STUDIES ON METABOLIC CHANGES IN LIVER OF TUMOUR-BEARING MICE

by

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A thesis submitted to the University of Glasgow for the Degree of Doctor of Philosophy in the Faculty of Medicine

February 1978

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LIST OF ABBREVIATIONS

ATP	Adenosine Triphosphate
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
CoASH	Coenzyme A
DMBA	Dimethylbenzathracene
LD_{50}	Lethal Dose Fifty
S.D.	Standard Deviation
S.E.M.	Standard Error of the Mean

ACKNOWLEDGEMENTS

I am greatly indebted to Sir Andrew Watt Kay for his advice and encouragement of this work. A debt of gratitude also to Professor K. C. Calman, who first suggested the need for such a study, and who has given freely of his advice at all stages of the investigation.

Several colleagues in the University of Glasgow and the Western Infirmary have also contributed with their time and advice. I thank Dr Ian More of the Pathology Department, who examined electron micrographs of tissue, and who guided me on the interpretation of the results. Also to Dr Leslie Fixter of the Biochemistry Department of the University, for helpful discussion on lipid metabolism, and who read appropriate sections of this thesis.

I am also greatly indebted to Dr Eric Newsholme, Department of Biochemistry, University of Oxford, who although not personally known by the author, has willingly given freely of his knowledge of the regulation of metabolism, and who at different periods gave encouragement.

Several members of the Technical Staff also contributed. Mr Eric Campbell, Senior Technician is thanked for his assistance and enthusiasm and also for carrying out the statistical analysis. Thanks are also expressed to Mrs M. Hughes for her help at different stages.

I am also indebted to Mr D. McSeveny, Senior Chief Technician, Pathology Department, Western Infirmary, for the processing of tissue for electron microscopy.

Finally a debt of thanks to Mr G. Donald and Staff for preparing diagrams and photographs and to Mrs M. Scott for her careful typing of the manuscript.

DECLARATION

Parts of this work have been presented at meetings of the Surgical Research Society and the British Association for Cancer Research and published as abstracts as follows:

1) Calman, K.C. & McAllister, R.A. (1975a) Metabolic abnormalities in tumour-bearing animals. British Journal of Surgery, 62, 161.
2) Calman, K.C. & McAllister, R.A. (1975b) Metabolic abnormalities in tumour-bearing animals. British Journal of Cancer, 32, 247.
3) McAllister, R.A., Soukop, M. & Calman, K.C. (1976) Metabolic changes in liver of tumour-bearing animals. British Journal of Cancer, 34, 312.
4) McAllister, R.A., Soukop, M. & Calman, K.C. (1977) Correction of changes in liver metabolites of mice following curative tumour resection. British Journal of Cancer, 36, 405.

Apart from the acknowledged collaboration, the original work reported in this thesis was performed solely by the author.

SUMMARY

In contrast to the widespread interest in metabolism of tumour cells, the effect of tumour growth on the metabolism of non-involved organs of the host has received little attention, although the importance of this is elucidating systemic effects of tumours is great.

This thesis therefore first set out to study the hepatic contents of the key regulatory intermediates acetyl CoA, CoA and citrate in liver of mice bearing either TLX-5 lymphoma, Sarcoma 180 or a C_3H transplantable mammary tumour. These studies showed that the hepatic contents of acetyl CoA and the free coenzyme were significantly decreased in liver of these animals, with concomitant increases in the citrate content. The direction of change in the hepatic contents of acetyl CoA and citrate were opposite to that found in starved tumour-free animals, and it was suggested that the increase in the hepatic content of citrate would favour gluconeogenesis by inhibition of phosphofructokinase.

The changes in the hepatic content of these metabolites occurred irrespective of the condition of the animal. Thus mice bearing TLX-5 lymphoma presented with anorexia, cachexia and hypothermia. There was also evidence that this tumour invaded liver. In contrast, both the mammary tumour and Sarcoma 180 remained locally malignant, and the animals showed no apparent ill effects of their tumour.

Study of the daily changes of these metabolites showed that these occurred at an early stage of tumour growth in mice bearing TLX-5 lymphoma. Early changes in spleen and thymus weights were also found. Later in a study adjacent to the main line of enquiry it was shown that in mice bearing Sarcoma 180, the onset of thymic atrophy could be correlated with the beginning of loss of body weight.

The administration of pantothenate to mice bearing Sarcoma 180 failed to revert the tumour-induced fall in the hepatic contents of free coenzyme A, but under these conditions a marked fall in body weight and spleen weight occurred without altering the rate of tumour growth. These data suggested some defect in the metabolism of pantothenate in mice bearing this tumour.

Further investigations showed that the hepatic contents of lactate, pyruvate, malate, α-oxoglutarate and oxaloacetate were altered in tumour-bearing mice. Calculation of the cytosolic NAD^+ / NADH ratios in these livers, showed that the cytoplasm was in a more reduced state than normal, and it was suggested that this might favour gluconeogenesis. Decreases in the hepatic contents of ATP, ADP and AMP were also found, but the ratios ATP/ADP and ATP/AMP did not change significantly in mice bearing Sarcoma 180, although the latter ratio fell significantly in mice bearing the lymphoma.

It was stressed however, that determination of the whole cell content of some of these metabolites

requires careful interpretation due to the well-recognised problem of compartmentation.

An increase in the lactate content of blood as a possible reason for the increase in the hepatic content of citrate in mice bearing either TLX-5 lymphoma or Sarcoma 180 was examined. In the former model blood lactate was not significantly altered, but in the latter, this showed significant decreases. In both models, blood glucose values were significantly decreased.

Although it was previously suggested that the increase in the hepatic content of citrate in these livers would favour gluconeogenesis, by citrate-inhibition of phosphofructokinase, the activity of the latter, as well as that of fructose-diphosphatase in liver of mice bearing TLX-5 lymphoma was not changed significantly. The conditions of the *in vitro* assay of these enzymes did not however necessarily reflect the conditions *in vivo*. It was also found that the hepatic contents of glucose-6-phosphate and fructose-6-phosphate were significantly decreased in mice bearing TLX-5 lymphoma. A decrease in the content of fructose-6-phosphate would also suggest a decrease in the activity of phosphofructokinase.

The possibility that the increased content of citrate in tumour bearing mice involved some disturbance of lipid metabolism due to inhibition of ATP-citrate lyase was next examined. These studies showed that the activity of this enzyme was significantly decreased in liver of mice bearing either TLX-5 lymphoma or

Sarcoma 180. The question then arose as to whether the tumour-induced changes in the hepatic contents of acetyl CoA and citrate could be altered by starvation of tumour-bearing mice. Under these conditions however, the hepatic contents of acetyl CoA did not increase, nor did a significant decrease in citrate occur, and in fact in starved mice bearing Sarcoma 180, the hepatic contents of citrate showed further increases.

The acetyl CoA/CoASH ratios in liver of fed mice bearing Sarcoma 180 were not significantly altered when compared to normal fed mice. Starvation of mice bearing this tumour increased the ratio acetyl CoA/ CoASH in a similar manner to that found in starved tumour-free mice. In contrast in fed mice bearing TLX-5 lymphoma, this ratio was increased significantly, but when these animals were starved, changes in the ratio did not occur. However there was no evidence of ketonuria in mice bearing either TLX-5 lymphoma or Sarcoma 180.

The increase in the hepatic content of citrate in fed or fasted tumour-bearing mice was considered to be of sufficient magnitude to activate acetyl CoA carboxylase, and stimulate fatty acid synthesis. It was also pointed out that the failure to reduce the hepatic contents of citrate by starvation of tumour-bearing mice could indicate that the regulation of fatty acid synthesis in liver of these animals was out of control.

Further investigations showed that in mice bearing TLX-5 lymphoma, there was a two-fold increase in weight

of epididymal adipose tissue, whereas in mice bearing the sarcoma, decreases were found. The hepatic content of triglycerides in the latter model also showed significant decreases. When both of these models were starved, the weight of the epididymal fat pads of mice bearing Sarcoma 180 decreased further. There were no significant changes however in the weight of the epididymal adipose tissues in starved mice bearing the lymphoma. These data suggested that the two tumours had different effects of fat mobilisation in their host, and this was discussed in terms of disturbances of hormonal activity in these animals.

The earlier observation that changes in the hepatic contents of acetyl CoA, CoASH and citrate occurred shortly following tumour implant suggested that products of tumour cells might be involved in the aetiology of these changes. This was supported by the observation that curative resection of a small localised mammary tumour could revert these changes to normal. Further, when the resection was unsuccessful the tumour-induced changes in the contents of these metabolites also recurred.

In support of these findings, it was also shown that changes in the hepatic contents of these metabolites could also be induced in normal mice following the intraperitoneal injection of a cell-free preparation of TLX-5 lymphoma cells, or in some instances non-viable tumour cells.

Finally, ultrastructural changes in liver of mice bearing Sarcoma were studied to examine whether

there was evidence of injury to mitochondria. These studies showed that mitochondria in these livers were swollen with loss of cristae. Other changes were also observed. It was also shown that these effects on mitochondria could also be induced in normal mice following the intraperitoneal injection of a cell-free preparation of TLX-5 lymphoma cells.

FOREWORD

'Tumour-host relations exist, they are part of the natural history of cancer and the host, and until an understanding of them is achieved only a partial knowledge of cancer will be attained'.

Begg (1958)

INTRODUCTION

General

It has long been recognised that the growth of malignant tumours in man, and experimental animals may cause marked alterations in the physiological state of their host, that cannot be attributed to mechanical interference with vital organs, or as a result of the secretion of hormones by the tumour cells. These systemic effects of a tumour are generally referred to as cachexia, a symptom complex that has been estimated to be the most frequent cause of death in cancer patients (Warren, 1932).

According to Theologides (1972), cancer cachexia, in patients is characterised by anorexia, loss of body weight with depletion of fat and other components, water and electrolyte abnormalities, and increased energy expenditure. Metabolic abnormalities such as hypoalbuminaemia (Mider, Alling and Morton, 1950; Winzler, 1953), hypoglycaemia (Marks, Steinke, Podolsky and Egdahl, 1974), and disturbances of lipid metabolism (Barclay, Skipski, Terebus-Kekish, Greene, Kaufman and Stock, 1970) have also been reported.

Clinical observations have shown that some patients may present with cachexia at an early stage of tumour growth. However, others may only become cachectic at the terminal stage of their illness.

In man and experimental animals it is well-recognised that different neoplasms may produce very different systemic effects on their host. Thus some

features of the cachexia syndrome may predominate over others. This is a complicating factor, not only in attempts to define the mechanisms involved, but also in defining which particular systemic effect may be of prime importance.

In spite of much investigation, the pathogenesis of cancer cachexia remains obscure. The problem of elucidating even one of the many systemic effects of a tumour on its host is reflected in a voluminous literature. Several authorities have discussed the problem in patients (Costa, 1963; De Wys, 1970; Theologides, 1972; Bondy, 1976) and excellent reviews have been written on the systemic effects of tumour growth in small animals (Haven and Bloor, 1956; Begg, 1958; Costa, 1963; Shapot, 1972). The immunological parameters of tumour-host relations have also been documented (Morton, 1973).

Without assuming too close a relationship between the systemic effects of transplantable and induced tumours in small animals, and those found in cancer patients, many similarities exist.

In tumour-bearing animals these include anorexia, loss of body weight with depletion of fat and nitrogen (Mider, Sherman and Morton, 1949; Haven, Bloor and Randall, 1949), extensive muscle wasting (Sherman, Morton and Mider, 1950), and increased energy expenditure (Mider, 1951; Pratt and Putney, 1958). Disturbances of lipid and carbohydrate metabolism have also been reported.

The described effects of alterations in lipid

metabolism include loss of body fat (Boyd, Connell and McEwan, 1952), hyperlipaemia (Begg and Lotz, 1956; Haven and Bloor, 1956) and alterations in the pattern of circulating lipoproteins (Creinin and Narayan, 1971; Cox and Gokcen, 1975). Although little is known of the mechanisms involved in these changes, it is likely that some can be attributed to anorexia (Costa, Lyles and Ullrich, 1976). It is known however that some tumour systems will induce loss of body fat in their host without inducing anorexia (Costa and Holland, 1962). Also as discussed later it has recently been proposed that the disturbances of lipid metabolism and decreased food intake in some tumour-bearing mice may be mediated by the release of a humoral factor from the tumour cells (Liebelt, Liebelt and Johnston, 1971).

Disturbances of carbohydrate metabolism in tumour-bearing animals has been recognised for many years, and have been discussed by Costa (1963), and more recently by Shapot (1972). These effects of a tumour include loss of liver glycogen (McFadzean and Jeung, 1956; Carey, Pretlow, Ezdinli and Holland, 1966), and hypoglycaemia (Mallick, Banerjee, and Shrivastava, 1968; Shapot and Blinov, 1974).

It seems likely that depletion of the glycogen reserves may involve stimulation by glucagon (Shapot, 1972), but the hypoglycaemia observed in some tumour-bearing animals is more difficult to explain. An excessive consumption of glucose by the tumour cells, which is utilised not only for the synthesis of amino acids (Campbell and Halliday, 1957) but also of pentose

of nucleic acids (Shapot, 1972), may be involved. However it is known that some tumour systems do not induce hypoglycaemia in the host, and this may involve an increase in gluconeogenesis in the host (Gutman, Thilo and Biran, 1969; Shapot and Blinov, 1974) to maintain the normal level of blood glucose.

Several studies have shown that growth of tumours in experimental animals may cause structural changes in organs distal to the tumour mass, and hypertrophy of spleen, liver and adrenals, and atrophy of the thymus have been well-documented (Haven and Bloor, 1956; Begg, 1958; Medigreceaunu, 1970; Theologides and Pegelow, 1970). These changes are found in the absence of metastases in these organs, and none are specific to the presence of a tumour. Several factors appear to be involved. They include as discussed in a later section of this thesis, not only the immunological response of the host, but also metabolic adaptation to the presence of a tumour.

In summary therefore, the systemic effects of a growing tumour in small animals may depend upon the tumour system used. Also associated pathological states such as hormonal imbalance, and alterations in the nutritional status of the host may be involved. There is also the possibility that some of these effects on the host are due to the release of products from the tumour cells.

The following sections now discuss in more detail some of the systemic effects of tumours that are pertinent to the studies to be presented in this thesis.

SOME SYSTEMIC EFFECTS OF TUMOURS ON THEIR HOST

Loss of Body Weight

Progressive weight loss is a frequent finding in cancer patients and tumour-bearing animals (Theologides, 1972). Although in some patients this can be attributed to anorexia, malabsorption, and increased energy expenditure, there is considerable evidence both from clinical observation in patients, and studies on experimental animals that other factors are involved (Theologides, 1972; Bondy, 1976). It is well-recognised for example that weight loss may be the first manifestation of malignant disease, and also weight loss may occur in patients with advanced cancer, and in whom the caloric intake is more than adequate. In such situations it has been proposed that toxic factors released from the tumour cells may be involved (Donovan, 1954; Begg, 1958; Gellhorn, 1970), but as discussed later, the release of toxic factors from tumour cells *in vivo* has been difficult to prove.

Attempts to induce weight gain in tumour-bearing rats by force-feeding have been extensively investigated (Begg and Dickenson, 1951; Stewart and Begg, 1953; Mider, 1953; Sugimura, Birnbaum, Winitz and Greenstein, 1959) but only a temporary improvement was achieved. In these animals such treatment can be considered unphysiological, and stress may influence the results. Intravenous feeding has also been studied in tumour-bearing animals, but according to one report (Cameron

and Pavlat, 1976) the additional nutrition evoked a greater rate of tumour growth. However in recent studies on the effect of intravenous hyperalimentation in tumour-bearing rats it has been shown that although the absolute weight of the tumour increased, the ratio of tumour weight to body weight did not alter significantly (Ota, Copeland, Strobel, Daly, Gum, Guinn and Dudrick, 1977).

Dudrick and his group have also shown that intravenous hyperalimentation may induce weight gain in some cancer patients (Copeland, MacFadyen and Dudrick, 1974).

The earlier studies leading to the concept that tumours act as nitrogen 'traps' and utilise nitrogen from the tissues of the host when the intake of dietary nitrogen is reduced have been reviewed by Mider (1953) and Costa (1963). It has also been recently postulated that tumours may 'trap' glucose and possibly vitamins as well (Shapot, 1972). Although there is some evidence of an increased uptake of glucose by tumours in vivo (Norman and Smith, 1956; Campbell and Halliday, 1957) as well as amino acids (Yamamota, Aikawa, Matsutaka, and Ishiwaka, 1974), the uptake of an essential nutrient such as a vitamin leading to its depletion in the host has not been documented.

Rivlin (1973) has investigated the metabolism of riboflavin in tumour-bearing animals. He has suggested that tumours may affect the metabolism of vitamins, and in studies to be presented later it is shown that treatment of mice bearing Sarcoma 180 with pantothenate

induces a marked loss of body weight. There was also a reversal of the hypertrophy of spleen in these animals. These studies are presented in Chapter 2. As discussed therein, Ghadially and Wiseman (1956), have reported that supplementation of the diet of rats bearing RD_3 sarcoma with methionine induces the tissues of the host to waste, and at the same time increases the rate of tumour growth. In our own studies however, tumour growth was not affected by treatment of the animals with the vitamin.

MORPHOLOGICAL CHANGES IN ORGANS OF THE HOST

Although earlier workers have reported on the hypertrophy of spleen, liver and adrenals and atrophy of thymus in tumour-bearing animals, in recent years attention has been limited.

These studies have been reviewed by Haven and Bloor (1956), and by Begg (1958) who has also discussed the associated histopathological changes in these organs.

Liver

Analysis of livers of tumour-bearing animals has shown that the water content (McEwen and Haven, 1941) as well as that of nitrogen (Sherman, Morton and Mider, 1950) is increased. The reason for these changes is unclear, but malnutrition is not responsible since in starved animals liver weight decreases (Harrison, 1953).

According to Wiseman and Ghadially (1958), the enlarged liver of the tumour-bearing host may reflect an increase in metabolic activity. This would be consistent with a previous report of increased mitotic activity in liver of tumour-bearing animals (Malmgren, 1956), as well as a more recent finding of increased synthesis of DNA (Rev-Kury, Kury and Friedell, 1966). Increases in both of these parameters may reflect increases in the activity of several enzymes involved in the synthesis of macromolecules (Herzfeld and Greengard, 1972).

Although explanations for the enlargement of liver of the tumour-bearing host remain speculative, it is of interest that Toporek (1972) has shown that there is a decrease in synthesis of serum proteins in liver of tumour-bearing rats, concomitant with an increased synthesis of liver proteins. It was also shown in these studies that both of these changes may involve a humoral factor from the tumour cells.

Spleen

Hypertrophy of spleen in tumour-bearing animals is a frequent finding (Stewart and Begg, 1953; Medigrecenaunu, 1970), and one of the most striking morphological changes seen.

Rapid enlargement of spleen has been reported to occur following tumour implant (Siegler and Koprowska, 1962), and this has also been observed in the present studies on tumour-bearing mice. It has also been found in normal animals following the injection of

* The hypertrophy is associated with histopathological changes including increases in the number of giant cells (Begg, 1952).

extracts of various tumours - the so-called 'toxohormone' - (Nakahara and Fukuoka, 1958; Kampschmidt, Adams and McCoy, 1959).

As described later, the present author and colleagues (McAllister, Soukop and Calman, 1977), have shown that the enlarged spleen of mice bearing small localised mammary tumours is reverted to normal following a curative resection. Also as described later, the injection of cell-free ascitic fluid from mice bearing TLX-5 lymphoma, into normal mice also induces hypertrophy of spleen (as well as thymic atrophy).

These observations support the view that hypertrophy of spleen in tumour-bearing animals is due to the immunological response (Konda, Nakao and Smith, 1973; Konda and Smith, 1973). They also suggest that products of tumour cells may be involved in this effect, and these are being investigated at present.

Thymus

Although atrophy of the thymus of tumour-bearing animals has been recognised for many years (Larionow, 1932; Fukuoka and Nakahara, 1952; Simu, Toma, Nestor and Rosculet, 1968; Ertl, 1973), very little is known of the mechanisms involved.

Ertl (1973) considers that thymic atrophy is related to hypertrophy of the adrenals but the latter is a much less frequent finding (Nakahara and Fukuoka, 1958) and thymic atrophy is diminished, but not abolished in adrenalectomised tumour-bearing rats

(Simu, Toma, Nestor and Rosculet, 1968).

Studies on mice bearing MT890 ascitic tumour have shown that the beginning of thymic atrophy in these animals corresponded with enlargement of spleen, and histopathological changes in liver (Siegler and Koprowska, 1962). Later Ertl (1973) showed that the commencement of thymic atrophy in rats bearing Walker 256 carcinoma could be related to the beginning of loss of body weight and progressive cachexia.

These findings suggest a close physiological relationship between thymic atrophy and some of the systemic effects of a tumour, and later it is shown that the above relationship between thymic atrophy and loss of body weight is also found in mice bearing Sarcoma 180 (Chapter 8).

Adrenals

The histological and histochemical changes that occur in the adrenal of some tumour-bearing animals have been extensively studied (Haven and Bloor, 1956; Ertl, 1973).

According to some reports, hyperfunction of the adrenals with hypersecretion of glucocorticoids can be detected during the early stages of tumour growth. In the latter stages, these changes lead to exhaustive insufficiency, with depletion of the glucocorticoid reserves (Allot and Skelton, 1960; Ertl, 1973). Not all authors are in agreement with these findings. For example, increased hypophyseo-adrenal function, and increase in the plasma

corticosterone levels have also been described in the advanced stages of tumour growth in rats (Nakata, Suematsu, Nakata, Matsumoto and Sakamoto, 1964).

The importance however of glucocorticoids in the enhancement of gluconeogenesis is well recognised (for reviews see Weber, 1968 and Exton, 1972). Such mechanisms include the induction of several amino-transferases in liver (Barnabel and Sereni, 1962; Estrada and Cordoba, 1963; Beato, Kalimi and Feigelson, 1972) and increased activity of several of these enzymes have also been reported in liver of tumour-bearing animals (Nakata, Suematsu, Nakata, Matsumoto and Sakomoto, 1964; Greengard, Baker and Friedell, 1967).

According to Shapot (1972), the hypersecretion of glucocorticoids may be involved in the enhancement of gluconeogenesis in tumour-bearing animals.

ULTRASTRUCTURAL CHANGES IN LIVER OF TUMOUR-BEARING ANIMALS

Several workers have described changes in the hepatocellular ultrastructures that frequently accompany the growth of localised subcutaneous neoplasm in small animals (Ghadially and Parry, 1965; Parry and Ghadially, 1966, 1967, 1969; Khandekar, Dardachte, Garg, Tuchweber and Kovacs, 1972; Bhawan, Friedell and Jacobs, 1975), but some of the findings differ according to the species of animal used, the type of tumour, and the condition of the animal at the time of the observations

(Khandekar, Dardachte, Garg, Tuchweber and Kovacs, 1972).

Parry and Ghadially (1966) studied rats bearing subcutaneous carcinogen-induced tumours when the animals were in the agonal phase, and showed that these changes included marked swelling of mitochondria indicative of an uncoupling of oxidative phosphorylation. However, according to Baldwin, George and Cunningham (1975), mitochondria from livers of rats bearing Walker 256 carcinoma at a stage when the animals remained healthy showed no differences in the rate of loss of respiratory control, although when the animals were near death, liver mitochondria could not catalyse ATP formation.

