latter possibility can be ruled out by the fact that the incorporation of glucose carbon into alanine, in malignant tissues, is higher than in the normal tissues. This is further substantiated by the experiments where the addition of glutamate, alanine, or NH_4Cl had no increasing effect on the formation of glutamate and aspartate in the tumour tissues. The evidence thus points to the possibility that the Tricarboxylic acid cycle may be operating at a slower rate in neoplastic tissues. While the values obtained for alanine are high, those for glutamate and aspartate are low. There is a gradual decrease in the formation of these three amino acids, from glucose, in the following order: alanine, glutamate and aspartate. Wenner et al. (1952) made a survey of the levels of various citric acid cycle enzymes, in tumours and reported that all enzymes except aconitase and α-ketoglutaric dehydrogenase were present at a comparable level with normal tissue. In view of these findings, coupled with those reported in Chapter I of the thesis, it seems probable that there is a partial malfunctioning of the citric acid cycle.

Studies on the effects of formate, pyruvate and cysteine on the operation of the hexose monophosphate shunt, in tumour tissues in general, and Ehrlich ascites cells in particular, indicate that the better the electron acceptor for TPNH, the more efficient is the stimulation in the

oxidation of glucose through the shunt, as indicated by the $C^{14}O_2$ production from glucose-1-C^{14}. These results may indicate that the slow operation of the hexose monophosphate shunt may not be due to a low level of the required enzyme systems but is rather due to a lack of electron acceptors to bring about the oxidation of TPNH to TPN. This idea is in conformity with the findings of Wenner et al. (1958) who reported that the addition of artificial electron acceptors, such as methylene blue, could also stimulate the hexose monophosphate shunt in Ehrlich ascites cells.

In conclusion it may be said that tumour tissues in vitro show a lower incorporation of glucose carbon into glutamate and aspartate than the normal tissues, and this may be attributed to a partial malfunctioning of the citric acid cycle. Tumour tissues, in general, cause in increased efflux of glutamine from brain cortex slices in vitro, which is subsequently broken down by the tumour tissues. This phenomenon could be due to a high rate of glucose utilisation coupled with a high glutaminase activity in tumour tissues. Efficient function of the hexose monophosphate shunt in tumours may be dependent upon the presence of artificial electron acceptors for the oxidation of TPNH.

CLAIMS FOR ORIGINAL RESEARCH

1. Tumour tissues, in general, have a lower incorporation of glucose carbon into glutamate and aspartate than do rat brain cortex slices and chick embryo *in vitro*.
2. 4-day old chick embryos incorporate glucose carbon into glutamate, glutamine, aspartate and γ-aminobutyric acid *in vitro*.
3. Addition of glutamate, alanine, NH_4Cl and pyridoxal have no effect on the amino acids formation, from glucose, by tumour tissues *in vitro*.
4. The presence of liquid Novikoff hepatoma in rats, has been shown to decrease the capacity of brain cortex slices to incorporate glucose carbon into glutamine *in vitro*.
5. When tumour tissues are incubated, *in vitro*, with normal rat brain cortex slices, in a medium containing 5 mM glucose, there is an apparent decrease in the incorporation of glucose carbon into glutamine, by the brain cortex slices.
6. The decreased formation of glutamine by rat brain cortex slices in the presence of neoplastic tissues has been shown to be due to an insufficiency of glucose in the medium, and to the high glutaminase activity of tumour tissues. These two conditions cause an efflux of glutamine from the brain.

7. Tumour tissues have been shown to have an increased incorporation of glucose carbon into alanine, glutamate and aspartate, upon incubation with rat brain cortex slices *in vitro*.

8. The addition of 1mM glutamine-U-C^{14} with 5mM glucose-U-C^{14}, in a medium containing Ehrlich ascites cells, gives an increased formation of glutamate, aspartate and alanine. This increase is of the same order as observed in the presence of rat brain cortex slices *in vitro*.

9. A dialysate, obtained by dialysing a suspension of Ehrlich cells against Krebs-Ringer phosphate medium, pH 7.4, for six hours in the cold, has also been shown to cause a decrease in the formation of glutamine, in rat brain cortex slices *in vitro*. The dialysate also causes an increase in the formation of ammonia in a medium containing brain cortex slices and 5mM glucose.

10. The dialysate has been shown to contain protein-like material and its activity of decreasing glutamine formation in brain cortex slices and increasing the ammonia level is thermolabile.

11. Hyaluronic acid and hyaluronidase do not have any effect on the amino acid formation, from glucose, by rat brain cortex slices *in vitro*.

12. In Ehrlich ascites cells, sodium formate stimulates the oxidation of glucose-1-C^{14} and not glucose-6-C^{14}. The increase in the oxidation of glucose-1-C^{14} results in a decreased incorporation of glucose-1-carbon into glycine, glutamate, aspartate and alanine.

13. The presence of malonate, at a concentration of 5 mM, inhibits the $C^{14}O_2$ production from glucose-6-C^{14}, but has no effect on the glucose-1-C^{14} oxidation by Ehrlich ascites cells, in a medium containing formate.

14. L-cysteine has been shown to stimulate the oxidation of glucose-1-C^{14} and not glucose-6-C^{14} in Ehrlich ascites cells. The increased oxidation of glucose-1-C^{14} results in a decreased incorporation of glucose-1-carbon into amino acids.

15. The presence of malonate does not affect the $C^{14}O_2$ production from glucose-1-C^{14} but inhibits the oxidation of glucose-6-C^{14} by Ehrlich ascites cells, in the presence of cysteine.

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