These findings on the biochemical evidence alone, were considered to indicate that ultra-structural changes in mitochondria were due to the degenerative process of dying, and not to the tumour per se.

However, other workers have reported that the growth of a transplantable mammary tumour in rats also induced ultra-structural changes in mitochondria in liver of the apparently healthy host (Khandekar, Dardachte, Garg, Tuchweber and Kovacs, 1972). These latter workers proposed that hepatic fine structural changes in these livers were due to a direct toxic effect of the tumour, a view also held by Bhawan and Friedell, 1975). Such proposals are of interest because in a previous study (Parry and Ghadially, 1970) it was found that treatment of normal rat with a highly purified toxohormone also induced changes in hepatocellular ultra-structures that paralled these

changes in animals hosting the subcutaneous tumour.

Later in Chapter 7, it is shown that growth of subcutaneous Sarcoma 180 in mice induces several ultrastructural changes in liver of these animals, that include swelling of mitochondria and loss of cristae. It is also shown therein, that such changes can also be induced in normal mice following the injection of cell-free ascitic fluid from mice bearing TLX-5 lymphoma.

EARLY SYSTEMIC EFFECTS OF TUMOUR GROWTH IN SMALL ANIMALS

In 1963, in a review on cancer cachexia, Costa wrote,

'Another point of importance is to establish whether the systemic effects of tumours are a continuous function of tumour mass, whether they are related to the rate of tumour growth, or are, rather, phenomena which reach their full expression at some time during the course of the neoplasm progressing thereafter as a function of time unaffected by subsequent behaviour of the tumour'.

Prior to that time, some observations had been made on early measurable changes that occurred in animals shortly after tumour implant. Pratt and Putney (1958) for instance reported increases in energy expenditure in rats shortly after tumour implant, and other workers (Haven, Bloor and Randall, 1951) showed that mobilisation of body fat occurs at

an early stage of growth of the Walker 256 carcinoma in rats, during the period when food intake remained normal, and before the carbohydrate and protein stores became depleted. In support of these observations, Costa and Holland (1962) described an early acute fat loss in mice bearing Krebs-2 carcinoma, that occurred during the first week and when the tumour was just palpable. They also found that an increase in oxygen consumption could be detected 12 hours after implantation of the tumour in these animals.

Since then, more recent studies have shown that changes occur in several serum lipid fractions three to four days after implantation of Ehrlich ascites tumour in mice (Creinin and Narayan, 1971), or following the injection of Simian virus 40 into Syrian hamsters (Cox and Gokcen, 1975).

That such early changes are not confined to disturbances of lipid metabolism is evidenced by the investigations of Herzfeld and Greengard (1972) who have shown that the activity of ornithine aminotransferase in liver of tumour-bearing rats shows a marked decrease in as short a time as four hours after tumour implant.

In Chapter 2 of this thesis, evidence is presented showing the 24 hours after tumour implant in mice significant changes occur in the hepatic contents of acetyl CoA and citrate. As discussed therein, the change in the acetyl CoA content of liver of these animals may be related to a report that appeared after our observations, showing that the administration of ^{14}C-tripalmitin to mice bearing Krebs-2 carcinoma,

resulted in a marked suppression of $^{14}CO_2$, that was detectable within 24 hours of tumour implant. It was also shown that this procedure may have potential significance in the detection of cancer in patients (Costa, Lyles and Ullrich, 1976).

A cautionary note however must be made as to the use of animal models in such studies, because as Liebelt et al. (1974) have pointed out, the sudden imposition of a rapidly growing population of tumour cells on a healthy and often young animal is of obvious marked contrast to the more chronic series of events that are found in the human situation.

This abrupt challenge to the host is no doubt responsible for the immunological response evidenced as already described by hypertrophy of spleen, but the other changes induced in the host, including our own observations of rapid changes in the hepatic content of regulatory intermediates are less understood.

The involvement of factors of tumour origin cannot be excluded from the aetiology of these early systemic effects of tumours, and as described in a later Chapter (6), we have found evidence of this in our own observations.

SOME MECHANISMS THAT HAVE BEEN PROPOSED IN THE PATHOGENESIS OF CANCER CACHEXIA

The literature on cancer cachexia contains many hypotheses (Costa, 1963; Gold, 1968; Theologides, 1972; Gold, 1974; Theologides, 1974).

The many similarities that exist between starvation and cancer cachexia, including weight loss and thymolymphatic atrophy have led to proposals that the large dietary demands of a tumour on its host may be responsible for loss of body weight and negative nitrogen balance. The force-feeding experiments on rats that have already been cited have shown however that malnutrition alone is not responsible (Theologides, 1972). Such evidence however has been obtained under unphysiological conditions (Costa, 1963), and is complicated by the fact that the nutritional status of the host is known not only to affect tumour growth (Tannenbaum and Silverstone, 1953; Ghadially and Wiseman, 1956; Henderson and Le Page, 1959; Cameron and Pavlat, 1974) but also to affect the metabolism of the tumour (Rosen, Budwick, Solomon and Nichol, 1961; Ota, Copeland, Strobel, Daly, Gum, Guinn and Dudrick, 1977). In the former instance, this effect may involve alterations in the immunological status of the host, which in both malnourished (Cannon, 1949; Ross and Bras, 1965) and tumour-bearing animals, changes are found.

A unique situation also exists between starvation and cancer cachexia, in that in starved animals liver weight decreases (Harrison, 1953) whereas in tumour-bearing animals increases in the weight of liver occur. It has already been noted that this may involve an increase in metabolism of liver in the tumour-bearing host (Wiseman and Ghadially, 1958). Also in starved tumour-free animals the metabolic rate decreases whereas in both cancer patients (Gellhorn and Holland, 1954; Theologides, 1972) and animals hosting tumours

increases are found (Pratt and Putney, 1958). Part at least of this increase in oxygen consumption by the tumour-bearing host may be due to the high energy demands of gluconeogenesis (Bondy, 1976).

The nutritional aspects of malignant disease have been discussed in detail by Tannenbaum and Silverstone (1953) and more recently by Copeland, MacFadyen and Dudrick (1974).

As discussed later in this section much attention has also been paid to the possibility that tumours secrete toxic products that perturb metabolism in the host (Nakahara and Fukuoka, 1958; Kampschmidt, 1970; Olivares, 1970).

It has also been proposed that tumours induce a greatly augmented pathway of gluconeogenesis in the host, and that such a mechanism is involved in the pathogenesis of cancer cachexia (Gold, 1968; Shapot, 1972; Gold, 1974).

The evidence for an increase in gluconeogenesis in the tumour-bearing host however is largely circumstantial.

Further, although an increase in Cori cycle activity in cancer patients occurs when compared to patients with other diseases (Reichard, Moury, Hochella, Patterson and Weinhouse, 1963), as Shapot and Blinov (1974) have pointed out no data on gluconeogenesis in tumour-bearing animals from lipid or amino acids can be found in the literature prior to that date.

Although several reports have described changes

in the activities of several enzymes in liver of tumour-bearing animals (Kampschmidt, Adams and McCoy, 1959; Suda, Tanaka, Sue, Harana and Morimura, 1966; Greengard, Baker and Friedell, 1967; Gutman, Thilo and Biran, 1969; Herzfeld and Greengard, 1972; Isohashi, Terada, Nakanishi and Sakamoto, 1976), very little attention has been paid to the regulation of intermediary metabolism in liver of tumour-bearing animals, although the potential importance of this in the pathogenesis of cancer cachexia is great.

It has been postulated however that in cancer cachexia such regulatory mechanisms are disrupted (Theologides, 1972). According to this suggestion tumours produce low molecular weight metabolites such as polypeptides and nucleotides that allosterically activate and inactivate various enzyme systems in the host. Alterations in various metabolic equilibria are thereby effected, and to quote, 'throws the metabolism of the host into a chaotic state'.

It has also been proposed (Marks, Steinke, Podalsky and Egdahl, 1974) that the hypoglycaemia in patients with large single neoplasms may be due to the release of tryptophan, a known inhibitor of gluconeogenesis (Ray, Foster and Lardy, 1966), or short chain polypeptides with a similar effect.

There was however little experimental evidence that tumour growth perturbed intermediary metabolism in liver of the host, by mechanisms involving alterations in key glycolytic intermediates or those involved in tricarboxylic acid cycle activity. Extensive

investigations were therefore begun on these lines (Calman and McAllister, 1975a, 1975b; McAllister, Soukop and Calman, 1976; McAllister, Soukop and Calman, 1977).

The possibility that some of these changes may be related to the production of factors from tumour cells was also investigated (McAllister, Soukop and Calman, 1977), and are presented later in this thesis.

As discussed later in this section, some of these studies had considerable bearing on the proposal (Gold, 1968, 1972; Shapot, 1972) that tumour growth induces an augmented pathway of gluconeogenesis in the host. These concepts will now be discussed.

(1) ENHANCEMENT OF GLUCONEOGENESIS IN THE TUMOUR BEARING HOST

The first suggestion that the growth of a tumour might induce changes in the pathways of intermediary metabolism in the host stemmed from the work of Fenninger and Mider (1954). They proposed that such alterations, 'forced' the host to use more expensive pathways in terms of energy and nitrogen metabolism. However, the mechanism involved in this effect was considered to be a selective removal of essential nutrients from the host by the tumour. and as discussed previously this has not been proved.

Later Gold (1968, 1972) postulated that the growth of a tumour induced a greatly augmented pathway of

gluconeogenesis in the host, the high energy demands of which depleted host energy reserves in terms of ATP. This he considers is due to increased gluconeogenesis from lactate produced by tumour glycolysis and other gluconeogenic substrates, mainly amino acids after their conversion to oxaloacetate.

However, although there is some evidence of increased Cori cycle activity in patients (Reichard, Moury, Hochella, Patterson and Weinhouse, 1963), the concept of Gold (1972) rests heavily on an ever increasing production of lactate from the tumour cells, and this is not a consistent finding in either patients (Bondy, 1976) or tumour-bearing animals (Greenstein, 1954; Costa, 1963). There is however evidence that the concentration of various amino acids in blood of some cancer patients (Kelley and Waisman, 1967) and tumour-bearing animals is increased (Wu and Bauer, 1960). An increased concentration of alanine, the predominant amino acid in hepatic gluconeogenesis (Felig, 1973) has also been reported in the blood of patients with advanced cancer, and evidence presented that this was derived from the breakdown of muscle proteins (De Wys, 1970).

Shapot (1972) however, has also implicated an increase in gluconeogenesis in the host as a major factor in the pathogenesis of cancer cachexia. According to him, this is due to the high rate of glucose consumption by the tumour cells, which effects increases in the gluconeogenic flux in the host in order to maintain normal blood glucose levels. In support of this

suggestion, Shapot and Blinov (1974) have shown that in mice bearing Sarcoma 180 which maintained normal levels of glucose in blood over the whole period of tumour growth, there was an increase in gluconeogenesis as measured by glucose formation from tyrosine-^{14}C.

In contrast, in mice bearing Ehrlich's ascites carcinoma, in which hypoglycaemia developed from the fifth day following tumour implant, there was no enhancement of endogenous glucose formation from labelled amino acids in these animals.

According to these workers the differences found in the gluconeogenic response in animals bearing Sarcoma 180 and the Ehrlich ascites tumour is due to differences in the response of the host tissues to glucocorticoids. Thus stimulation of gluconeogenesis by the administration of cortisol failed to increase blood glucose levels in mice bearing the ascites tumour. However, when large doses of cortisol were given, or when the animals were subjected to stress, normal blood glucose values were found. In contrast, the gluconeogenic response to cortisol in mice bearing Sarcoma 180 was similar to that found in normal animals, and in fact, in the tumour-bearing group, slight hyperglycaemia resulted (Shapot and Blinov, 1974).

The concepts of both Gold (1968, 1974) and Shapot (1972) implicating increased gluconeogenesis in the pathogenesis of cancer cachexia would necessitate that there would be an increase in the activity of key enzymes in liver towards gluconeogenesis, but the evidence for this is conflicting.

Although this will be discussed in more detail later, here it may be mentioned for instance that Suda, Tanaka, Sue, Harano and Morimura (1966) have reported a switch in enzymic activity to a predominantly glycolytic pathway in liver of rats hosting the Walker 256 carcinoma, while other workers in studies on rats bearing what was described as a non-metastasising sarcoma have reported increases in activity of gluconeogenic enzymes (Gutman, Thilo and Biran, 1969).

Shapot and Blinov (1974) however consider that previous reports (Greengard, Baker, and Friedell, 1967) of increases in activity of tryptophan oxidase and tyrosine transaminase in liver of tumour-bearing rats would support their findings discussed above, but as others have pointed out, (Herzfeld and Greengard, 1972) these enzymes are particularly sensitive to glucocorticoids, and the changes in activity may well have been due to stress.

In studies to be reported in this thesis, we have shown that the activities of phosphofructokinase and fructose-1,6-diphosphatase are not altered in liver of tumour-bearing mice. We have also previously reported (Calman and McAllister, 1975a), that in these animals, there are changes in the hepatic content of acetyl CoA and citrate that are opposite to those found in animals under gluconeogenic conditions. However, as discussed later, an increased citrate content in liver in vivo could favour gluconeogenesis by inhibition of phosphofructokinase. In tumour-

bearing mice decreases in the oxalaoacetate content of liver were also found, and we have suggested that this might be due to increased synthesis of glucose. In support of these suggestions, it was also shown that the cytoplasm of liver cells in these animals was in a more reduced state than normal, and this might favour gluconeogenesis (Chapter 3 p. 96).

In attempts to elucidate several of the above changes in the hepatic contents of these metabolites, attention was then directed towards establishing whether such changes could be induced in liver of normal animals by the injection of preparations of tumour cells. Several previous reports had described various effects that could be induced in normal animals following the injection of either tumour extracts, or non-viable tumour cells, and these will be discussed briefly.

(2) PRODUCTS OF TUMOUR ORIGIN AFFECTING HOST METABOLISM

Some mention has already been made in this presentation that some of the systemic effects of a tumour may be due to products of the tumour cells. The concept of a cancer toxin is an old one and has received considerable support by the isolation of the so-called 'Toxohormone' from cancer tissue by Nakahara and Fukuoka (1949) which when injected into normal mice stimulated some of the systemic effects of tumours in vivo, such as depression of liver catalase activity and decrease in serum iron

(Kampschmidt, Adams and McCoy, 1959), and also induced hypertrophy of spleen liver and adrenals and thymic atrophy (Nakahara and Fukuoka, 1958).

However none of these effects are tumour-specific, and both a decrease in catalase activity in liver (Miller, 1948) as well as thymolymphatic atrophy are also found in starved tumour-free animals (MacFarlane, 1971).

The literature on 'Toxohormone' is extensive, and has been reviewed by Nakahara and Fukuoka (1958) and others (Costa, 1962; Kampschmidt, 1970). Nowadays, however there is doubt as to whether this product has any relationship to the products of tumour cells in vivo. Probably the main argument against the production of 'Toxohormone' by tumour cells, is the fact that repeated injections are necessary to maintain effects such as depression of activity of liver catalase, or serum iron (Kampschmidt, Adams and McCoy, 1959; Fukuda, Okada, Akikawa, Matsuoa and Urushizaki, 1966). Thus the normal recipient appears to form some resistance to this material in contrast to the irreversible systemic effects induced by tumours in vivo.

However, in recent years there is an increasing body of evidence that both human and murine cancer cells produce biologically active products that affect the growth characteristics of normal cells (Argyris and Argyris, 1962; Rounds, 1970; Rubin, 1970; Nair and DeOme, 1973). The secretion of ectopic hormones by some human cancers is also recognised (Gellhorn, 1970).

It has also been reported (Suda, Tanaka, Sue,

Harano and Morimura, 1966), that growth of Ehrlich ascites tumour in mice induces a deviation of key enzymes in liver towards glycolysis, and that the factor responsible is transmitted by the blood. It has also been demonstrated, that some of these changes may also be induced in normal animals following the injection of various fractions from sonically disrupted tumour cells (Tanaka, Yanagi, Miyahara, Kaku, Imamura, Taniuchi and Suda, 1972).

According to Costa and Holland (1962), the early acute fat loss in mice bearing Krebs-2 carcinoma can also be induced in normal animals following the injection of non-viable tumour cells. This they consider was due to the presence of a virus in their tumour cell preparations. However Liebelt and coworkers (1974) have shown that the injection of saline extracts of a CBA 2663 stomach tumour of mice into normal mice induced significant increases in the steroid, steroid esters and total lipid content in serum from these animals.

The growth of this tumour in CBA mice did not induce anorexia, and the animals maintained a relatively constant body mass, but a shift in composition, as evidenced by a decrease in lipid and increase in non-lipid compartments was found. However when the tumour was transplanted into experimentally obese mice, a marked decrease in body weight that included loss of body fat, occurred.

These data suggested that this tumour produced a

lipid-mobilising factor, and Liebelt et al. (1974) consider that this is responsible for the triad of decreased food intake, lipid depletion and hyperlipaemia in tumour-bearing animals.

In recent studies, the present author and colleagues (McAllister, Soukop and Calman, 1977), have shown that the injection of cell-free ascitic fluid from TLX-5 lymphoma into normal mice effected alterations in the hepatic content of acetyl CoA and citrate in these animals, and thereby induced changes previously found (McAllister and Calman, 1975a) in liver of tumour-bearing mice. Such changes in normal mice also followed the injection of non-viable TLX-5 lymphoma cells, and in these animals daily changes in the triglyceride content of liver were found that also mimicked such changes in mice hosting this tumour.

Good experimental evidence that some of the systemic effects of tumours involve the release of products of the tumour cells is also to be found where such effects can be reversed following curative resection. These include reversal of the increase in activity of serum aldolase that is found in rats hosting the Walker 256 carcinoma or Sarcoma 39 (Sibley and Lehninger, 1949). More recently, Shikasaka and Fujii (1975) have found that the elevated level of thymidine kinase activity in rats bearing Yoshida sarcoma, decreases after removal of the tumour.

Some of the present author's observations on the effects of curative resection on some of the systemic effects of tumours in mice have already been described.

In recent studies we have also shown (McAllister, Soukop and Calman, 1977) that alterations in the hepatic contents of acetyl CoA, free CoA and citrate that accompany growth of tumours in mice can be reverted to normal in mice bearing small localised mammary tumours by curative resection. Further, when this procedure was unsuccessful, abnormalities in the hepatic contents of these metabolites again occurred.

PLAN OF THE THESIS

This thesis reports a study of metabolic changes in livers of tumour-bearing mice.

It consists of six Chapters describing these changes, the attempts that were made to revert some of them to normal, and the experimental approaches carried out to define their aetiology. The final Chapter is a brief note on observations that were made on these animals outwith the main line of enquiry.

Since the majority of the studies presented here are based on a new approach to the problem of systemic effects of a tumour on its host, background information is limited. Therefore for purposes of clarity, each Chapter begins with its own introduction, and ends with a discussion of the findings reported therein.

CHAPTER I

MATERIALS AND METHODS

(1) ANIMALS

In the majority of the studies to be reported here, male CBA mice were used. These were from an inbred strain maintained in the Animal House of the University Department of Surgery, by conventional inbred mating systems.

Where studies were made on mice bearing a C_3H transplantable mammary tumour, then this strain of animal was used. These were obtained from an inbred strain maintained in the Glasgow Institute of Radiotherapeutics and Oncology, Belvidere Hospital, Glasgow, by courtesy of Dr A. H. W. Nias.

Unless otherwise stated, all CBA strain mice were fed a standard cubed diet (Breeding Diet Oxoid) whereas the C_3H strain mice were fed Diet 41 (Oxoid). Water was given to all animals add lib.

Prior to the start of an experiment, using mice which were to be inoculated with either TLX-5 lymphoma or Sarcoma 180, groups of 12 or 20 animals were taken, at three to four months of age, then weight-matched and ear marked. One of each matched pair serving as a control while the other received the tumour under study.

After selection of animals in this fashion, they were allowed to acclimatise themselves to their new surroundings for two to three days. This was found

to counteract weight loss that occurs rapidly in mice when transferred from one environment to another.

(2) TUMOURS USED

(a) Sarcoma 180

According to Stewart, Snell, Dunham and Schlyen (1959), Sarcoma 180 is an undifferentiated tumour that was originally found in the right axillary region of a white male mouse in the Crocker Laboratory New York in 1914. In the literature it is therefore sometimes referred to as Crocker Sarcoma 180.

Although the tumour can be transplanted by the intraperitoneal, subcutaneous or intramuscular route, in the present studies subcutaneous inoculation has been used.

According to Austin and Glaser (1970), both the sex of the mouse, its body weight at transplant and the temperature of the environment have an effect on the growth of Sarcoma 180. For this reason, only male mice were used, and the temperature of their environment maintained as far as possible at $27^{o}C$.

Sarcoma 180 has been reported to invade surrounding tissues (Stewart et al. 1959). In the present studies only liver of mice bearing this tumour was used for the study of concentrations of metabolites, and enzymic activity. Routine histological examinations of these livers showed no evidence of metastases.

(b) TLX-5 Lymphoma

This tumour was first induced by Dr D. I. Connell at the Institute of Cancer Research, London by whole body X-ray irradiation of CBA mice and transplantation of the enlarged thymuses into male CBA mice. In studies to be presented here mice received the ascites cells intraperitoneally, and histological examination of liver by Professor K. C. Calman showed evidence of metastases. In our experience 2×10^6 ascites cells injected i.p. killed the animals in about eight days.

(c) Transplantable C_3H Mammary Tumour

The above tumour was originally a spontaneous tumour found in a mouse by Dr A. H. W. Nias in 1973. Since then it has been extensively studied in his Laboratory in the Glasgow Institute of Radiotherapeutics and Oncology. The tumour has been found to be locally malignant with no evidence of metastases in lungs, liver and spleen.

(3) SOURCES OF TUMOURS USED

At the start of these investigations male mice bearing either TLX-5 lymphoma or Sarcoma180 were obtained from the Chester Beatty Research Institute London. The former was in ascites form, and the latter had been implanted subcutaneously in the subscapular region.

Thereafter the tumours were passaged in animals

in the Department of Surgery, except on odd occasions when animals had died, before routine passage could be carried out, and the facilities offered by the Chester Beatty Research Institute were again called upon.

As already stated, male C_3H mice bearing this tumour were supplied by courtesy of Dr A. H. W. Nias. These had received a tumour cell suspension of 10^6 cells in 0.05 cm^3 of Minimum Essential Medium, Hank's Salt Solution, (Flow Laboratories) containing horse serum, glutamine and non-essential amino acids.

The tumour cells in these mice had been injected subcutaneously in the midline of the back at the level of the abdomen.

(4) ROUTINE PASSAGE OF TUMOURS

Routine passage of the TLX-5 lymphoma and Sarcoma 180 were carried out at regular intervals as follows.

(a) TLX-5 Lymphoma

Mice bearing this tumour in ascites form, were sacrificed and the ascitic fluid removed from the peritoneal cavity, as sterile as possible. The fluid was first mixed with Hank's Balanced Salt Medium (Flow Laboratories), and the number of tumour cells counted using an Improved Neubauer counting Chamber.

Dilutions were then made with the same medium, to give a cell population of 2×10^6 per 0.5 cm^3. This was then injected into male mice by the intra-

peritoneal route under light ether anaesthesia. These animals were then sacrificed about six to seven days later, and the procedure repeated.

(b) Sarcoma 180

The following procedure was adopted both for routine passage of the tumour, as well as when setting up mice for purposes of study.

Animals bearing this tumour were sacrificed by cervical dislocation, and the subcutaneous tumour growing in the subscapular position removed under sterile conditions. The tumour mass was then divided in two, and areas of necrosis dissected out. Portions of the tumour, free of the necrotic area were then chopped up on a glass plate with a little Hank's Balanced Salt Medium. One gram of the minced tumour was then rapidly weighed, mixed with 1.0 cm^3 of the same medium, then filtered through a gauze swab. 0.25 cm^3 of the tumour suspension was then injected subcutaneously into mice in the subscapular position under ether anaesthesia. For routine passage purposes, this procedure was carried out after every eight to 10 days.

(5) CONTROL GROUPS

Considerable care was taken in the choice of adequate controls. In studies on mice bearing Sarcoma 180, normal mice received the same volume of Hank's Balanced Salt Medium by the same route.

In some experiments, in mice bearing TLX-5 lymphoma, normal control mice received 2 x 10^6 spleen

cells from normal CBA mice. For this purpose spleens were removed at sacrifice, and placed in small wells made of fine mesh metal gauze. About 1 cm^3 of the Balanced Salt Medium was added, and the spleen pushed through the gauze, using a sterile glass rod. The spleen cells so obtained were collected, suitable aliquots removed and the number of cells counted as before. This was then diluted with the same medium usually to give a cell population of 2×10^6 in 0.5 cm^3 of the medium which was administered intraperitoneally.

As described later, in some experiments, CBA strain mice were challenged with spleen cells from A strain mice.

In other studies, including those on mice bearing the C_3H mammary tumour, normal, weight and age-matched mice were used as controls.

(6) CONDITION OF ANIMALS

Mice bearing the TLX-5 lymphoma became cachectic after about five to six days of tumour growth.

However as Liebelt et al. (1974) have pointed out, little attention has been paid to the clinical course of tumour growth in small animals, and we have therefore aimed at defining cachexia in these animal models. This was characterised by anorexia, lassitude, and changes in the texture of the fur. Figure 1 shows some of these changes in a mouse bearing TLX-5 lymphoma.

It was also observed that in these animals there

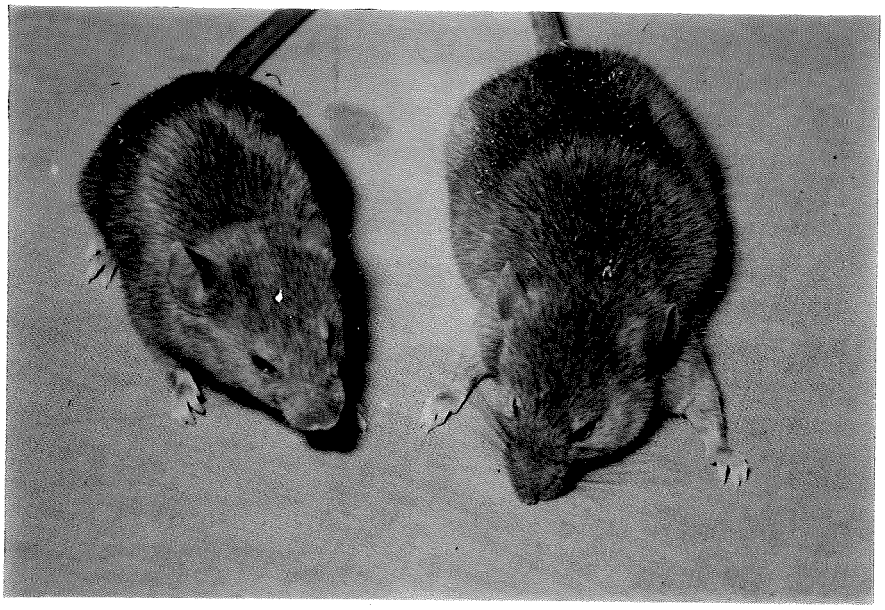


FIGURE 1 Normal male mouse (left) and male mouse bearing TLX-5 lymphoma (right) seven days after inoculation with 2×10^6 tumour cells i.p. The tumour-bearing animal presents with cachexia, as evidenced by lassitude and changes in the texture of the fur.

were marked decreases in body temperature*, and Figure 2 shows these changes when recorded over the seven day period of tumour growth. It will be seen that a marked fall occurred on the fifth day following tumour implant, and thereafter a steady fall in body temperature occurred until the study was terminated.

In contrast, mice bearing either Sarcoma 180 or the transplantable C_3H mammary tumour showed few ill effects of their tumours, and as shown later mice bearing Sarcoma 180 continued to eat normally even when bearing large tumours.

(7) SACRIFICE OF ANIMALS

Herrera and Freinkel (1968) have reported increases in the hepatic content of acetyl CoA in rats that had been killed by either Nembutal or ether, and for this reason, in the present studies, mice were killed by cervical dislocation.

* As determined by a thermocouple placed in the inguinal region. As can be seen, body temperature was elevated during the first five days following tumour implant. This would be consistent with an elevated basal metabolic rate, that has been reported in rats shortly following tumour implant (Pratt and Putney, 1958).

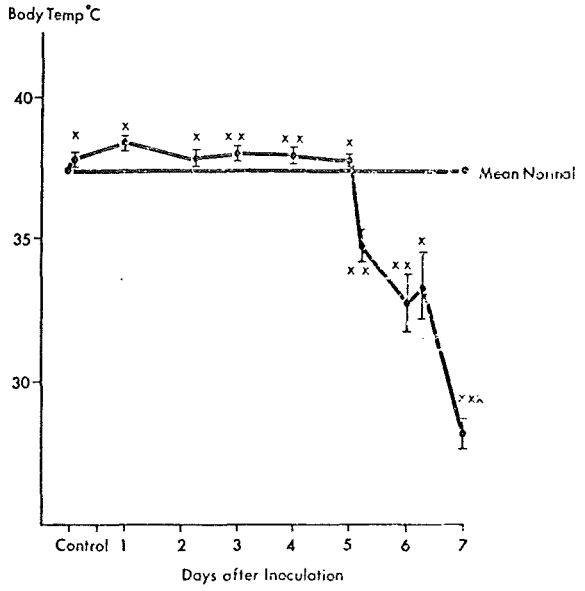


FIGURE 2 Changes in body temperature following the injection of 2×10^6 TLX-5 lymphoma cells i.p. Six animals were studied in each group.

SECTION I

PREPARATION OF TISSUE AND ENZYMATIC DETERMINATION OF METABOLITES

(1) PREPARATION OF TISSUE FOR ASSAY OF METABOLITES

(a) The Technique Used for Freeze-Clamping Mouse Liver

The labile nature of many of the metabolites studied, necessitated the use of the 'freeze-stop' technique developed by Bucher et al. (1964).

For this purpose, the liver is clamped between two aluminium plates to produce a thin layer of tissue, the temperature of which, according to Bergmeyer (1965) reaches - 160°C in 0.5 seconds.

The tongs used in the present studies are shown in Figure 3 and were constructed from Cheetle forceps, by bolting aluminium plates measuring 6 cm long by 5 cm wide on to the jaws of the forceps. The aluminium plates should be about 4-5 mm thick to give the necessary rigidity.

Prior to the procedure, the tongs were immersed in liquid nitrogen contained in aluminium trays, and stoneware pestles and mortars pre-cooled in the same manner.

Pre-weighed glass homogenising vessels (Jencons) with a capacity of 15 cm^3, (152 mm long; internal diameter 15 mm) were placed in Dewar Flasks containing Dry-Kold, and kept there for at least an hour before use.

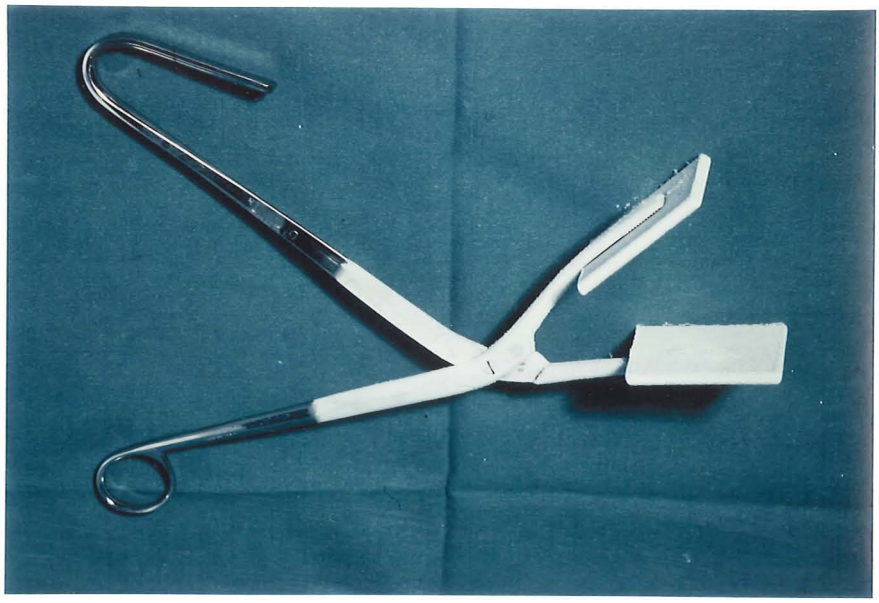


FIGURE 3 Tongs used for freeze-clamping of mouse liver.

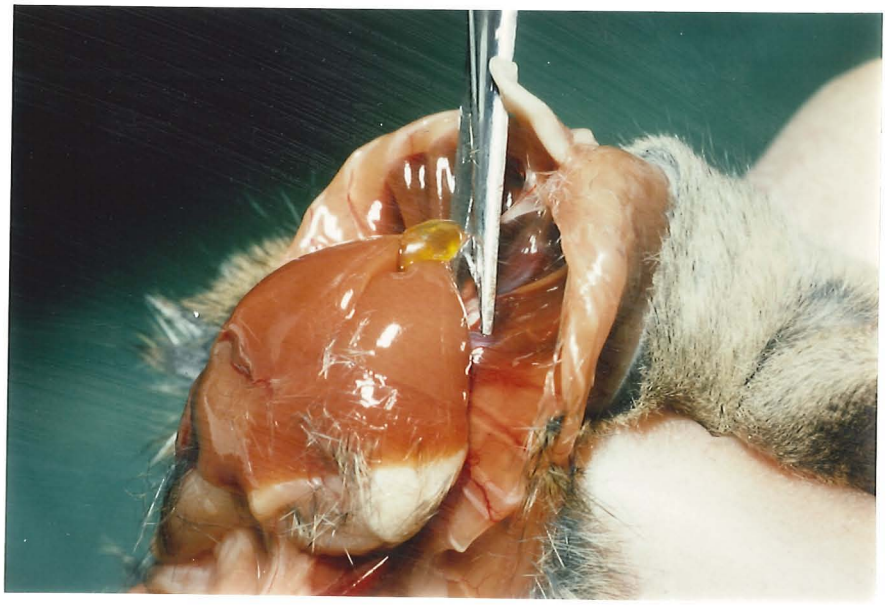


FIGURE 4 Removal of liver prior to freeze-clamping.

Once the equipment was assembled, the mouse was killed by cervical dislocation, and the liver rapidly exposed by a transverse upper abdominal incision.

The ligament attaching the liver to the diaphragm was cut (Figure 4) and the whole organ dropped onto the pre-cooled tongs (Figure 5) held by another operator, who then immediately clamps them and immerses the whole into liquid nitrogen.

At this stage, the tissue may be kept in the liquid nitrogen for a reasonable period, but in any event, as an additional precaution, it was kept there for at least five minutes.

It may be noted here, that the sizes given for the aluminium plates are such, that once the liver is clamped, the tissue does not extrude beyond the edges of the plates. However with larger animals such as the rat, the liver tissue would extrude beyond the edges of clamps of these dimensions, and in such an instance, the non-clamped tissue would require to be cut off and discarded.

Figure 6 shows a freeze-clamped liver, and illustrates the very thin layer of tissue obtained by this technique.

The frozen liver is then reduced to a fine powder using the pre-cooled mortar and pestle.

Considerable care has to be taken at this stage to prevent the tissue thawing out, and this is best prevented, by firstly crushing the tissue for a few

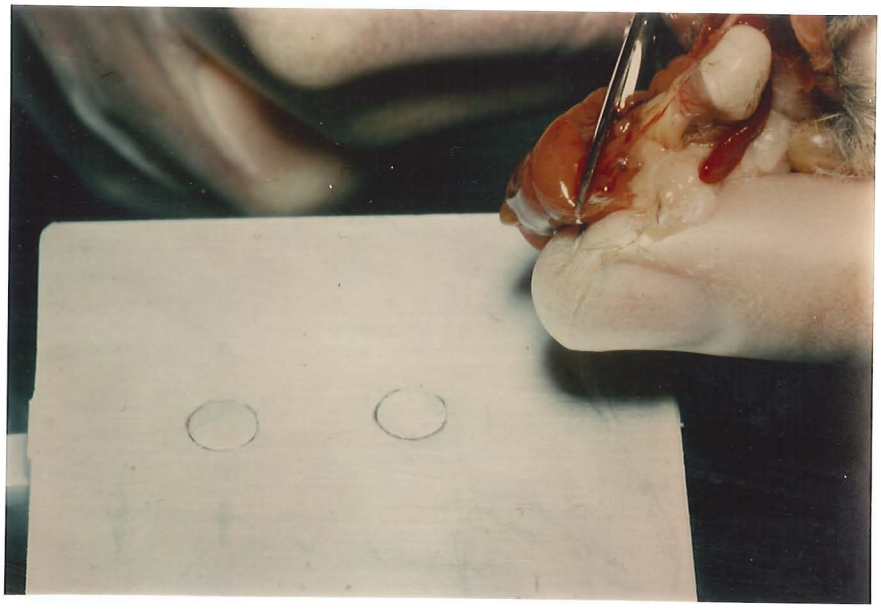


FIGURE 5 Liver about to be dropped onto the lower plate of the pre-cooled tongs.

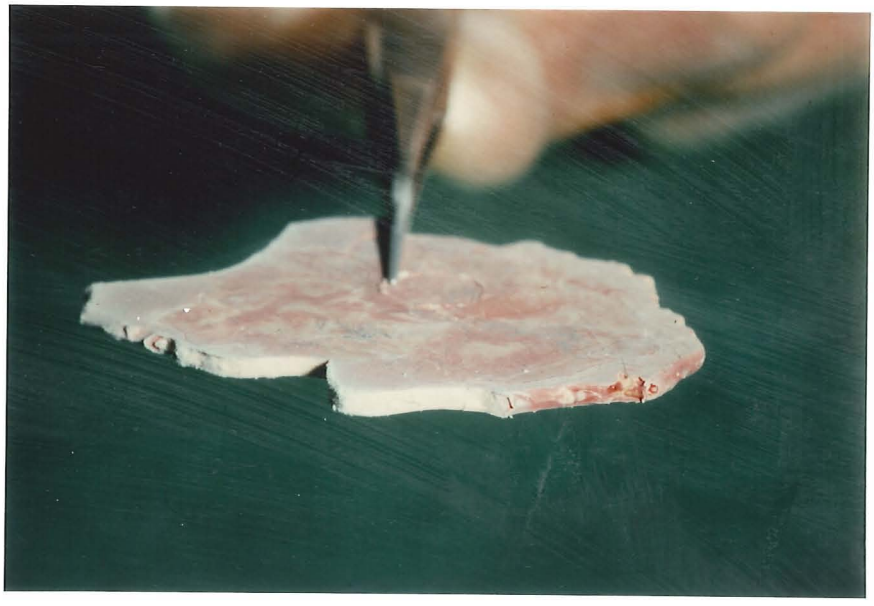


FIGURE 6 Thin wedge of liver obtained after freeze-clamping.

seconds, then immediately either adding liquid nitrogen to it, or returning the mortar and pestle to the tray of liquid nitrogen. The final stage of powdering the tissue is then carried out, but again with frequent cooling in between. The powdered liver is then transferred to a pre-weighed homogenising vessel using a spatula that has been pre-cooled in liquid nitrogen, whereas the vessel itself should be pre-cooled in Dry-Kold. After a rapid re-weigh, the vessel is returned immediately to the Dewar Flask.

(b) Extraction of Metabolites

Perchloric acid is used to precipitate proteins and extract the metabolites from the frozen liver. Although the amount of liver taken varies, as does the volume and molarity of the perchloric acid used, the basic technique is the same, and will be described here in order to avoid repetition. The exact amounts of tissue required will be given later.

The extraction of metabolites with perchloric acid and the subsequent homogenisation step, is the stage where things can go wrong, and considerable care must be paid to the prevention of the tissue thawing out before the perchloric acid precipitates the proteins.

In the present studies, the homogeniser used was the conventional Potter and Elvehjem (1936) type and consisted of a teflon pestle (Jencons) driven by a 1/10th horse power motor (Anderman) which gives a maximum speed of approximately 6000 r.p.m.

For convenience, the pestle can be stored in a deep-freeze (- 25°C) or stored in a Dewar Flask containing Dry-Kold, then removed immediately before use.

The required volume of ice cold perchloric acid is then added to the powdered tissue in the homogenising vessel, mixed rapidly with a glass rod that has been cooled in ice, then the vessel is placed in crushed ice.

The mixture is then immediately homogenised using two series of passes, with 30 seconds between each.

The homogenate is then centrifuged at 3000 g for 10 minutes and the supernatant removed. At this stage some methods call for a double extraction of the tissue with a further aliquot of perchloric acid, and where necessary this is carried out as described later.

The next stage is the removal of perchloric acid from the tissue extract by precipitating it as the potassium salt with either potassium hydroxide or potassium carbonate. In the present studies, the supernatant was brought to the required pH (given later) using a dual purpose micro-electrode and an accurate pH meter (Research Model; Electronic Instruments). The temperature of the sample being compensated by appropriate adjustment of the instrument.

This stage, although appearing elementary requires considerable care in order to avoid over-shooting the required pH, and subsequent loss of the sample. For this reason, we titrate the sample during constant

magnetic stirring, with in some cases three different strengths of the appropriate base, one of low molarity being used to attain the final adjustment.

Once the pH has been adjusted, the sample is then cooled in ice, for 10 minutes and the potassium perchlorate removed by centrifugation.

(2) METHODS USED FOR THE DETERMINATION OF METABOLITES

Considerable attention was paid to the selection of suitable methods for the determination of the hepatic content of metabolites* under investigation, as well as to methods for the assay of enzymic activities.

The majority of the procedures used are those given by Bergmeyer (1965) in his book, 'Enzymatic Methods of Analysis', which has achieved the status of a classic in this field.

Since such procedures involve measurement of changes in the absorbance of NADH or NADPH, it should be noted here, that the extinction coefficient of NADH and NADPH used in the calculations was 6.22 at 340 nm and 3.30 at 366 nm and not the revised values given by McComb et al. (1976).

Although these workers have shown such revisions are necessary, since for example, an increase in temperature will decrease the molar absorptivities, as will an increase in ionic strength, the revised values have so far not been currently applied.

* Recovery values for metabolites added to perchloric acid extracts of mouse liver were satisfactory. Although few values for the hepatic contents of these metabolites in the mouse have appeared in the literature, where available these were generally found to be in agreement with those found in the present study.

Further, the aim of the present studies has involved studying changes in the hepatic content of metabolites, and activities of some enzymes induced by tumour growth in the host, and these data have been compared against the values found in normal control groups.

Although the procedures to be described give in most instances an estimate of at least two metabolites on the one liver extract, by the use of accurate timing, and the appropriate enzyme it is of course possible to carry out several determinations on the one sample. This is an obvious advantage in saving time and labour, but also more important limits the number of animals used. It is also possible to store the frozen liver powder in Dry-Kold for up to 14 days without loss of pyruvate, oxaloacetate, citrate, α-oxoglutarate, acetyl CoA and CoASH, and data showing this is presented later.

(a) Determination of 'Total' CoA, Acetyl CoA and CoASH

Many of the available methods for the determination of CoA are either non-specific or suffer the disadvantage of being open to interference from other tissue constituents (Michal and Bergmeyer, 1965).

The following method of Mollering and Bergmeyer (1975) was therefore used throughout, since it is highly specific for acetyl CoA and the free coenzyme.

The method gives a measurement of the 'Total' CoA, that is the sum of acetyl CoA, free CoA (CoASH) and

(7) Standard Coenzyme A:

5.0 mg CoA are dissolved in 5 cm^3 deionised water. The exact content is then determined by the end-point method using phosphotransacetylase as described later. The solution is then diluted accordingly to give a working standard containing 10 μg CoA/cm^3 ≡ 0.653 nmol in 0.05 cm^3.

(8) Phosphotransacetylase suspension (PTA) 1000 U/cm^3.

(9) Malate dehydrogenase suspension (MDH) 1400 U/cm^3.

(10) Citrate synthase suspension (CS) 140 U/cm^3.

Stability of Reagents

All solutions and suspensions except potassium carbonate were kept refrigerated at 4°C. Fresh solutions of triethanolmine/malate buffer; NAD/acetylphosphate; DTT and N-Ethyl maleimide should be prepared daily, whereas the standard CoA must be used within a few hours of preparation.

According to the manufacturer (Boehringer Mannheim) the PTA suspension is stable for six months; whereas the MDH and CS suspensions are stable for up to 12 months.

Procedure

5.0 cm^3 of ice-cold perchloric acid (1 mol/l) is added to 200-300 mg frozen liver in a homogenising vessel and the homogenisation carried out as described previously.

The mixture is then centrifuged at 3000 g for 10 minutes, and the supernatant removed.

The pH is then brought to 6.0 using 2.5 mol/l, potassium carbonate to begin with, then, 0.25 mol/l, the final adjustment being made with 0.025 mol/l.

The mixture is then decanted into a centrifuge tube using the minimum of deionised water to effect the transfer, then cooled in ice for 10 minutes.

It is then centrifuged for 10 minutes at 300 g, and the volume of the supernatant noted.

Conditions - Wavelength 340 nm. Path length 1 cm. Temperature 25°C. Readings are made against air.

The following are added to two cuvettes, one for the determination of the 'Total' CoA, and the other for the determination of acetyl CoA plus oxidised CoA.

	'Total' CoA	Acetyl CoA + Oxidised CoA
Supernatant	0.05 cm^3	0.05 cm^3
N-ethyl maleimide	-	0.05 cm^3
Deionised water	0.30 cm^3	0.20 cm^3

After mixing, the cuvettes are maintained at 25°C for 10 minutes. The following are then added:

Triethanolamine/malate	1.5 cm^3	1.5 cm^3
Dithiothreitol	0.05 cm^3	0.10 cm^3

A further incubation at 25°C for 15 minutes is made and the following added:

	'Total' CoA	Acetyl CoA + Oxidised CoA
NAD/Acetylphosphate	0.20 cm^3	0.20 cm^3
MDH	0.015 cm^3	0.015 cm^3
PTA	0.015 cm^3	0.015 cm^3

The course of the reaction is then followed until it stops (usually five minutes). 0.02 cm^3 of the C.S. suspension is then added to each.

After rapid mixing the course of the reaction is followed for 17 minutes at one minute intervals. The absorbance at two minutes and at 17 minutes is noted from the tracing and the difference between the two (ΔA) determined. Each day, prior to the determination of the hepatic content of the coenzyme, a blank was set up consisting of 0.05 cm^3 deionised water in place of the supernatant, as were three standards consisting of 0.05, 0.03 and 0.01 cm^3 of the working standard made to a volume of 0.05 cm^3. The amounts of free coenzyme A present were 0.653, 0.391 and 0.130 nmol CoASH respectively.

As noted previously, the standard solution must be prepared fresh, and the exact content determined by means of the end-point method using phosphotransacetylase.

This was carried out as follows:

Determination of Free CoA with Phosphotransacetylase (PTA: E.C.2.3.1.8)

Conditions - Light path 1 cm using silica cuvettes. Temperature of assay 25°C. Readings are made against air at a wavelength of 233 nm.

Reagents

(1) Tris (hydroxymethyl) -aminomethane, (0.1 mol/l) pH 7.6.

(2) Acetylphosphate, (0.1 mol/l).

(3) PTA, Crystalline suspension in 3.2 mol/l ammonium sulphate, Approximately 1000 U/mg.

Procedure

The following are placed in the cuvette in the following order:

Tris Buffer, pH 7.6	2.79 cm^3
Acetylphosphate	0.10 cm^3
Sample	0.10 cm^3

After mixing, the initial absorbance (A_1) is read, then the reaction is started by the addition of 0.01 cm^3 PTA suspension.

Again after mixing, the reaction is followed until it stops (usually in two minutes), and the absorbance (A_2) is read.

A blank is also carried through the procedure and consists of 0.1 cm^3 of the tris buffer in place of the sample.

Calculation

The content of free coenzyme A in the standard solution is given by:

$$\Delta A \times 5.18 = \text{mg coenzyme A}/cm^3$$

where Δ A is, derived from:

Δ A = $(A_2 - A_1)$ of the sample - $(A_2 - A_1)$ of the blank.

The content of 'Total' coenzyme A in liver is given by:

(1) $\frac{\Delta \text{ A Sample} - \Delta \text{ A Blank}}{\Delta \text{ A Standard} - \Delta \text{ A Blank}}$ x Concentration of standard (nmol) x $\frac{1}{0.05}$ = nmol 'Total' CoA/cm^3 of the supernatant

(2) $\frac{\text{nmol 'Total' CoA/cm}^3 \text{ x Supernatant Volume x 1000}}{\text{mg/Powdered frozen liver}}$ = nmol 'Total' CoA/g liver (wet wt.).

Similarly, the content of acetyl CoA (+ oxidised) is derived as above by substituting the appropriate absorbance. The content of the free coenzyme A is then derived by subtraction of this value from the 'Total'.

Notes

Linearity of the Reaction

Figure 7 shows the linearity obtained when the three standards were put through the complete method used for the assay of coenzyme A in liver.

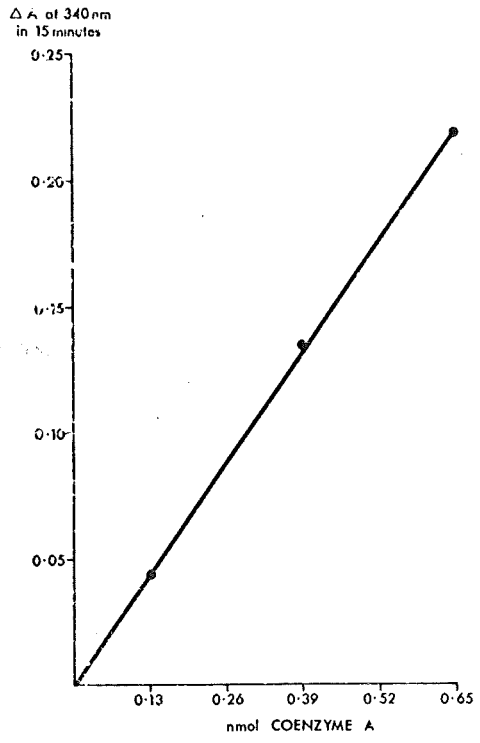


FIGURE 7 Linearity of the reaction in the determination of Coenzyme A.

(b) Determination of Pyruvate, Citrate and α-Oxoglutarate

Reagents

(1) Perchloric acid, (0.6 mol/l).

(2) Citrate standard solution.
2.1015 g citric acid are dissolved in approximately 80 cm^3 deionised water and the pH adjusted to 7.5 with sodium hydroxide (10 mol/l) before making to 100 cm^3 with deionised water. This solution is then diluted 1/500 to give 37.827 µg citrate/ 0.9 cm^3 (0.2 mmol/l).

(3) Zinc chloride, (0.03 mol/l).

(4) Triethanolamine buffer, (0.1 mol/l).
1.856 g triethanolamine hydrochloride are dissolved in approximately 80 cm^3 deionised water. The pH is adjusted to 7.6 with sodium hydroxide (1 mol/l) before making to 100 cm^3 with deionised water.

(5) β-nicotinamide adenine dinucleotide, reduced form (β-NADH), disodium salt, (0.01 mol/l). 1.28 cm^3 sodium hydrogen carbonate, (0.12 mol/l) are added to a pre-weighed vial containing 10 mg β-NADH (Sigma, London).

(6) Lactate dehydrogenase suspension (LDH), 598 U/cm^3.

(7) Malate dehydrogenase suspension (MDH), 1405 U/cm^3.

(8) Citrate lyase suspension (CL), 100 U/cm^3.

(9) Glutamate dehydrogenase suspension (GLDH), 240 U/cm^3.

Stability of Reagents

All solutions and suspensions are kept refrigerated at 4°C. The citrate standard solution, zinc chloride, perchloric acid and triethanolamine buffer are stable for 12 months whereas the β-NADH solution must be used within a few hours of preparation.

According to the manufacturers (Boehringer Mannheim) the LDH and MDH suspensions are stable for several months but the CL suspension loses about 10 per cent of its activity within one month.

Procedure

4.0 cm^3 ice-cold perchloric acid is added to approximately 750 mg frozen powdered liver in a pre-cooled homogenising vessel, well mixed then homogenised.

The homogenate is then transferred to a centrifuge tube together with washings (1 cm^3 of perchloric acid). If more than 750 mg of tissue have been used, then an extra 0.5 cm^3 of perchloric acid for every additional 100 mg, or proportion thereof, is used to wash out the homogenising vessel.

The combined mixture is then cooled in ice for about three minutes, then centrifuged at 3000 g for 10 minutes, then again cooled in ice for about five minutes, before decanting the supernatant into a small beaker.

The pH is adjusted to 7.5 using potassium hydroxide (2 mol/l) with constant stirring using a magnetic stirrer. To avoid over-titration, the former strength of the alkali should only be used until the pH is

about 4.0, then 0.1 molar potassium hydroxide should be used thereafter.

The mixture is then cooled in ice for 10 minutes then centrifuged (3000 g for 10 minutes) and the volume of the supernatant noted.

Conditions - Wavelength 366 nm; path length 1 cm. Temperature 25°C. Measurements are made against air.

(c) Determination of Pyruvate and Citrate (Moellering and Gruber, 1966)

The following are pipetted into cuvettes:

Triethanolamine buffer	2.0 cm^3
β-NADH	0.06 cm^3
Zinc Chloride (0.03 mol/l)	0.01 cm^3
Supernatant	0.9 cm^3

After mixing, the absorbance of the mixture is read until it stabilises, and the final absorbance (A_1) noted.

Next, 0.01 cm^3 LDH suspension is mixed in, and the change in absorbance recorded at one minute intervals, until the reaction stops, usually in about five minutes. Again the final absorbance is noted (A_2).

Note

A standard solution of citrate is included with each set of samples, and consists of 0.9 cm^3 of the diluted standard equivalent to 0.18 μmol citrate, in

place of 0.9 cm^3 of the supernatant.

(d) Determination of α-Oxoglutarate (Bergmeyer and Bernt, 1965)

The conditions for the assay of α-oxoglutarate are the same, except that the wavelength is changed to 340 nm.

The following are added to cuvettes:

Supernatant	3.0 cm^3
β-NADH (0.01 mol/l)	0.04 cm^3

The absorbance is then recorded until it stabilises and the value at this stage noted (A_4).

0.04 cm^3 GLDH suspension is mixed in, and the change in absorbance recorded at one minute intervals until the reaction stops. If as happens on occasions, the reaction continues beyond 10 minutes, the readings are plotted and extrapolated from zero time. The absorbance is again noted (A_5).

For the determination of α-oxoglutarate the blank consists of 3.0 cm^3 of deionised water in place of the supernatant.

Calculation

Pyruvate

$\Delta A = (A_1 - A_2)$

$$\Delta A \times \frac{1003 \times \text{Supernatant Volume}(cm^3)}{\text{mg Liver Taken}} = \frac{\mu\text{mol pyruvate/g}}{\text{liver (wet weight)}}$$

Citrate

$$\Delta A = (A_2 - A_3)$$

$$\frac{\Delta A \times 1010 \times \text{Supernatant Volume}(cm^3)}{\text{mg Liver Taken}} = \frac{\mu mol \text{ citrate/g}}{\text{liver (wet weight)}}$$

α-Oxoglutarate

$$\Delta A = (A_4 - A_5) - \text{E blank}$$

$$\frac{\Delta A \times 165 \times \text{Supernatant Volume}(cm^3)}{\text{mg Liver Taken}} = \frac{\mu mol \alpha\text{-oxoglutarate/}}{\text{g liver (wet weight)}}$$

(e) Determination of Lactate and Malate (Hohorst, 1965a, b)

Reagents

(1) Perchloric acid, (0.6 mol/l).

(2) Potassium carbonate, (5 mol/l; 0.5 mol/l and 0.05 mol/l).

(3) Hydrazine-glycine buffer, (0.4 mol/l hydrazine sulphate, 1.0 mol/l glycine) pH 9.5. 7.5 g glycine, 5.2 g hydrazine sulphate and 0.2 g ethylene-diamine-tetra-acetic acid (EDTA) are suspended in about 40 cm^3 deionised water before adding 51 cm^3 sodium hydroxide, (2 mol/l). After adjusting to pH 9.5 the buffer is made to 100 cm^3 with deionised water.

(4) Nicotinamide adenine dinucleotide (5×10^{-2} mol/l).

(5) Lactate dehydrogenase (LDH), - 1375 U/cm^3.

(6) Malate dehydrogenase (MDH), - 6000 U/cm^3.

Stability of Reagents

All solutions and suspensions are stored, stoppered in a refrigerator at 4°C. The enzyme suspensions are stable for several weeks as is the NAD which does not require to be neutralised due to the high buffering capacity of the hydrazine-glycine buffer. The buffer used is stable for only one week.

Procedure

At least 400 mg frozen powdered liver is transferred to a cold homogenising vessel and perchloric acid (0.6 mol/l) added at a concentration of 1 cm^3 for every 140 mg tissue.

The mixture is then homogenised as already detailed, and transferred to a centrifuge tube, the suspension remaining in the homogenising vessel being washed out with 0.5 cm^3 of the perchloric acid.

The mixture is then allowed to reach room temperature before centrifuging at 3000 g for 10 minutes. The supernatant is then decanted into a small beaker, and cooled to 4°C, then the pH is adjusted to 3.5 by the addition of potassium carbonate with constant stirring, using a magnetic stirrer. The adjustment of the pH is best begun using drops of the strongest solution of potassium carbonate (5 mol/l), followed by the other two (0.5 mol/l and 0.05 mol/l respectively).

The mixture is then left in ice for 10 minutes, and the precipitate of potassium perchlorate removed by centrifugation at 3000 g for 10 minutes.

The volume of the supernatant is then measured.

Conditions - Wavelength, 340 nm. Path length 1 cm. Temperature 25°C. Readings are made against air.

The following are pipetted into the cuvettes in the following order:

Hydrazine-Glycine Buffer	1.35 cm^3
NAD (5×10^{-2} mol/l)	1.5 cm^3
Supernatant	0.3 cm^3
Deionised Water	1.2 cm^3

After mixing, and allowing to stabilise the absorbance (A_1) is read.

0.03 cm^3 of the LDH suspension is then mixed in, and the change in absorbance read at one minute intervals, until the reaction stops, usually in about five minutes. At this stage, the absorbance A_2 is taken.

Blank - A blank consisting of 0.3 cm^3 of deionised water in place of the supernatant is taken through the procedure.

The malate content of the sample is then determined as follows:

Hydrazine-Glycine Buffer	1.35 cm^3
NAD	0.15 cm^3
Supernatant	1.5 cm^3

The absorbance is then recorded until it stabilises, and is then taken (A_3).

The malate dehydrogenase suspension is then mixed in and the change in absorbance measured at one minute intervals until the reaction stops (usually in about five minutes); the final absorbance (A_4) is then taken.

Calculations

(1) Lactate

$$\frac{\Delta A(\text{Test}) - \Delta A(\text{Blank}) \times 1623.8 \times \text{Supernatant Volume}}{\text{mg Liver Taken}} =$$

μmol lactate/g liver (wet weight)

Where, $\Delta A = A_2 - A_1$

(2) Malate

$$\frac{\Delta A(\text{Test}) - \Delta A(\text{Blank}) \times 324.7 \times \text{Supernatant Volume}}{\text{mg Liver Taken}} =$$

μmol malate/g liver (wet weight)

Where, $\Delta A = A_4 - A_3$

(f) Determination of Glucose-6-Phosphate and Fructose-6-Phosphate

Procedure according to Hohorst (1965c)

Reagents

(1) Potassium carbonate, (2.5 mol/l).

(2) Perchloric acid, (0.6 mol/l).

(3) Triethanolamine buffer, pH 7.6 (0.4 mol/l).

(4) Magnesium chloride, (0.5 mol/l).

(5) Nicotinamide adenine dinucleotide phosphate (NADP), approximately 5 mmol/l.

(6) Glucose-6-phosphate dehydrogenase (G6PDH), - 900 U/cm^3.

(7) Phosphoglucose isomerase (PGI), - 0.2 mg protein/ cm^3.

Stability of Reagents

All solutions and suspensions are kept refrigerated at 4^oC. Under these conditions all are stable for several weeks.

Procedure

5.0 cm^3 ice-cold perchloric acid (0.6 mol/l) are added to 800 - 900 mg of the frozen, powdered liver, in a pre-cooled homogenising vessel, then homogenised according to the standard procedure.

The homogenate is decanted into a centrifuge tube, and centrifuged for 10 minutes at 3000 g.

The supernatant is then transferred to a 25 cm^3 beaker and the latter placed in ice.

One cm^3 of the perchloric acid and 1 cm^3 of deionised water are then used to wash out the homogenising vessel, and the washings added to the pellet remaining after the centrifugation step.

After thorough mixing, the mixture is left to

cool in ice for five minutes then centrifuged. The supernatant is then combined with the previous one in the beaker, and pH is raised to 3.5 using 2.5 molar potassium carbonate.

The mixture is then decanted into a centrifuge tube, using the minimum amount of deionised water to effect the transfer, and then cooled in ice for 10 minutes.

After centrifuging for another 10 minutes at 3000 g, the volume of the supernatant is noted.

The glucose-6-phosphate and fructose-6-phosphate content is then determined as follows

Conditions - Wavelength 340 nm. Path length 1 cm. Temperature 25°C.

Readings are made against a cuvette containing triethanolamine buffer.

The following are placed in a cuvette:

Triethanolamine buffer	1.0 cm^3
Supernatant	1.5 cm^3
NADP (5 mmol/l)	0.02 cm^3
Magnesium chloride (0.5 mol/l)	0.02 cm^3

After mixing, the absorbance is measured until a constant reading is obtained (A_1).

0.01 cm^3 of the G-6-PDH suspension is mixed in, and the changes in absorbance recorded at one minute

intervals until the reaction stops (usually about five minutes). This absorbance (A_2) is then read.

Then 0.01 cm^3 PGI suspension is added, and the change in absorbance recorded at one minute intervals until the reaction stops (usually in about five minutes). The final absorbance (A_3) is then noted.

Calculations

The glucose-6-phosphate content is given by

$$\frac{\Delta A \times 273.3 \times \text{Volume of Supernatant}}{\text{mg Liver Taken}} =$$

μmol glucose-6-phosphate/g liver (wet weight)

Where $\Delta A = A_2 - A_1$

Similarly the fructose-6-phosphate is given by

$$\frac{\Delta A \times 274.4 \times \text{Volume of Supernatant}}{\text{mg Liver Taken}} =$$

μmol fructose-6-phosphate/g liver (wet weight)

Where $\Delta A = A_3 - A_2$

(g) Determination of the Triglyceride Content of Liver

Triglycerides were extracted from livers of mice by the method of Wheeldon and Collins (1957). After saponification of an aliquot of the extract, the glycerol content is determined enzymatically using the Test Combination of Boehringer Mannheim.

Extraction of Triglycerides

About 500 mg of liver is taken and approximately 5 cm^3 of chloroform-methanol (2:1 v/v) are added. The mixture is homogenised until a fine suspension of the tissue is obtained. The homogenate is then transferred with washings using the same solvent mixture to glass-stoppered test tubes, and the volume made to about 12 cm^3. After thorough mixing the samples were placed in a refrigerator overnight. The extract is then filtered into flasks, and the precipitate on the filter paper is then washed twice with about 10 cm^3 of the same chloroform-methanol mixture.

The combined extracts are then taken to dryness in a rotary evaporator under mild suction at a temperature not exceeding 37°C. About 10 cm^3 of ether-ethanol (1:1 v/v) is then added and evaporated to dryness as before. This was found to remove traces of water.

The dried residue is then dissolved in about 2 cm^3 of chloroform-methanol (2:1 v/v), the flask washed out with the same solvent and the volume made to 5.0 cm^3.

Saponification of an aliquot (usually 0.1 cm^3) was then carried out. The saponification step and subsequent determination of the free glycerol content was then determined enzymatically according to the manufacturer's instructions.

Other Determinations

The following were also determined using the Test-Combinations of Boehringer Mannheim :

(a) Blood Glucose and Lactate.

(b) ATP, ADP and AMP of mouse liver after freeze-clamping and extraction of the metabolites with perchloric acid.

(h) Hepatic Contents of Metabolites Derived by Calculations

The cytosolic $(NAD^+)/(NADH)$ ratios were derived by calculation as was the oxaloacetate content, by methods described in the text.

(i) Stability of Various Metabolites in Freeze-Clamped Livers of Normal Mice

The data shown in Table I were obtained from an experiment in which livers of normal mice were freeze-clamped, powdered and combined to form a pool of frozen liver. Aliquots were immediately taken and the contents of pyruvate, oxaloacetate, citrate, α-oxoglutarate, acetyl CoA and CoASH determined. The remainder of the powdered liver being kept in a Dewar Flask containing Dry-Kold.

The analyses were then repeated at intervals up to 14 days.

As shown in Table I, there were no significant changes in the hepatic contents of these metabolites after 14 days of storage of the powdered liver under these conditions.

TABLE 1

THE EFFECT OF STORAGE IN DRY-KOLD ON THE HEPATIC CONTENTS OF METABOLITES IN FREEZE CLAMPED LIVER OF NORMAL MICE

Conditions	Hepatic Content Of Metabolites					
	Pyruvate μmol/g	Oxaloacetate μmol/g	Citrate μmol/g	α-Oxoglutarate μmol/g	Ac CoA nmol/g	CoASH nmol/g
Base Levels	0.198±0.014	0.027±0.007	0.161±0.005	0.155±0.010	66.83±2.39	63.02±2.62
After 14 Days Storage in Dry Ice	0.200±0.014	0.023±0.006	0.154±0.008	0.163±0.011	66.26±3.65	66.83±3.31
P	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

N.S. = Not Significant

Values are in terms of mean (±S.E.M.) of six determinations.

(j) Expression of Results

The hepatic contents of metabolites under study were expressed in terms of the appropriate concentration per g of liver wet weight. Where changes in total liver weight had occurred, then in some instances values were calculated in terms of whole liver content.

(k) Statistical Evaluation of Results

All results were expressed as the mean, plus or minus the standard error of the mean, or in a few instances the standard deviation.

Significance was calculated on the basis of Student's t (P) test.

SECTION II

DETERMINATION OF ENZYMIC ACTIVITIES

(a) Determination of Activity of Phosphofructokinase and Fructose,1,6-Diphosphatase in Mouse Liver

Procedure according to Dr E A Newsholme
(Personal communication)

Reagents

Enzymes used were supplied by Boehringer Mannheim.

(1) Extraction Mixture

This contained, triethanolamine (50 mmol/l); sucrose (250 mmol/l); E.D.T.A. (5 mmol/l); magnesium chloride (10 mmol/l); mercaptoethanol (30 mmol/l) at pH 7.5.

(2) Fructose Diphosphatase Assay Mixture

Tris buffer (61.2 mmol/l); E.D.T.A. (1.22 mmol/l); $MgCl_2$ 6 H_2O (2.44 mmol/l); $MnCl_2$ (1.22 mmol/l); mercaptoethanol (30.6 mmol/l). At pH 7.5.

(3) Nicotinamide adenine dinucleotide reduced form (10 mmol/l).

(4) ATP (30.5 mmol/l). Neutralised with potassium bicarbonate (1 mmol/l).

(5) AMP (60.9 mmol/l).

(6) Aldolase (100 ug/0.01 cm^3).

(7) Glycerol-3-phosphate dehydrogenase. Ten µg in 0.01 cm^3.

(8) Triose phosphate isomerase. Ten µg in 0.01 cm^3.

(9) Fructose-6-phosphate (60.9 mmol/l).

(10) PFK Assay Mixture

This contained tris buffer (63.4 mmol/l); KCl (253.7 mmol/l); mercapotethanol (12.7 mmol/l) at pH 7.5.

(11) NADP (15.3 mmol/l).

(12) Creatine phosphate (30.6 mmol/l).

(13) Glucose-6-phosphate dehydrogenase (10 μg/0.01 cm^3).

(14) Creatine kinase (100 μg/0.01 cm^3).

(15) Myokinase (Adenylate kinase); 10 μg/0.01 cm^3.

(16) Phosphoglucose isomerase (10 μg/0.01 cm^3).

(17) Fructose,1,6-diphosphate (3.1 mmol/l).

(18) Antimycin (4.6 mmol/l ethanol).

Procedure

Liver is removed, weighed and kept cool on ice. It is then chopped into small pieces, and homogenised in 10 cm^3 of the extraction mixture per g of liver. The homogenate is then centrifuged at 11,000 g for 20 minutes at 0°C, then 0.75 cm^3 of the supernatant is pipetted into a 10 cm^3 volumetric flask and 0.015 cm^3 of the antimycin added. The volume is then made to 10 cm^3 with the extraction mixture and 0.20 cm^3 of this used for the assays.

(1) Assay of Phosphofructokinase Activity

Conditions

Readings are made at 340 nm against air. Temperature, 25°C.

The following are pipetted into cuvettes:

PFK assay mixture	2.4 cm^3
NADH	0.1 cm^3
ATP	0.1 cm^3
AMP	0.1 cm^3
Extract	0.2 cm^3
Aldolase	0.015 cm^3
Glycerol-3-phosphate dehydrogenase	0.015 cm^3
Triose phosphate isomerase	0.015 cm^3

After mixing, the absorbancy at 340 nm is recorded for 10 minutes.

(2) Assay of Fructose Diphosphatase Activity

Conditions are as described above. The following are pipetted into cuvettes:

FDP'ase extraction mixture	2.5 cm^3
NADP	0.1 cm^3
Creatine phosphate	0.1 cm^3
Extract	0.2 cm^3
Glucose-6-phosphate dehydrogenase	0.015 cm^3
Creatine kinase	0.015 cm^3

Myokinase	0.015 cm^3
Phosphoglucose isomerase	0.015 cm^3

The absorbance is then read at 340 nm until it stabilises, fructose,1,6-diphosphate (0.1 cm^3) is mixed in and the readings continued for 10 minutes.

The activity is then calculated in terms of μmol/minute/g liver.

(b) Determination of Catalase Activity in Liver

The tissue is homogenised in the presence of a nonionic detergent (Triton X) according to the method of Cohen, Dembiec and Marcus (1970). The catalase activity of the supernatant is then determined by the method of Aebi (1975).

Reagents

(1) Stock Isotonic Buffer

Sodium chloride (18 g), Na_2HPO_4 (2.73 g) and NaH_2PO_4 $2H_2O$ (0.486 g) are dissolved in deionised water, the pH adjusted to 7.4, and the solution made to 200 cm^3.

(2) Working Buffer pH 7.4

The above is diluted 9 in 100 with deionised water.

(3) 10% v/v Triton X in the above buffer.

(4) Sodium Perborate, pH 7.0 (100 mmol/l)

7.694 g of the perborate are dissolved 20 cm^3 hydrochloric acid (1.5 v/v), and about 400 cm^3 of deionised water added. The pH is adjusted to 7.0 and the volume made to 500 cm^3.

(5) Phosphate Buffer, pH 7.0 (50 mmol/l)

This was prepared by mixing potassium dihydrogen phosphate (0.05 mmol/l) and disodium hydrogen phosphate (15.5 mmol/l) in the proportions 1:1.55.

(6) Potassium Permanganate (10 mmol/l).

Preparation of Tissue

Approximately 500 mg liver is taken and added to 5 cm^3 ice-cold isotonic buffer, homogenised to give a fine suspension (Ultra-Turrox) then centrifuged at 700 g for five to 10 minutes. The supernatant is removed, and 3 cm^3 of it placed in ice for 30 minutes. Next, 0.03 cm^3 of Triton X in the buffer at pH 7.4 is added to give a final concentration of one percent. After mixing well, 1 cm^3 of the solution is removed and made to 100 cm^3 with ice-cold isotonic buffer.

One cm^3 of the mixture is used for the assay.

A water bath, thermostatically controlled at 20°C and fitted with a tray containing 25 cm^3 flat-bottomed flasks is used.

The following are pipetted successively into the flasks:

	Blank	Test
Phosphate buffer (50 mmol/l, pH 7.)	1.0 cm^3	1.0 cm^3
Sodium perborate (100 mmol/l, pH 7.)	3.0 cm^3	3.0 cm^3

These are then pre-incubated at 20°C for 10 minutes, then the following are added:

Water	1.0 cm^3	-
Sample	-	1.0 cm^3

The shaker, and a stopwatch are immediately started, and then the reaction stopped immediately after 30 seconds by the rapid addition of 3.0 cm^3 sulphuric acid (1 mol/l).

The remaining perborate is then determined by back-titration with potassium permanganate (10 mmol/l).

Calculation of Activity

The concentration of perborate at the beginning of the reaction is 37.5 mmol/l.

The activity is then expressed in terms of the number of milliequivalents of sodium perborate decomposed per mg liver (wet weight).

(c) Determination of Activity of Citrate Cleavage Enzyme

Procedure according to Kornacker and Lowenstein (1965a)

Reagents

(1) Reaction Mixture

This contained ATP (disodium salt) 5 mmol/l; $MgCl_2$ (5 mmol/l); coenzyme A about (0.56 mmol/l); reduced glutathione (10 mmol/l); citrate (trisodium salt) 5 mmol/l. Hydroxylamine HCl (500 mmol/l) neutralised with sodium hydroxide. The solution is prepared prior to use.

(2) Ferric Chloride Reagent

Ferric chloride (2 g) and trichloroacetic acid (6.7 g) are dissolved in HCl (0.8 mol/l) and made to 100 cm^3.

Procedure

Liver (approx. 1 g) is removed rapidly and homogenised in ice-cold sucrose (3 cm^3 of 0.25 mol/l), then centrifuged at about 4,500 g at 0^oC for 10 minutes. The supernatant is then removed and recentrifuged for 30 minutes at 0^oC at 59,000 g.

An aliquot of the supernatant is then removed and the protein content determined by the method of Lowry, Rosebrough, Farr and Randall (1951). During the estimation, the remainder of the supernatant must be kept at 0^oC.

The supernatant is then diluted to give a protein concentration in the range 0.2 to 6 mg per assay. Then 0.1 cm^3 of this is taken and made to 1.0 cm^3 with the reaction mixture, and incubated for 40 minutes at 37°C.

At the end of this time, the reaction is stopped by the addition of 3.0 cm^3 of the ferric chloride reagent, and the mixture centrifuged for about five minutes at 4,000 g.

The supernatant is then removed with a Pasteur pipette, and the absorbance determined at 510 nm against a blank, consisting of 0.1 cm^3 of the diluted supernatant, plus 0.9 cm^3 of the reaction mixture following immediately by 3 cm^3 of the ferric chloride reagent.

Values for the amount of citrate cleaved in the reaction are extrapolated from a calibration curve prepared by taking up to 500 ug acetohydroxamic acid in a volume of 1 cm^3 and adding 3 cm^3 of the ferric chloride reagent. The activity is then calculated in terms of µmol/mg protein/hour.

Other Techniques Used

(a) Electron Microscopy

Pieces of liver were minced in glutaraldehyde in phosphate buffer, then post-osmicated, dehydrated and embedded in Araldite. They were then sectioned on and LKB ultratone, stained with uranyl acetate and lead citrate, and examined in a Philips 301 G electron microscope.

CHAPTER 2

METABOLIC CHANGES IN LIVER OF TUMOUR-BEARING MICE

(1) The Hepatic Contents of Acetyl CoA, Free CoA and Citrate

Although changes in the hepatic contents of acetyl CoA and citrate have been extensively investigated in rats following gluconeogenic stimuli such as starvation and the induction of diabetes (Start and Newsholme, 1968; Herrera and Freinkel, 1968) as well as following treatment with certain hormones (Hornbrook, Burch and Lowry, 1965; Greenbaum, Gumaa and McLean, 1971; Exton and Harper, 1972), little attention has been paid to the content of these regulatory intermediates in liver of tumour-bearing animals.

In an early study, (Shils, Friedland, Fine and Shapiro, 1956), the hepatic content of CoA and various vitamins in liver of tumour-bearing mice were determined, but values for the levels of these metabolites in normal livers were not included.

Later, other workers (Mascitelli, Coriandoli, Boldrini and Citterio 1958) in a study of the effect of isoniazid on the CoA and pyridoxine content of tissues of tumour-bearing rats, reported that an increase in CoA and decrease in pyridoxine occurred following treatment with the drug. However normal rats were not used as

controls in these investigations, and the authors used a non-specific method of assay (Kaplan and Lipmann, 1948) for CoA, and in fact little can be made of the findings of these workers.

More recently, Rapp (1973) studied the CoA content of liver, adrenals, spleen and kidney of rats and mice bearing a variety of different tumours, as well as in liver of patients with carcinoma of the colon. In all of the tissues studied, marked decreases in the CoA content were found.

Rapp (1973) however used a non-specific chemical method of assay (Handschumacher, Mueller and Strong, 1951) based on the procedure of Kaplan and Lipmann (1948), that also measures acetyl CoA, acyl thiol esters of CoA and various precursors in the biosynthesis of CoA (Novelli, Schmetz and Kaplan, 1954; Cavallini, Mondovi, De Marco and Ferro-Luzzi, 1959).

Consequently it is not possible to interpret his data in terms of the actual form of the coenzyme present.

Rapp (1973) however, although offering no interpretation of his findings, suggested that the decrease in the content of CoA in tissues of the tumour-bearing host, might be of significance in the deleterious effects of a tumour on its host.

There is also a lack of information on the citrate content of tissues of tumour-bearing animals. In an early study, it was reported (Haven, Randall and Bloor, 1949) that in rats bearing the Walker 256 carcinoma, the citrate content of various organs was in general, higher than those found in a control group of animals

in which the tumour had not taken.

In the present investigation the acetyl CoA, free CoA and citrate content of liver of tumour-bearing mice has been studied using specific enzymatic methods of assay on freeze-clamped liver of these animals.

Part of this study has already been presented elsewhere (Calman and McAllister, 1975a, 1975b; McAllister, Soukop and Calman, 1976).

Materials and Methods

Mice bearing either Sarcoma 180, TLX-5 lymphoma or a transplantable C_3H mammary tumour were used. Details as to tumour transplant, and control groups used have already been described in detail. The stages of tumour growth at which the studies were made are detailed in the present study.

The coenzyme A and citrate content of freeze-clamped liver were determined as described in detail in Chapter 1 of this thesis.

Body weights, total liver weight, spleen and thymus weights were also recorded. Where applicable, tumours were dissected free from connective tissue and the weight determined.

Expression of Results

The hepatic contents of the metabolites under study were expressed in terms of concentration per g of liver wet weight. Where alterations in liver weight had occurred, the validity of the results were

checked also by calculating in terms of whole liver content. As would be expected, in some instances this resulted in alterations in the degree of statistical significance.

Results

The data presented in Figure 8 shows the daily changes in the hepatic contents of acetyl CoA, CoASH and 'Total' CoA in livers of mice after inoculation of 2×10^6 TLX-5 lymphoma cells i.p. Twenty-four hours later, there were significant reductions in the hepatic content of both metabolites. During the six days of tumour growth, the CoASH content remained significantly lower than that of the controls. Two days after tumour implant the acetyl CoA content returned to normal levels, and remained so until the fifth and sixth days when significant depressions again occurred. In these experiments, and with this size of inoculum, the mice became cachectic after about six days of tumour growth. Total CoA, that is the sum of acetyl CoA, oxidised CoA and reduced CoA, remained at significantly low levels over the period of the experiment.

In these studies, control mice received 2×10^6 spleen cells from animals of the same inbred strain. In order to obviate a possible allogeneic effect of the tumour cells, normal male CBA strain mice were

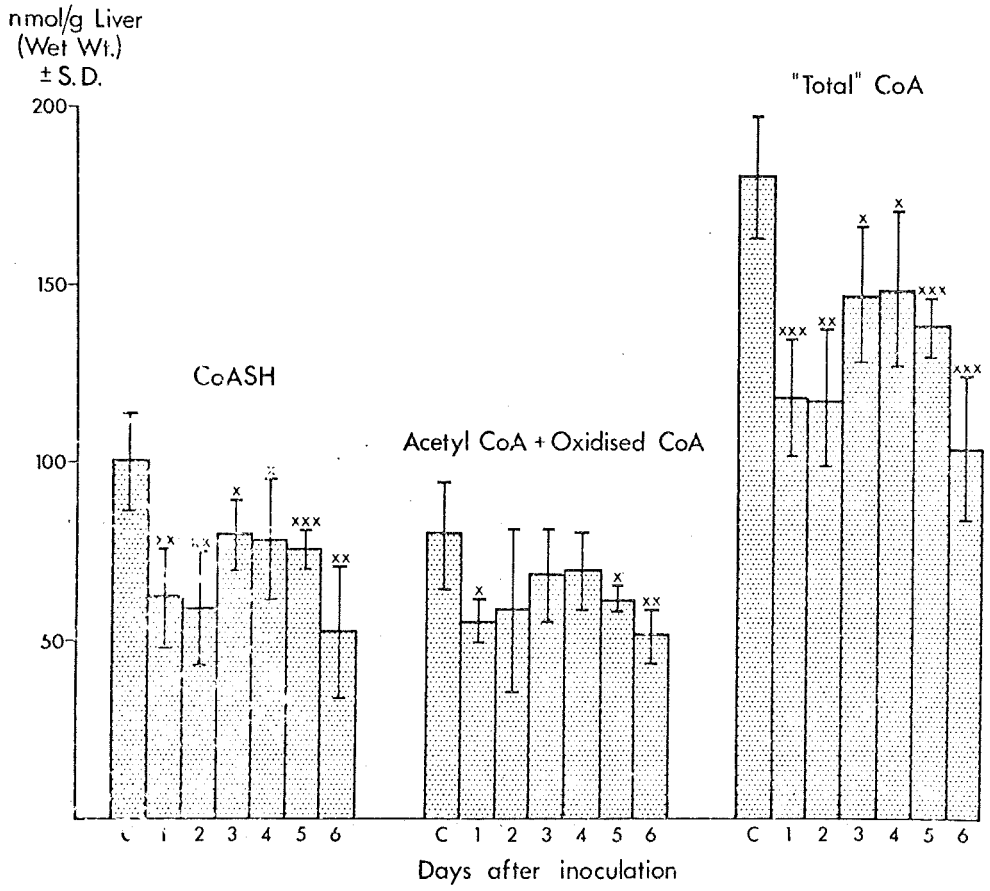


FIGURE 3 Changes in the hepatic content of CoASH, Acetyl CoA and 'Total' CoA in mice following the i.p. injection of 2×10^6 TLX-5 lymphoma cells. Control animals received 2×10^6 spleen cells from CBA mice in Hank's Balanced Salt Medium. Results are means of six animals in each group.

* = $P < 0.05$ ** = $P < 0.01$ *** = $P < 0.001$

challenged by i.p. injection of 2×10^6 spleen cells from male, A strain mice. Determination of the CoA content of livers in these animals at periods of up to eight days after injection, showed no significant differences from normal.

Data derived from a separate experiment (Figure 9) using mice that had received 2×10^6 TLX-5 lymphoma cells i.p., showed that significant increases in the citrate content of liver occurred on the first and second day following the injection. On the third and fourth day, this had reached normal values, but on the seventh day of tumour growth, marked increases ($P < 0.001$) occurred.

Because of the large number of tumour cells injected into these animals, less acute studies were made on mice that had received 2×10^3 cells by the same route. Three days later, the acetyl CoA content of liver decreased significantly (Table 2). In contrast to that found with the larger inoculum, this remained at a low level over the entire period of the experiment. The CoASH content of these livers did not alter significantly from the control values, until the tenth day when the animals were markedly cachectic and the experiment was terminated. 'Total' CoA was significantly decreased on the second, third and sixth day of tumour growth. On the eighth day this had returned to normal values, but on day 10, significant depressions again occurred. It should be noted however that the significance of changes in the 'Total' CoA content, which is derived from the sum of acetyl CoA and the reduced form of the coenzyme are entirely

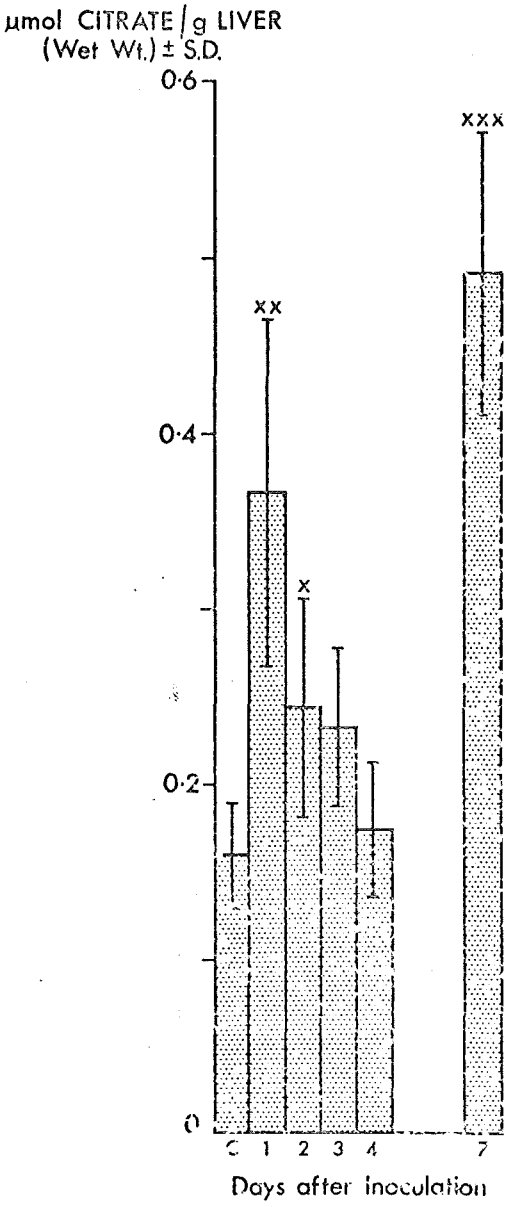


FIGURE 9 Changes in the hepatic content of citrate in mice following the i.p. injection of 2×10^6 TLX-5 lymphoma cells. Results are means $\pm$ S.D. of six animals in each group.

TABLE 2

THE ACETYL CoA, CoASH, 'TOTAL' CoA AND CITRATE CONTENT OF LIVERS OF MICE FOLLOWING I.P. INJECTION OF 2×10^3 TLX-5 LYMPHOMA CELLS

Days After Implant	Acetyl CoA nanomol/g	P	CoASH nanomol/g	P	'Total' CoA nanomol/g	P	Citrate µmol/g	P
0 (Controls)	77.05±12.55	-	84.30±14.26	-	161.35±22.09	-	0.16±0.04	-
1	71.05±9.83	N.S.	97.93±8.01	N.S.	168.99±14.15	N.S.	0.45±0.10	< 0.001
2	69.79±10.97	N.S.	60.35±10.86	N.S.	130.14± 8.09	< 0.02	0.45±0.07	< 0.001
3	53.55± 9.80	< 0.01	74.13± 2.86	N.S.	127.68± 7.68	< 0.01	0.33±0.04	< 0.001
6	52.82± 7.79	< 0.01	73.42±14.58	N.S.	126.25±14.50	< 0.02	0.27±0.08	< 0.05
8	57.15±12.88	< 0.05	78.26±12.25	N.S.	135.41±15.44	N.S.	0.25±0.09	N.S.
10	60.44±4.00	< 0.02	58.75±14.85	< 0.05	119.16±14.17	< 0.01	0.25±0.13	N.S.

Values given are means ±S.D. Six animals were studied in each group.

P = Statistical evaluation by unpaired 't' test.

N.S. = Not Significant.

dependent therefore on the degree of change in the content of the two.

The citrate content of these livers also changed in a direction opposite to those found in normal animals under gluconeogenic conditions, with marked increases occurring on the first three days following tumour implant. On the sixth day, when the next determinations were made this was still significantly increased when compared to the controls (Table 2). Thereafter normal values were found on the eighth and tenth days of tumour growth.

In animals that received 2×10^3 TLX-5 lymphoma cells, symptoms of cachexia usually appeared about the seventh to eighth day of tumour growth, although there were some variations as would be expected between different groups. The fact that early changes in CoA and citrate occurred in liver after the injection of the tumour cells, and before visible signs of cachexia became evident, suggested that cachexia was not a prerequisite for these metabolic abnormalities.

Changes in Spleen, Thymus and Body Weights

The influence of this tumour on the spleen, thymus, liver and body weights in mice is shown in Table 3. Six days after the injection of 2×10^6 TLX-5 lymphoma cells i.p., body and thymus weights had decreased significantly. Spleen weights increased significantly compared to control animals, but the mean liver weight did not change.

Studies of the daily changes in thymus and spleen

TABLE 3

CHANGES IN BODY WEIGHT, SPLEEN, THYMUS AND LIVER WEIGHTS IN TUMOUR-BEARING MICE

	Controls	Tests (TLX-5) Starting	Final	% Change	P	Controls	Tests (Sarcoma 180) Starting	Final*	% Change	P
Body Weight (g)	30.46±1.17	33.76±2.38	31.81±2.63	-5.8	<0.01	26.35±2.48	25.23±1.76	20.09±1.69	-20.4	<0.01
Liver Weight (g)	1.51±0.12	-	1.62±0.16	+6.7	N.S.	1.51±0.11	-	1.23±0.08	-18.5	<0.01
Spleen Weight (mg)	76.72±11.67	-	178.7±25.51	+132	<0.001	92.55±14.83	-	156.20±25.98	+68.8	<0.001
Thymus Weight (mg)	25.52±6.88	-	11.53±3.80	-54	<0.01	23.98±7.28	-	6.90±4.50	-71.2	<0.01

Values are in terms of mean ±S.D. of the six animals in each group.

P = Difference from Controls by Student's 't' test for statistical comparison of organ weights. Body weight changes according to paired 't' test.

N.S. = Not Significant.

* Mean Body Weight at sacrifice minus tumour weight.

Mice bearing TLX-5 lymphoma were sacrificed after six days of tumour growth.

Sarcoma 180 mice were sacrificed 15 days after implant.

weights in mice from the previous experiment that had received 2 x 10^3 TLX-5 lymphoma cells i.p., showed that the mean thymus weights decreased significantly 24 hours later, and this continued over the 10 days of tumour growth (Figure 10). Also shown is the essentially similar pattern that occurred in thymus weight changes in mice that had received 2 x 10^5 tumour cells i.p. In contrast, the animals that had received this number of cells showed increases in mean spleen weights after three days of tumour growth, compared to six days in those that received 2 x 10^3 tumour cells (Figure 11). Mice bearing Sarcoma 180 showed a 20 percent reduction in body weight with significant decreases in thymus weight. Liver and spleen weights in these animals increased significantly (Table 3).

Food and Water Intake in Tumour-Bearing Mice

Although the pattern of change in the hepatic contents of acetyl CoA and citrate in these animals were not indicative of an increased supply of fatty acids to liver as a result of starvation, the mean food and water intake in mice bearing TLX-5 lymphoma was studied. Also included were a group of mice bearing Sarcoma 180, a tumour that had been found as described later also to induce similar changes in the CoA and citrate content of host liver.

One day after the i.p. injection of 2 x 10^6 TLX-5 lymphoma cells, the amount of food and water consumed by the animals fell to 48 and 15 percent respectively of normal, (Table 4) with further decreases on the

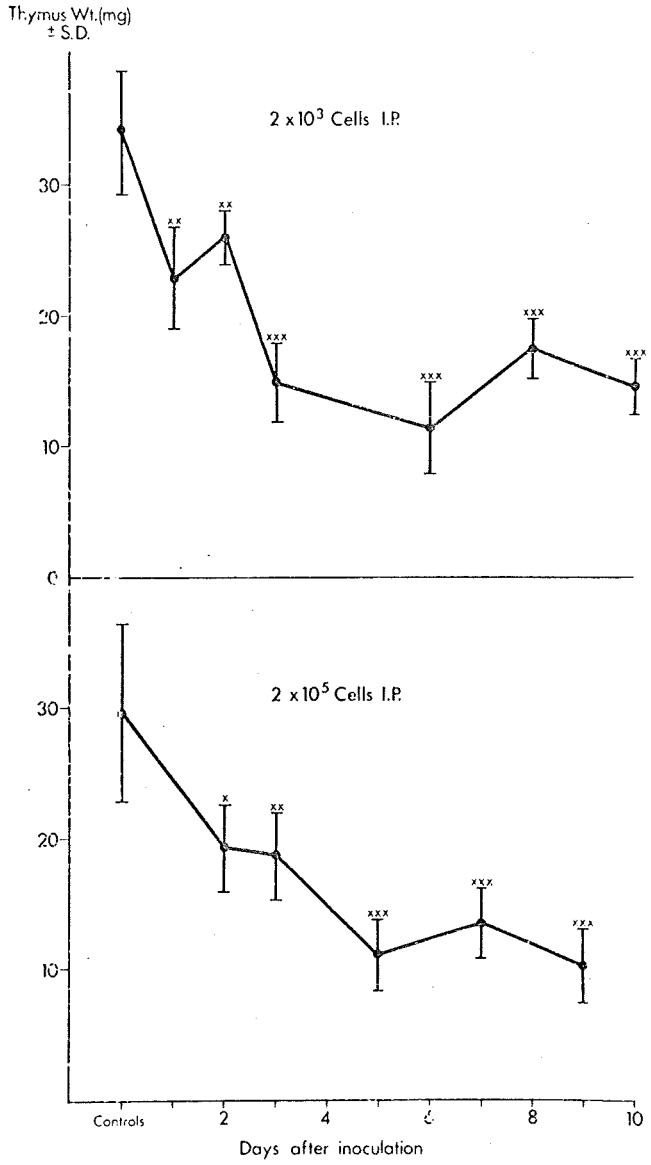


FIGURE 10 Changes in the mean thymus weights of mice following the i.p. injection of 2×10^3 or 2×10^5 TLX-5 lymphoma cells. Six animals were studied in each group.

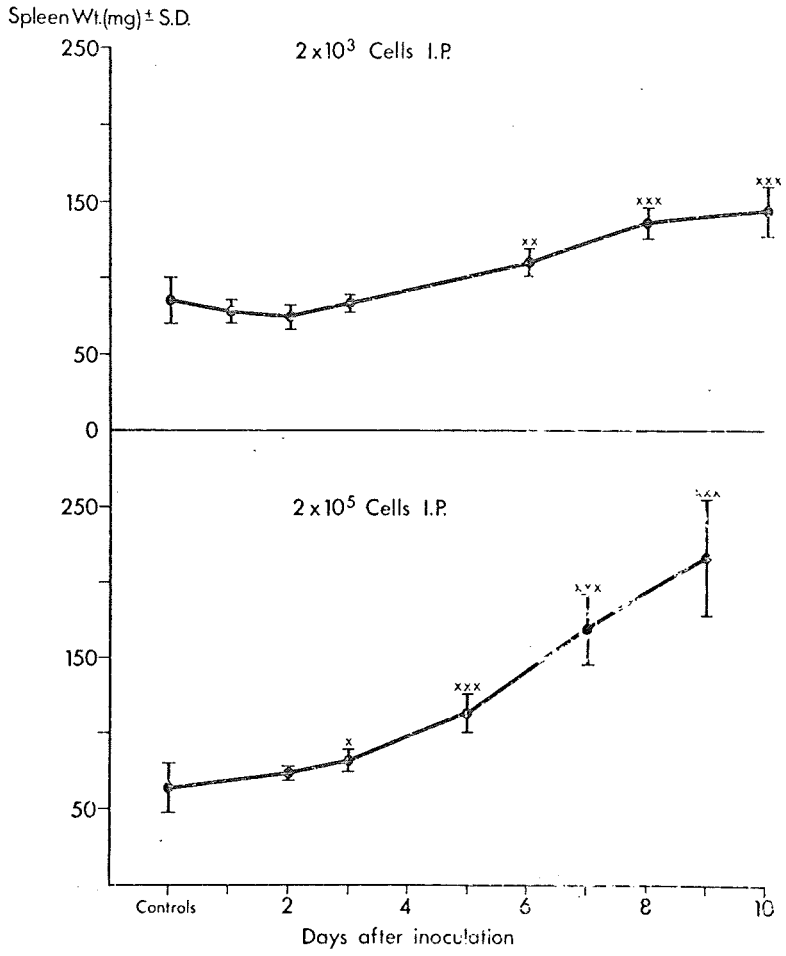


FIGURE 11 Increases in the spleen weights of mice following the i.p. injection of 2×10^3 or 2×10^5 TLX-5 lymphoma cells. Results are means of six animals studied in each group.

TABLE 4

FOOD AND WATER CONSUMPTION OF MICE BEARING TLX-5 LYMPHOMA OR SARCOMA 180

Days after inoculation	TLX-5 Lymphoma		Sarcoma 180	
	Food Intake % Difference	Water Intake % Difference	Food Intake % Difference	Water Intake % Difference
1	-48	-15	+13	+40
2	-68	-82	-38	-38
3	-33	-15	-66	-55
4	-36	- 6	-48	-34
5	-70	-33	- 8	+ 4
6	-70	-68	- 3	+ 9
7	-87	-91	+ 1	+ 8
8			+15	+18
9			+ 8	0
10			+10	+16
11			0	+ 8
12			+ 3	+17
13			- 1	+ 4
14			- 5	+12
15			+13	- 6

Results expressed as percent difference compared to normal. Six animals were studied in each group.

second day. Thereafter there was some improvement on the following two days, but the overall picture was one of anorexia over the seven days of tumour growth.

Mice bearing Sarcoma 180 showed no change in the amount of food and water consumed on the day following implant. On the two to four day period food consumption dropped by 38, 66 and 48 percent of normal. Very similar decreases in water intake occurred at this time. Thereafter both the food and water consumption increased to about normal values, and this continued over the 15 days of tumour growth. On termination of the experiment on day 15, the mean tumour weight of these animals was 3.44 g $\pm$ S.D. 1.02. In spite of the burden of their tumour these animals remained reasonably fit and active, and did not present symptoms of cachexia.

Results with Mice Bearing a C_3H Mammary Tumour or Sarcoma 180

In order to establish that changes in the hepatic contents of CoA and citrate were due to a systemic of the tumour on its host, and not a result of tumour dissemination, the studies were extended using mice bearing either a transplantable C_3H mammary tumour that was known not to disseminate or Sarcoma 180. In the latter group histological examination of liver showed no evidence of tumour involvement.

Highly significant reductions ($P < 0.001$) in the hepatic contents of both acetyl CoA and CoASH were

found in mice bearing the mammary tumour (Figure 12). The citrate content of these livers showed significant increases (Figure 13). In these animals, the mean tumour weight was 16.12 mg $\pm$ S.D. 3.42 at which stage they were only just palpable *in situ*. Spleen weights had increased significantly ($P < 0.001$) and thymus weights had decreased ($P < 0.001$) but no change in liver weight was found. Body weights in these animals were not recorded.

The increased citrate content of livers of animals bearing the small mammary tumour contrasted markedly with those bearing Sarcoma 180 (mean tumour weight 0.5 g $\pm$ S.D. 0.32) in which the content of this metabolite in liver remained at normal levels (Figure 13). Although the 'Total' CoA and acetyl CoA had decreased significantly, the CoASH content of these livers remained at normal levels (Figure 14).

The possibility that there might be some correlation between the weight of the tumour and alterations in the hepatic content of citrate was examined in another experiment, with mice in which the mean tumour weight was 4.42 g $\pm$ S.D. 1.23. Table 5 shows that in these animals, the hepatic citrate content was increased by 102 percent over that of the controls. Marked reductions ($P < 0.001$) were also found in the 'Total' CoA, acetyl CoA and the reduced coenzyme. Further studies however are necessary to establish whether indeed such a correlation exists.

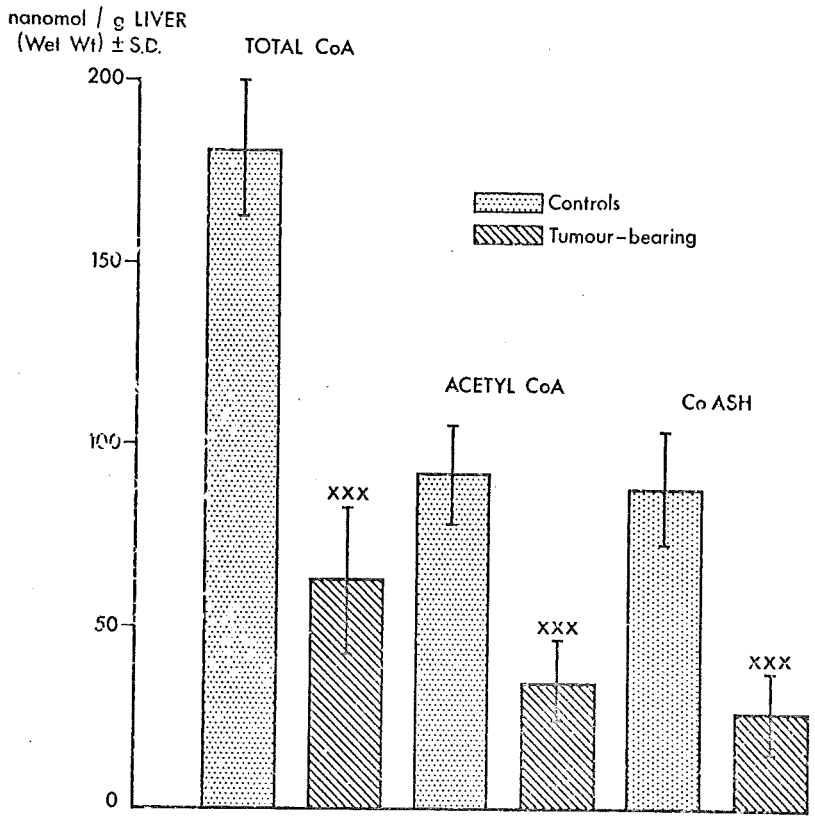


FIGURE 12 The 'Total' CoA, Acetyl CoA and CoASH content of livers of mice bearing a C_3H mammary tumour. Results are the means of six animals.

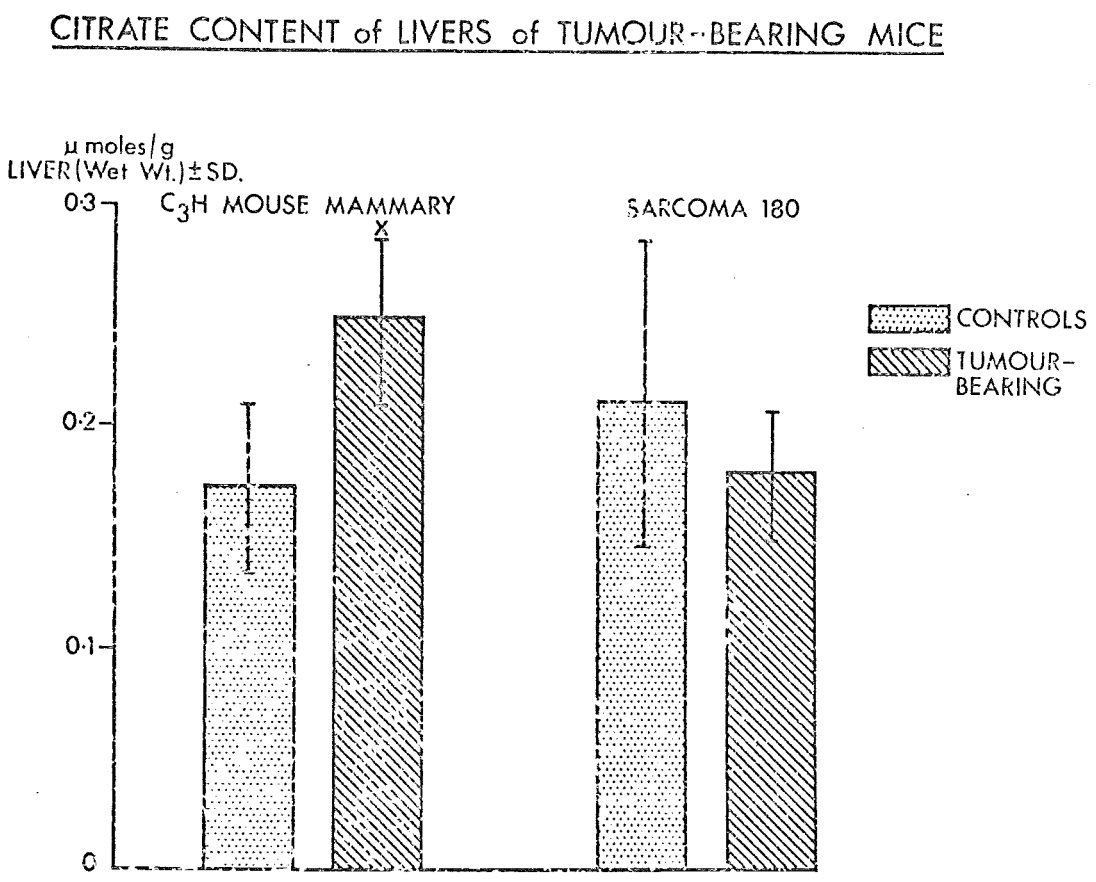


FIGURE 13 The hepatic citrate content of mice bearing a C_3H mammary tumour or Sarcoma 180. Results are the means of six animals studied in each group.

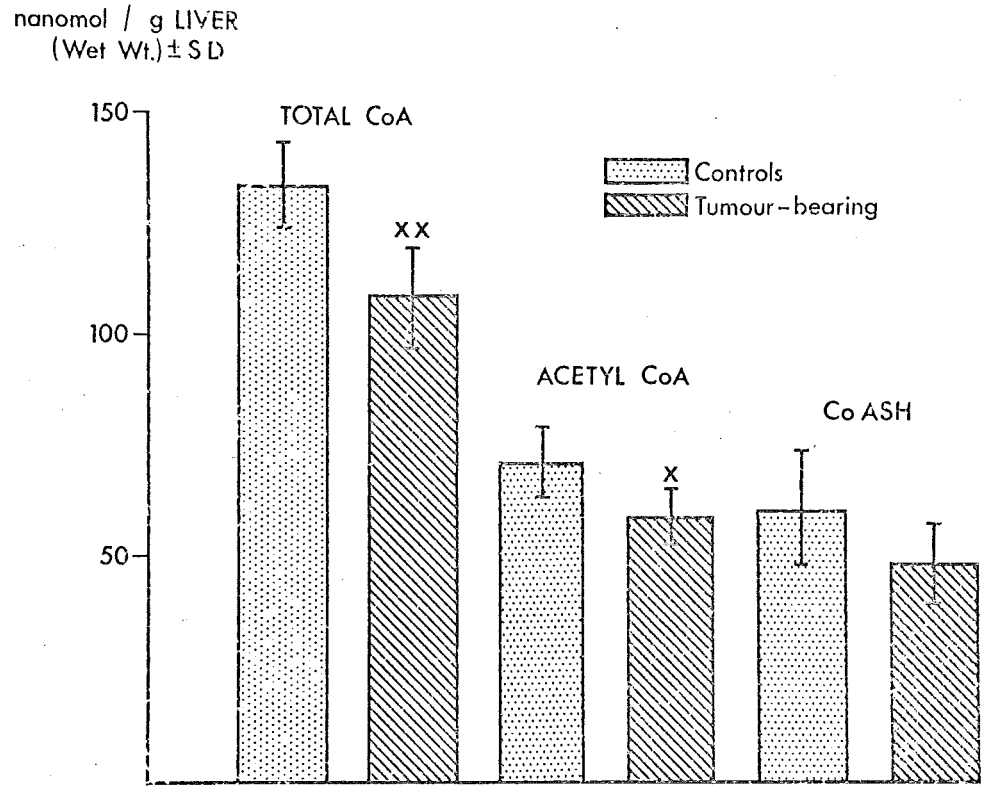


FIGURE 14 The 'Total' CoA, Acetyl CoA and CoASH content of livers of mice bearing Sarcoma 180. Mean tumour weight = 0.5 g $\pm$ S.D. 0.82. Results are means of six control animals and six tumour-bearers.

TABLE 5

EFFECT OF SARCOMA 180 ON THE CONTENTS OF ACETYL CoA, CoASH, 'TOTAL' CoA AND CITRATE IN MOUSE LIVER

	Acetyl CoA -	CoASH (nanomol/g Wet Weight)	'Total' CoA -	Citrate µmol/g Wet Wt.
Controls	69.39±15.07	68.29±10.0	137.59±22.70	0.57±0.10
Tumour-Bearers	35.14± 7.0	20.93± 8.16	56.08±14.54	1.15±0.45
% Change	-49	-69	-59	+102
P	< 0.001	< 0.001	< 0.001	< 0.02

Results expressed as means ±S.D. Six animals were studied in each group.

P = Statistical evaluation by unpaired 't' test.

Discussion

These studies have shown that tumour-growth in experimental animals effects decreases in the acetyl CoA, and CoASH content of liver, with concomitant increases in the citrate content. The direction of change in acetyl CoA and citrate are opposite to those found in normal animals under conditions of forced-gluconeogenesis as has been reported by several workers in the starved and acute alloxan diabetic rat (Wieland, 1966; Start and Newsholme, 1968). The data available at the present time precludes an interpretation of the mechanisms underlying the alterations in the level of these metabolites in liver of the tumour-bearing host. Investigations of these changes over several days of tumour growth in livers of mice bearing TLX-5 lymphoma showed that they occurred 24 hours after inoculation of the animals with 2×10^6 tumour cells. This suggested a possible allogeneic effect of the tumour cells, which however was not supported by our finding normal values in the hepatic contents of these metabolites in normal CBA strain mice that had been challenged with spleen cells from an A strain animal. Mice bearing the lymphoma also showed significant decreases in the mean thymus weight 24 hours after tumour implant.

The decreases in the acetyl CoA, and the increased citrate content of liver in these animals, could not be correlated with their physical condition since they occurred in mice that were anorexic, and showed symptoms of cachexia, as seen in those bearing TLX-5

lymphoma, or in those in which the tumour was small and remained localised as found in those bearing the small mammary tumour, which at that stage of growth had no apparent effect on the host. Although similar findings were made in mice bearing Sarcoma 180, there was some evidence to show that the size of the tumour might be a factor in the degree of change in the CoASH and citrate content of liver.

When the number of TLX-5 lymphoma cells injected had been reduced by one thousandth, the onset of changes in the hepatic contents of acetyl CoA and CoASH, but not citrate were delayed. At present we do not have a satisfactory explanation for the early changes in the level of these metabolites, a factor or factors released from the tumour cells cannot be excluded from the aetiology.

However, in addition to the latter suggestion, there are several other possible explanations that might be involved in these changes, and although these are mainly speculative at this stage, they offer possible approaches to the aetiology of these changes.

For instance increases in the hepatic content of citrate have been reported in rats, in which rapid glucose synthesis had been induced by the intraperitoneal injection of lactate (Hornbrook, Burch and Lowry, 1965). Although this increase in the hepatic citrate content was obtained using a dose of 1.5 g/Kg body weight, the possibility exists that an increase in the lactate content of blood of tumour-bearing mice may be involved in the accumulation of citrate in liver of

these animals, and this has been studied as reported in Chapter 3 of this thesis.

An increase in the hepatic content of citrate has also been shown to occur in normal rats following the intraperitoneal injection of L-tryptophan (Ray, Foster and Lardy, 1966). The mechanism involves inhibition of gluconeogenesis, because under these conditions, phosphoenolpyruvate carboxykinase is non-functional.

The accumulation of citrate in livers of tumour-bearing mice would be expected to inhibit phosphofructokinase since this metabolite is one of several modifiers of the enzyme (Start and Newsholme, 1970; Kemp, 1971). This would reduce the glycolytic flux, and favour gluconeogenesis, since the enzyme is rate-limiting in glycolysis (Scrutton and Utter, 1968). It is of interest however, that this mechanism does not regulate gluconeogenesis in liver of normal animals, since under gluconeogenic conditions e.g. starvation, the hepatic content of citrate decreases (Start and Newsholme, 1968; Herrera and Freinkel, 1968). It does however regulate gluconeogenesis in kidney cortex of normal animals, because under conditions of starvation, the citrate content of this tissue increases (Underwood and Newsholme, 1967), with concomitant inhibition of phosphofructokinase.

Since citrate is a substrate for fatty acid synthesis, following its cleavage to acetyl CoA and oxaloacetate in the cytosol by ATP-citrate lyase (Spencer and Lowenstein, 1962; Lowenstein, 1968),

the accumulation of citrate in liver of these tumour-bearing mice might reflect some abnormality of lipid metabolism in these animals, which as already noted is often perturbed in animals bearing tumours (Haven and Bloor, 1956; Costa and Holland, 1962; Cox and Gokcen, 1975). At this stage of the present study, we considered that increases in the hepatic content of citrate might therefore involve a decreased activity of the citrate-cleavage enzyme in liver of these tumour-bearing mice, and this possibility is examined later in this thesis (Chapter 4). As discussed therein, it is of interest that increases in the hepatic content of citrate are also found in rats under conditions of an increased rate of fatty acid synthesis, e.g. in starved animals refed glucose (Newsholme and Start, 1972).

Why the free coenzyme A content of liver of tumour-bearing mice should also be decreased is not clear at present. According to Brown (1959), the initial stage in the biosynthesis of coenzyme A, involves the formation of 4-phosphopantotheine, from pantothenate and L-cysteine, and as would be expected, decreases in the hepatic content of the coenzyme are found in pantothenate-deficient animals (Olson and Kaplan, 1948). However, other nutritional factors may also be involved, since decreases in the hepatic contents of CoA have also been reported in rats fed protein-deficient diets (Abiko, 1975), and later in the present thesis, it is shown that the hepatic contents of the coenzyme are decreased significantly, in starved, tumour-free mice (Chapter 4). The metabolic

implications of changes in the ratio acetyl CoA/CoASH in liver of fed and fasted tumour-bearing mice are also discussed later.

At the present stage of the investigation it was however of interest to determine whether the administration of pantothenate to tumour-bearing mice would increase the hepatic contents of the free coenzyme, and these studies will now be reported.

SOME OBSERVATIONS ON THE EFFECT OF THE ADMINISTRATION OF PANTOTHENATE TO TUMOUR-BEARING MICE

Procedure

Weight matched CBA mice, 4.5 to 5 months old were taken and placed in cages in groups of six. A measured volume of water (usually 50 cm^3) in a standard type water bottle was placed in position, and food provided ad lib. At the same time each day, the mean amount of water taken by each group was determined. This was continued for a period of five days.

The animals were then separated into three groups. The first group (Group 1) received Sarcoma 180 subcutaneously in the subscapular position under ether anaesthesia as described previously. The second group (Group 2) also received the tumour, but at the time of tumour implant, 0.5 cm^3 of saline containing 2 mg sodium pantothenate was given intraperitoneally. The third group (Group 3) were injected subcutaneously

in the subscapular position with 0.5 cm^3 Hank's Balanced Salt Medium, and also injected intraperitoneally with the same amount of saline containing pantothenate.

The animals were then housed in groups of six to a cage. All of those that had received pantothenate intraperitoneally (tumour-free and tumour-implanted groups) were allowed access to adequate amounts of water containing 0.02 mg calcium pantothenate per cm^3, which was prepared afresh daily. The other group of tumour-bearing mice received water without the vitamin.

The mean fluid intake of the three experimental groups were determined daily. Six mice were selected from each group on the 2nd, 4th, 8th, 10th and 15th day following tumour implant, and the body weight determined. The animals were then killed by cervical dislocation, and the hepatic contents of acetyl CoA and CoASH estimated. Spleen weight was also measured, as was the weight of the tumour, once this had become measurable.

The fluid intake of the remaining mice in each of the three experimental groups was measured daily for up to 15 days, when the experiment was terminated.

RESULTS

As shown in Table 6 and, as noted previously in this Chapter the mean fluid intake of mice fell on the second and third day following tumour implant, apparently moreso in mice that had also received pantothenate at the same time.

TABLE 6

APPROXIMATE FLUID AND PANTOTHENATE INTAKE OF NORMAL MICE AND MICE BEARING SARCOMA 180

Days Before Tumour Implant	Group (1)	Group (2)	Group (3)	Group (1)	Group (2)	Group (3)
	Mean Water Intake			Mean Pantothenate Intake (mg)		
1	3.9	3.9	4.0	-	-	-
2	3.45	3.7	4.0	-	-	-
3	3.95	3.8	3.6	-	-	-
4	3.6	3.7	4.2	-	-	-
5	3.6	3.8	3.8	-	-	-
Days After Implant						
1.	5.1	5.2	3.7	-	0.104	0.074
2	2.3	1.6	3.0	-	0.052	0.060
3	2.6	1.4	2.8	-	0.028	0.056
4	2.4	1.6	3.2	-	0.032	0.064
5	3.8	3.4	3.4	-	0.068	0.068
6	4.0	3.5	3.8	-	0.070	0.076
7	3.9	3.6	3.8	-	0.072	0.076
8	5.1	3.8	3.5	-	0.076	0.070
9	3.7	4.2	3.4	-	0.084	0.068
10	4.3	4.3	3.7	-	0.086	0.074
11	4.2	4.0	4.2	-	0.080	0.084
12	4.7	4.6	3.7	-	0.092	0.074
13	3.8	4.3	3.3	-	0.086	0.066
14	5.0	4.9	4.6	-	0.098	0.092
15	3.4	4.2	3.6	-	0.084	0.072
Total	-	-	-	-	1.09	1.07
Mean	-	-	-	-	0.072	0.071

Thereafter all groups ingested about the same amount of water, showing that those that had received the vitamin tolerated this treatment.

The amount of pantothenate ingested per mouse averaged 0.07 mg per diem, and when the total amount taken by mouth over the 15 days of the study was tabulated, this was the same in both the tumour-free group, and those bearing the tumour. It must be stressed however that for obvious reasons these amounts quoted must be considered to be very approximate. It might be argued that repeated injections of the vitamin might have been a better experimental approach, but it was considered that this treatment, particularly in mice bearing a growing tumour, might influence the results as a consequence of trauma.

(1) Changes in Body Weight

The most striking feature was a marked decrease in body weight of tumour-bearing mice that had received pantothenate, when compared to tumour-bearing controls (Figure 15), whereas in normal mice that had received the vitamin, this treatment had no effect on body weight over the period of the experiment. That the marked decreases in body weight in the pantotheante treated animals was not due to an increased rate of tumour-growth, is clearly shown in Figure 16, where it will be seen that the growth rate of the tumour in these animals was similar to that found in the untreated tumour-bearing group.

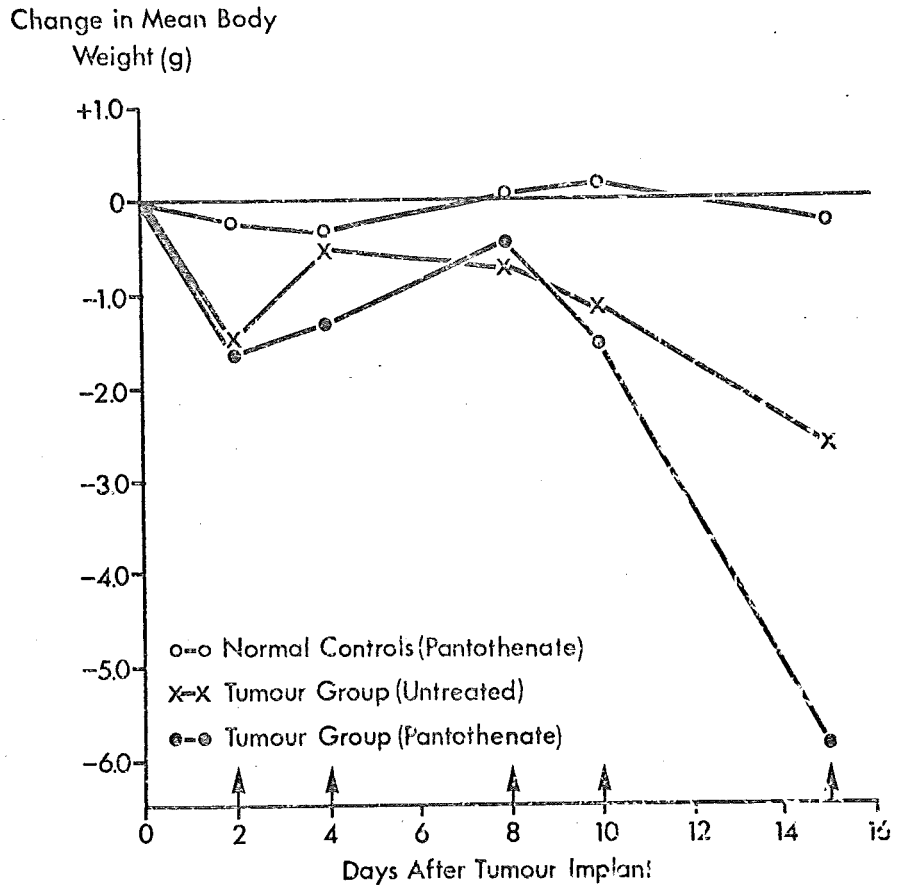


FIGURE 15 Changes in body weight induced by the administration of pantothenate to mice bearing Sarcoma 180. Six animals were used for each time interval.

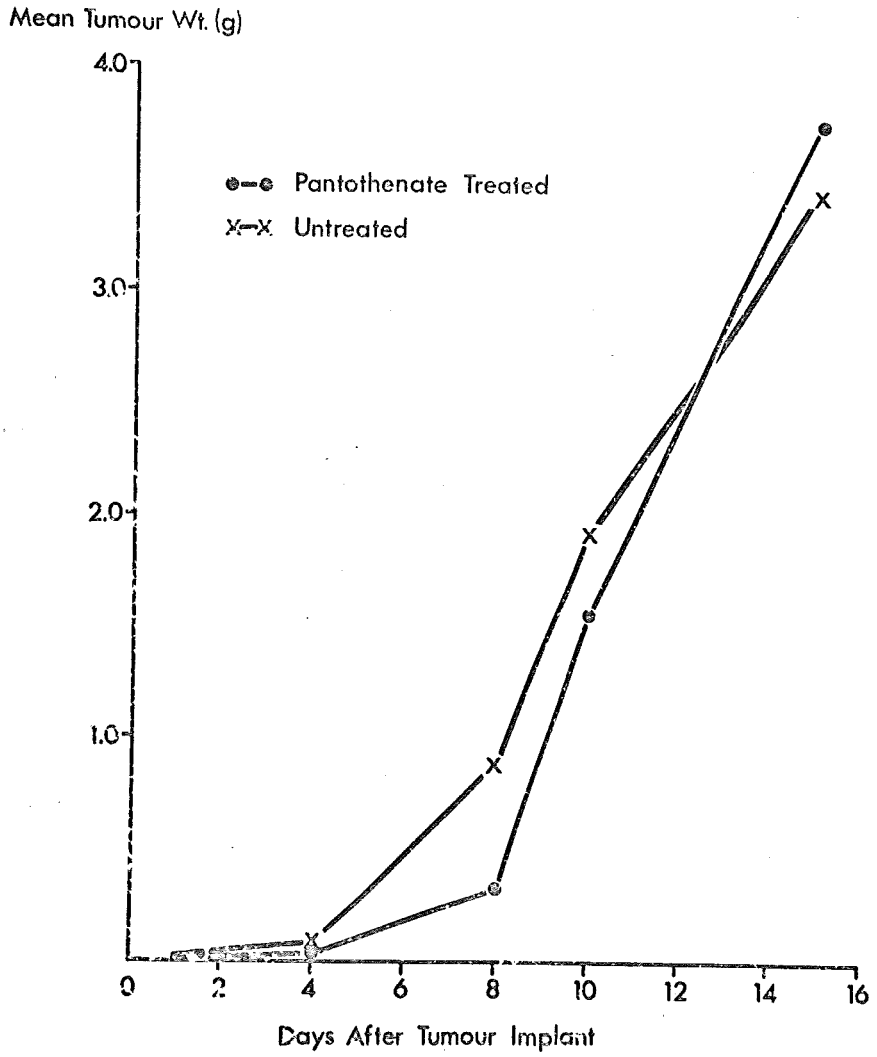


FIGURE 16 Growth curve of Sarcoma 180 in pantothenate-treated and untreated mice. Six animals were used for each time interval.

(2) Changes in Spleen Weight

As shown in Figure 17, the mean spleen weight of mice bearing Sarcoma 180, increased in parallel with a similar group that had received pantothenate, although the mean spleen weight in the latter group showed a greater increase by the eighth day following tumour implant. On the 15th day however, a marked decrease in spleen weight of the pantothenate-treated mice bearing the tumour occurred.

(3) Changes in the Hepatic Contents of Acetyl CoA and CoASH

(a) Acetyl CoA

As shown in Table 7, the hepatic contents of this metabolite in pantothenate-treated, and untreated tumour-bearing mice were very similar when measured at intervals up to 15 days following tumour implant. When these data were compared to normal mice that had received the vitamin, both tumour-bearing groups showed significant decreases in the hepatic content of acetyl CoA on the second and 15th day following the implant.

(b) CoASH

A similar picture occurred in the changes in the hepatic content of the free coenzyme in these livers (Table 8). Again, there was very little difference in the content of this metabolite in liver of tumour-bearing mice, when compared to a similar group that

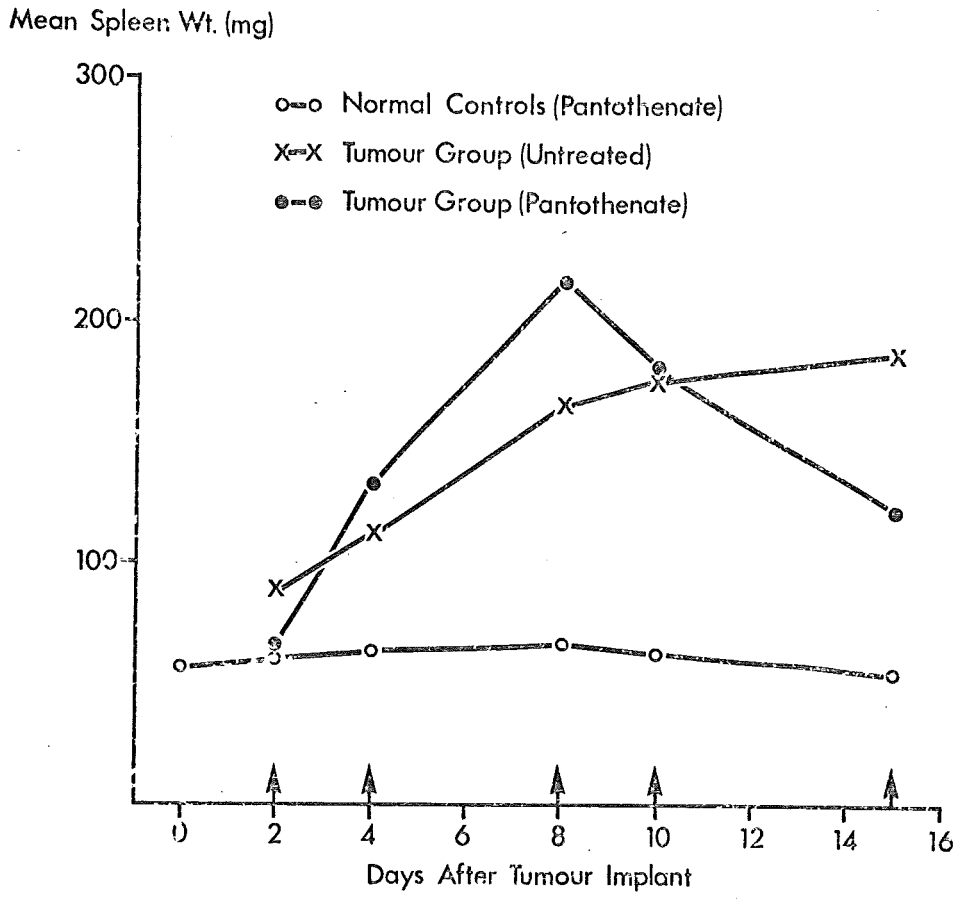


FIGURE 17 Changes in spleen weight induced by the administration of pantothenate to mice bearing Sarcoma 180. Six animals were used for each time interval.

TABLE 7

EFFECT OF ADMINISTRATION OF PANTOTHENATE ON THE ACETYL CoA CONTENT OF LIVER OF NORMAL MICE AND MICE BEARING SARCOMA 180

Day	Acetyl Coenzyme A (nmol/g Liver Wet Weight)		
	Controls (Pantothenate)	Tumour Group (Untreated)	Tumour Group (Pantothenate)
2	58.75 ± 2.18	43.10 ± 2.02^{a}	47.57 ± 2.10^{c}
4	59.18 ± 2.00	57.28 ± 2.56	58.15 ± 3.47
8	48.82 ± 2.85	57.27 ± 3.88	58.99 ± 3.29
10	61.02 ± 3.08	59.65 ± 2.61	62.96 ± 2.83
15	52.92 ± 2.68	30.79 ± 2.78^{b}	34.12 ± 1.66^{d}

Six mice were studied in each group. Values are means $\pm$ S.E.M.

a = Statistically significant ($P < 0.01$) when compared to controls.

b = Statistically significant ($P < 0.001$) when compared to controls.

c = Statistically significant ($P < 0.02$) when compared to controls.

d = Statistically significant ($P < 0.001$) when compared to controls.

TABLE 8

EFFECT OF ADMINISTRATION OF PANTOTHENATE ON THE FREE COENZYME A CONTENT OF LIVER OF NORMAL MICE AND MICE BEARING SARCOMA 180

Day	CoASH (nmol/g Liver Wet Weight)		
	Controls (Pantothenate)	Tumour Group (Untreated)	Tumour Group (Pantothenate)
2	68.98 ± 0.79	41.89 ± 5.99^{a}	29.28 ± 3.61^{c}
4	56.26 ± 4.05	62.56 ± 7.74	59.82 ± 7.57
8	59.76 ± 3.13	54.72 ± 5.13	54.90 ± 5.97
10	66.11 ± 2.45	59.68 ± 9.25	57.25 ± 5.56
15	61.29 ± 4.31	30.69 ± 1.88^{b}	25.49 ± 4.24^{d}

Values are the mean of six mice studied in each group $\pm$ S.E.M.

a = Statistically significant ($P < 0.01$) when compared to controls.

b = Statistically significant ($P < 0.001$) when compared to controls.

c and d = Statistically significant ($P < 0.001$) when compared to controls.

had received pantothenate. Also, in parallel with the changes found in the hepatic contents of acetyl CoA, the free coenzyme A content fell significantly on the second and 15th day following tumour implant, in both pantothenate-treated and untreated mice.

Since these data on the hepatic contents of acetyl CoA and CoASH in the tumour-bearing groups had been compared as shown (Tables 7 and 8), to normal mice that had received pantothenate, we compared the values found in the latter group to those of normal untreated mice. Although the data is not shown, there ~~was~~ were no significant changes in the free coenzyme A content of liver of pantothenate-treated mice, when compared to normal mice. However, those that had received pantothenate showed significant decreases in the hepatic content of acetyl CoA on the eighth day ($P < 0.02$) and also on the 15th day ($P < 0.05$). No explanation can be forwarded for this, but this does not obviate the finding that the administration of pantothenate to mice bearing Sarcoma 180 does not counteract the fall in the hepatic contents of either acetyl CoA or CoASH induced by the tumour.

DISCUSSION

Selection of mice at different periods up to 15 days, for the determination of acetyl CoA, CoASH, and the other parameters studied, meant in effect that these animals had received different amounts of pantothenate. However in normal mice that had received the vitamin, the hepatic contents of CoASH and acetyl

CoA on the 15th day were very similar to those found on the second day. By comparing the values found in the hepatic content of the free coenzyme in these animals to normal untreated controls, there was no significant differences found as a result of treatment with pantothenate. Therefore the pool size of the free coenzyme in liver was not altered by the administration of pantothenate, at least by the amounts given here. It was noted however that in these livers, there was a small but significant fall in the content of acetyl CoA on the eighth and 15th day.

The findings however showed that the fall in the hepatic contents of CoASH and acetyl CoA in tumour bearing mice could not be normalised by the administration of pantothenate, even on the 15th day, when the intake of the vitamin was maximal.

The marked loss of body weight, and spleen weight that occurred in mice bearing Sarcoma 180 following the administration of pantothenate cannot be explained at present. However, this could not be related to a greater rate of tumour-growth in mice receiving the vitamin. It may also be noted, that the amount of pantothenate administered i.p., plus the amount taken orally, was very much lower than the LD_{50} for this compound in mice, which has been reported to be 5 - 7.5 g/Kg body weight when given intraperitoneally (Knott, Tsao, McCutcheon, Cheldelin and King, 1957).

At this stage, we can only tentatively suggest that the administration of pantothenate to mice bearing Sarcoma 180 has some toxic effect, perhaps mediated

via some defect in the metabolism of the vitamin in these animals. Of interest in this connection are the observations of Wiseman and Ghadially (1958), who showed that supplementation of the diet of mice bearing RD_3 sarcoma, with four percent methionine increased the rate of tumour growth, and at the same time caused the tissues of the host to waste. The rationale behind these studies, was the previous observations by these workers, showing that excess methionine reduced the ability of normal tissues to take up other amino acids, but not the tumour cells in vitro. They suggest that as a consequence, the tumour is provided with a readier source of amino acids for growth (Wiseman and Ghadially, 1955a, 1955b; Wiseman and Ghadially, 1956; Wiseman and Ghadially, 1958).

It is also of interest that the methionine content of the diet has been claimed (Abiko, 1975), to be the most influential factor in the decreased content of coenzyme A in liver of rats fed low-protein diets.

Clearly much further work is necessary on the metabolism of coenzyme A in liver of tumour-bearing animals to elucidate the mechanisms involved in the findings here reported.

CHAPTER 3

FURTHER STUDIES ON METABOLIC CHANGES IN LIVER OF TUMOUR-BEARING MICE

Introduction

In the previous Chapter, several questions were raised as to the aetiology of changes in the hepatic content of acetyl CoA, CoASH and citrate in liver of tumour-bearing mice, and some possible mechanisms were proposed to account for these changes.

In the present study, attempts have been made to clarify some of these proposals, as well as gain further insight into the metabolic events taking place in these livers as a result of tumour growth in the host.

Part of these studies have been presented elsewhere (Calman and McAllister, 1975a, 1975b; McAllister, Soukop and Calman, 1976).

Methods and Materials

Mice bearing either TLX-5 lymphoma, Sarcoma 180 or a transplantable C_3H mammary tumour were used as previously.

Details as to the stage of tumour growth at which the determinations were made, are described where appropriate in the text.

Metabolites Studied

Lactate, pyruvate, malate, citrate α-oxoglutarate, ATP, ADP, AMP, fructose-6-phosphate and glucose-6-phosphate were determined on freeze-clamped livers as described previously.

The very low level of oxaloacetate in mouse liver, made the enzymatic determination of this metabolite unreliable. Values were therefore obtained by calculation*, as were the NAD^+ / NADH ratios, according to the procedures of Williamson, Lund and Krebs (1967).

(a) The Cytoplasmic NAD^+ / NADH Ratio

The lactate and pyruvate content of freeze-clamped livers were determined enzymatically, and the following applied:

$$\frac{\text{Pyruvate}}{\text{Lactate}} \times \frac{I}{K_{eq.}} = \frac{NAD^+}{NADH}$$

where $K_{eq.}$ is the equilibrium constant of lactate dehydrogenase, which is equal to 1.1×10^{-4}.

(b) The Cytoplasmic Oxaloacetate Content*

The malate content of freeze-clamped livers was determined enzymatically, and using the NAD^+ / NADH ratio calculated as above, the oxaloacetate content was determined using the formula,

*The reservations as to this approach are discussed on page 106.

$$\text{Oxaloacetate} = \text{Malate} \times \text{NAD}^+ / \text{NADH} \times K_{eq.}$$

where $K_{eq.}$ is the equilibrium constant of malate dehydrogenase which is equal to 2.75×10^{-5}.

(c) Enzymes Studied

In separate experiments, the activity of phosphofructokinase and fructose-1, 6-diphosphatase in liver of normal and tumour-bearing animals were determined.

(d) Others

The lactate and glucose content of blood from control and tumour-bearing mice were determined as described previously.

Since in these animals, livers had first to be freeze-clamped, blood was collected immediately afterwards by nicking the aorta, and withdrawing the blood rapidly from the abdominal cavity with a syringe containing heparin. Measured aliquots were immediately deproteinised by the appropriate method and the assay carried out immediately.

Results

The data shown in Figure 18, shows the significant decreases that occurred in the hepatic contents of lactate, pyruvate and malate in mice bearing TLX-5 lymphoma. In mice bearing Sarcoma 180, that at autopsy gave a mean tumour weight of 4.42 $\pm$ S.E.M. 0.55 g after 15 days of tumour-growth, the lactate

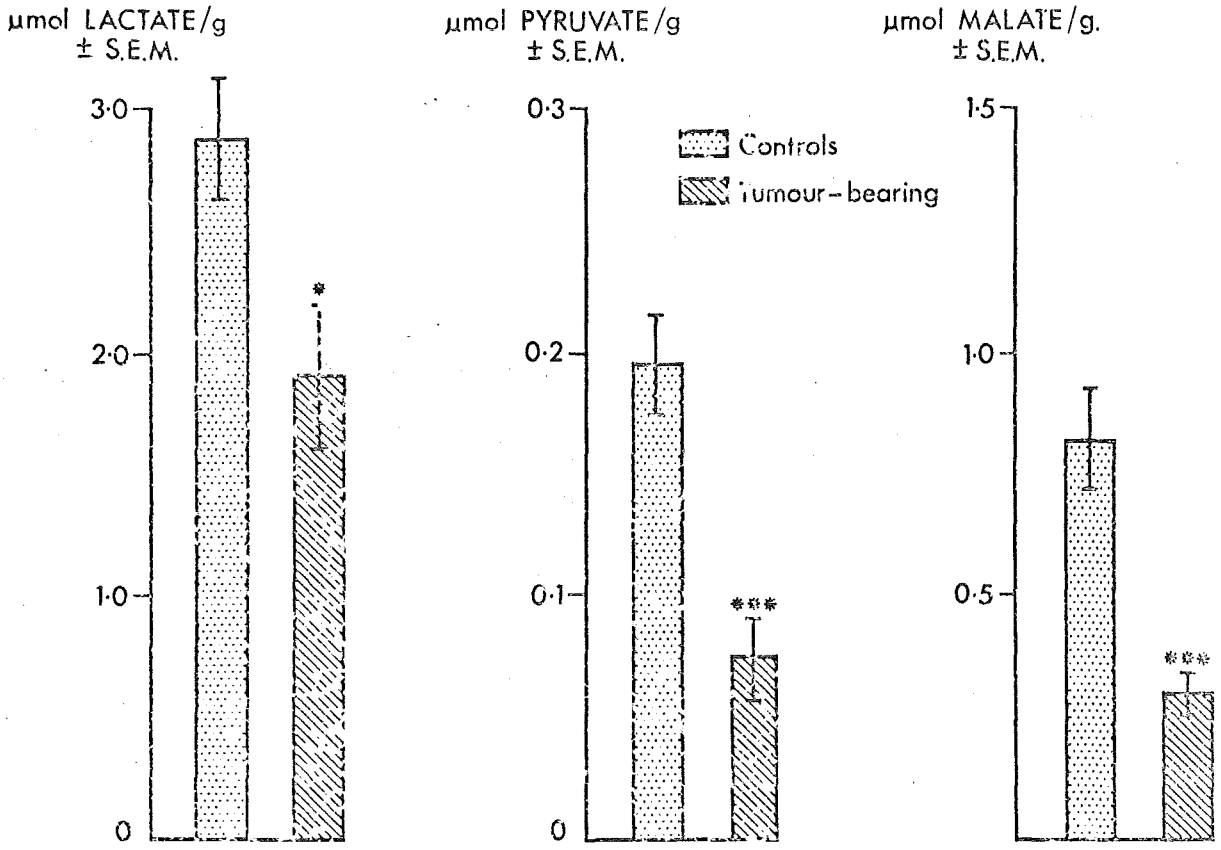


FIGURE 18 The lactate, pyruvate and malate content of livers of mice bearing TLX-5 lymphoma after seven days of tumour growth. Results are means of six animals in each group.

content of liver fell below normal, but not significantly so when compared to controls. There were highly significant reductions in the pyruvate content of these livers, and the malate content was also significantly depressed (Figure 19). The hepatic content of the latter was also significantly reduced ($P < 0.001$) in mice bearing the C_3H mammary tumour. In this group, the mean tumour was 0.73 $\pm$ S.E.M. 0.19 g. The lactate content of livers of these animals remained at normal values, and although the pyruvate content was decreased, this did not reach statistical significance (Figure 20).

Determination of the α-oxoglutarate content of livers of mice bearing these tumours showed a 53 percent decrease in those bearing TLX-5 lymphoma which was highly significant ($P < 0.001$). In mice bearing Sarcoma 180, with a mean tumour weight of 0.50 $\pm$ S.E.M. 0.35 g, there was an 18 percent decrease in the α-oxoglutarate content of liver, but this was not statistically significant. In contrast, in another series of mice bearing this tumour, in which the mean tumour weight was 4.27 $\pm$ S.E.M. 0.22 g after 15 days of tumour growth, there was a 60 percent reduction in the hepatic content of α-oxoglutarate ($P < 0.01$). These data are shown in Table 9, where it can also be seen that the α-oxoglutarate content of livers of mice bearing the C_3H mammary tumour, did not alter in either of the two groups of animals studied, and in which the mean tumour weights were 0.19 $\pm$ S.E.M. 0.04 g and 0.55 $\pm$ S.E.M. 0.12 g respectively.

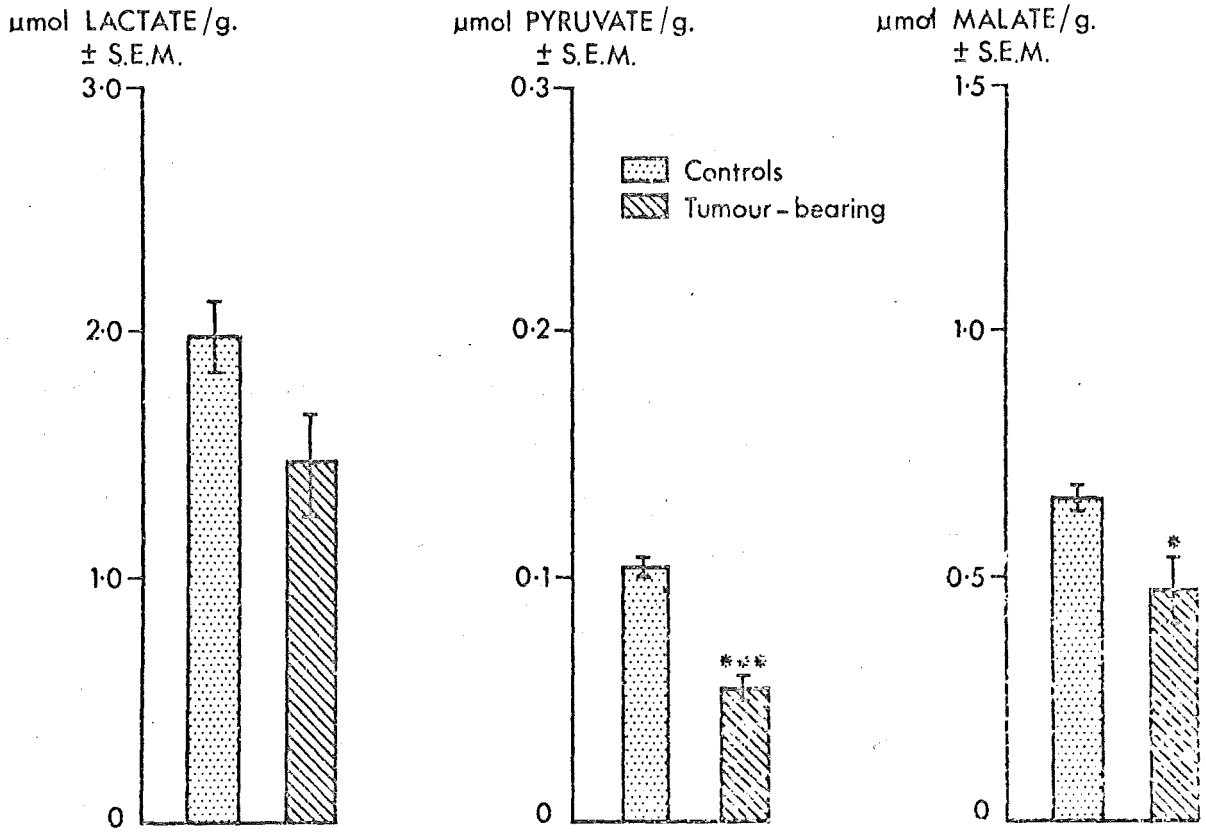


FIGURE 19 The lactate, pyruvate and malate content of livers of mice bearing Sarcoma 180. Results are means of six animals in each group.

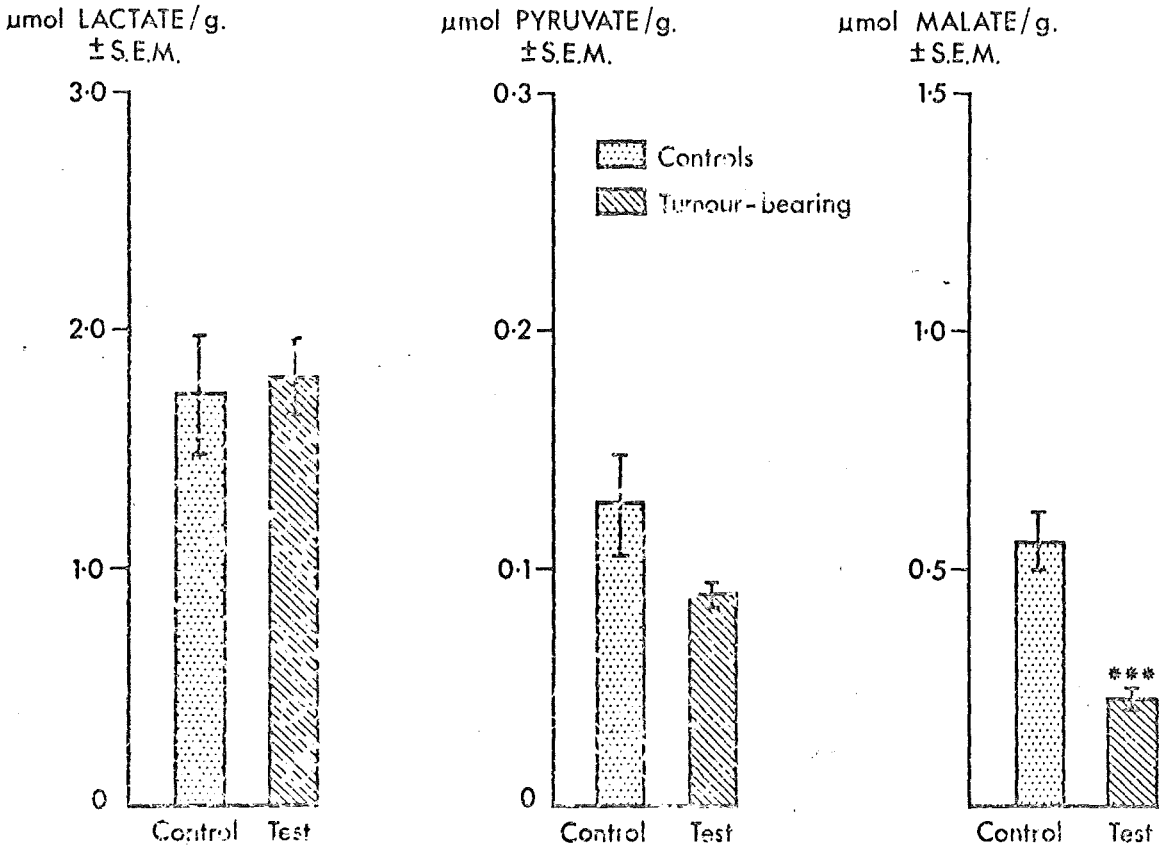


FIGURE 20 The lactate, pyruvate and malate content of livers of mice bearing a C_3H transplantable mammary tumour. Results are means of six animals in each group.

The data given in Figures 18, 19 and 20 for the lactate, pyruvate and malate content of livers of these mice was then used to calculate the lactate/pyruvate ratios, the cytoplasmic NAD^+ / NADH ratios and the cytoplasmic oxaloacetate content by the methods already described. Table 10 shows that significant increases in the lactate/pyruvate ratio occurred in livers of mice bearing either of the three tumours studied. In these experiments, the data shown in Table 10 for mice bearing Sarcoma 180, were derived from the group in which the mean tumour weight was 4.42 $\pm$ S.E.M. 0.55 g and in those bearing the mammary tumour, which gave a mean tumour weight of 0.73 $\pm$ S.E.M. 0.19 g; as is also shown in Table 10, the NAD^+ / NADH ratios in livers of these animals decreased significantly indicating, as discussed later, that the cytoplasm of the liver cell in these tumour-bearing mice was in a more reduced state than normal. The calculated values for the oxaloacetate content of these livers also showed significant decreases when compared to control values.

As shown in Table 11, the hepatic content of glucose-6-phosphate and fructose-6-phosphate were significantly decreased in mice bearing TLX-5 lymphoma. For various reasons it was not possible to determine the hepatic content of these metabolites in liver of animals bearing the other tumour systems.

Determination of the activity of phosphofructo-kinase and fructose-1, 6-diphosphatase in liver of mice bearing TLX-5 lymphoma showed that the activities of the enzymes were not altered from normal values (Table 12).

TABLE 9

THE ∝-OXOGLUTARATE CONTENT OF LIVERS OF TUMOUR-BEARING ANIMALS

Conditions	∝-Oxoglutarate μmol/g ± S.E.M.	% Change	P
Controls	0.146 ± 0.01		
TLX-5 Lymphoma	0.069 ± 0.009	-53	< 0.001
Controls	0.106 ± 0.01		
Sarcoma 180. Mean Tumour Wt. 0.50 ± S.E.M. 0.35g	0.086 ± 0.020	-18	N.S.
Controls	0.23 ± 0.03		
Sarcoma 180. Mean Tumour Wt. 4.27 ± S.E.M. 0.24g	0.09 ± 0.008	-60	< 0.01
Controls	0.113 ± 0.007		
C_3H Mammary Tumour. Mean Wt.=0.19 ± S.E.M. 0.04g	0.118 ± 0.074	+5	N.S.
Controls	0.097 ± 0.021		
C_3H Mammary Tumour. Mean Wt.=0.55 ± S.E.M. 0.12g	0.097 ± 0.017	NIL	N.S.

P = Statistical evaluation by unpaired 't' test.

N.S. = Not Significant.

Six animals were studied in each group.

TABLE 10

THE LACTATE/PYRUVATE RATIOS, REDOX STATE OF FREE NICOTINAMIDE ADENINE DINUCLEOTIDES AND OXALOACETATE CONTENT OF LIVERS OF TUMOUR-BEARING MICE

Conditions	Lactate/Pyruvate	NAD^+ / NADH	Oxaloacetate µmol/g
Controls	15.04±1.34 (6)	620±59 (6)	0.014±0.002 (6)
TLX-5 Lymphoma	25.53±2.18 (6)	365±35 (6)	0.003±0.0004 (6)
P	< 0.01	< 0.01	< 0.01
Controls	19.21±1.30 (6)	477±32 (6)	0.009±0.0007 (6)
Sarcoma	29.28±2.90 (6)	308±35 (6)	0.004±0.0006 (6)
P	< 0.02	< 0.01	< 0.001
Controls	12.08±0.75 (6)	757±46 (6)	0.012±0.0018 (6)
C_3H Mammary	20.33±1.59 (6)	456±41 (6)	0.003±0.003 (6)
Tumour	< 0.001	< 0.001	< 0.001

Values are means ± S.E.M. numbers in parenthesis are number of animals used. P = Statistical evaluation by unpaired 't' test. Cytosolic NAD^+ / NADH ratio and oxaloacetate content calculated according to the methods given in the text.

Mean tumour weights - Sarcoma 180 = 4.42g±S.E.M. 0.55g.

Transplantable C_3H Mammary tumour = 0.73g±S.E.M. 0.19g.

TABLE 11

THE GLUCOSE-6-PHOSPHATE AND FRUCTOSE-6-PHOSPHATE CONTENT OF LIVER OF MICE BEARING TLX-5 LYMPHOMA

HEPATIC CONTENT
(μmol/g Wet Wt.)

Conditions	Glucose-6-Phosphate	Fructose-6-Phosphate
Controls	0.280 ± 0.036 (10)	0.131 ± 0.020 (10)
Tumour-Bearers	0.100 ± 0.008 (9)	0.057 ± 0.004 (9)
P	< 0.001	< 0.01

Figures in parenthesis are number of animals studied.
Results expressed as means $\pm$ S.E.M.

TABLE 12

THE ACTIVITIES OF FRUCTOSE DIPHOSPHATASE AND PHOSPHOFRUCTOKINASE IN LIVER OF MICE BEARING TLX-5 LYMPHOMA

Conditions	Fructose Diphosphatase μmol/min/g	Phosphofructokinase μmol/min/g
Controls	7.51 ± 0.24 (6)	0.67 ± 0.017 (6)
TLX-5 Lymphoma	7.39 ± 0.17 (6)	0.75 ± 0.29 (6)
P	N.S.	N.S.

Values given are means $\pm$ S.E.M. Numbers in parentheses are numbers of animals used.

N.S. = Not significant.

Blood Glucose Values in Mice Bearing Either Sarcoma 180 or TLX-5 Lymphoma

The data given in Table 13 shows that growth of TLX-5 lymphoma or Sarcoma 180, resulted in marked decreases in the glucose content of blood in the host, but in mice bearing the mammary tumour, this did not alter significantly.

Blood Lactate Values

These determinations were only made on mice bearing either the TLX-5 lymphoma or Sarcoma 180. As shown in Table 14, the lactate content of blood of mice bearing TLX-5 lymphoma showed no significant change when compared to controls, but in mice bearing Sarcoma 180, significant decreases in the blood lactate values occurred.

Hepatic Content of Adenine Nucleotides

As shown in Table 15 there were significant decreases in the hepatic contents of ATP and ADP in mice bearing either TLX-5 lymphoma, or Sarcoma 180. The AMP content of these livers was only significantly decreased in mice bearing the Sarcoma. However, the ATP/ADP ratios were not significantly altered in both models, and the ATP/AMP ratio showed a significant decrease only in mice bearing the lymphoma.

The data also shows the 'energy charge' in these livers which was calculated from $E = \frac{1}{2} \left(\frac{ADP + 2\ ATP}{Total}\right)$, as described by Atkinson (1969). This value (E) was not significantly altered in mice bearing Sarcoma 180

TABLE 13

BLOOD GLUCOSE VALUES IN TUMOUR-BEARING MICE

Conditions	Blood Glucose mmol/l $\pm$ S.E.M.
Normal Controls (CBA)	12.23 ± 0.718 (6)
TLX-5 Lymphoma	4.88 ± 0.087 (6)
P	< 0.001
Normal Controls (C_3H)	10.87 ± 0.820 (6)
C_3H Mammary Tumour	13.90 ± 1.120 (6)
P	N.S.
Controls (CBA)	14.77 ± 0.565 (6)
Sarcoma (180)	9.67 ± 0.617 (6)
P	< 0.001

N.S. = Not Significant.

Numbers in parenthesis = Number of animals in each group.

TABLE 14

THE LACTATE CONTENT OF BLOOD OF TUMOUR-BEARING MICE

Conditions	Blood Lactate mmol/l ± S.E.M.	P
Controls	5.44±0.44 (4)	N.S.
TLX-5 Lymphoma	5.25±0.40 (6)	
Controls	5.39±0.12 (6)	< 0.001
Sarcoma 180	3.99±0.24 (6)	

Numbers in parentheses are numbers of animals studied.
N.S. = Not significant.

TABLE 15

THE ADENINE NUCLEOTIDE CONTENT OF LIVER OF TUMOUR-BEARING MICE

Conditions	Hepatic Content (μmol/g Liver Wet Wt.)			ATP/ADP	ATP/AMP	Energy Charge
	ATP	ADP	AMP			
Controls	1.55±0.03	1.09±0.026	0.355±0.01	1.42	4.39	0.699
Sarcoma 180	1.33±0.04	0.92±0.036	0.283±0.018	1.45	4.78	0.707
P	< 0.01	< 0.01	< 0.01	N.S.	N.S.	N.S.
Controls	2.04±0.09	1.50±0.028	0.448±0.018	1.36	4.57	0.700
TLX-5 Lymphoma	1.70±0.069	1.34±0.040	0.450±0.017	1.26	3.79	0.678
P	< 0.02	< 0.02	N.S.	N.S.	< 0.01	N.S.

Six animals were studied in each group. The energy charge was calculated as described in the text.

Mice bearing TLX-5 lymphoma were studied after eight days of tumour growth and those bearing Sarcoma 180 after 14 days of tumour growth.

Results expressed as means ± S.E.M.

or TLX-5 lymphoma, when compared to controls.

Discussion

These experiments show that highly significant reductions occur in the pyruvate content of livers of mice bearing either TLX-5 lymphoma or Sarcoma 180. The lactate content of these livers only decreased significantly in those bearing TLX-5 lymphoma. As described previously however, this tumour invades liver, and this may well cloud the picture of changes in the hepatic content of this metabolite.

The lactate/pyruvate ratios in livers of mice bearing either of the three tumours studied shows significant increases. Previous workers have shown that this ratio is only slightly increased in normal fasted rats, and raised three to five-fold in acute alloxan diabetes in these animals (Williamson, Lund and Krebs, 1967), changes that can be attributed to a decrease in glycolytic flux under these conditions. Since we have previously shown that mice bearing TLX-5 lymphoma are anorexic, whereas those bearing Sarcoma 180 are not, these increased lactate/pyruvate ratios cannot be due solely to reductions in food intake. Since pyruvate is the second major source of acetyl CoA in liver (Lowenstein, 1971) a fall in the hepatic content of this metabolite in liver of tumour-bearing mice, would be consistent with a decrease in the acetyl CoA content of livers of mice bearing these tumours that we observed previously. It is difficult however to assess the degree of reduction in the level of

pyruvate that would be necessary for this effect to be achieved, particularly since the pyruvate content of livers of mice bearing the C_3H mammary tumour, although decreased did not reach statistical significance. Further, in the normal fasted rat, the increased fatty acid supply to liver results in significant increases in the acetyl CoA content.

Alterations in the substrate pair lactate/pyruvate, in livers of these mice due to alterations in the activity of lactate dehydrogenase would appear unlikely since it has been shown (Manso, Sugiura and Wroblewski, 1958) that the activity of this enzyme is not altered in livers of mice bearing a variety of tumours, including Sarcoma 180. Similar findings for the specific activity, and distribution pattern of this enzyme in liver of rats hosting Morris hepatoma 16 have also been reported (Cornbleet, Vorbeck, Lucas, Esterly, Morris and Martin, 1974). We consider therefore that a decreased content of pyruvate in liver of mice bearing either TLX-5 lymphoma or Sarcoma 180, that we have reported here, would be consistent with a previous report of increased activity of gluconeogenic enzymes in livers of rats bearing transplantable tumours (Gutman, Thilo and Biran, 1969). There is also evidence that three-carbon fragments of the oxidative breakdown of glucose are actively utilised in the synthesis of tumour proteins (Shapot, 1972).

The decreases in the hepatic content of α oxoglutarate that were found in the present study, amounted to 53 percent in those bearing TLX-5 lymphoma ($P < 0.001$),

18 percent in those bearing Sarcoma 180, with a mean tumour weight of 0.50 g, and 60 percent in those mice bearing the larger Sarcoma (mean wt. 4.42 g). Whether there is any relationship to the size of the Sarcoma, and the degree of reduction in the hepatic content of this metabolite remains to be elucidated. It is of interest however that in mice bearing Sarcoma 180, which gave a mean tumour weight of 0.50 g, the citrate content of liver did not increase, whereas in those hosting Sarcoma 180, with a mean tumour weight of 4.42 g, the hepatic content of this metabolite increased significnatly.

The central position of α-oxoglutarate in intermediary metabolism, where it provides a link between the oxidative break-down of carbohydrate and fats, and the metabolism of amino acids (Tager, De Haan and Slater, 1969), indicates that several possible mechanisms could be responsible for its depletion in livers of these tumour-bearing mice. The occurrence in liver of several α-oxoglutarate-linked transaminases, raises the question of whether increased substrate utilisation due to increased activity of these enzymes is involved. Kampschmidt (1960) has reported increased activity of tyrosine transaminase in livers of rats bearing the Walker carcinosarcoma 256. The interpretation of such data is, however complicated, since the activity of these enzymes are sensitive to protein intake, as well as adrenal function (Levine, 1964). Significant decreases in the α-oxoglutarate content of livers of fasted and acute alloxan/diabetes rats have also been reported (Williamson, Lund and

Krebs, 1967) in which conditions, the activity of the tricarboxylic acid cycle is depressed (Start and Newsholme, 1968). In these gluconeogenic conditions however, the citrate content of liver is decreased (Herrera and Freinkel, 1968; Start and Newsholme, 1968), whereas in liver of tumour-bearing mice, the citrate content is significantly increased. Since the formation of α-oxoglutarate in the tricarboxylic acid cycle takes place in the sequence, citrate-isocitrate-α-oxoglutarate, and isocitrate is in equilibrium with citrate (Atkinson, 1968) an increased citrate content in livers of these tumour-bearing mice, and a decreased α-oxoglutarate content, could suggest that isocitrate dehydrogenase is inhibited. Altered tricarboxylic acid cycle activity in liver with accumulation of citrate and isocitrate as a result of inhibition of this enzyme by ammonia has been reported previously in arginine-deficient rats (Prior and Visek, 1973) and in ammonia intoxication (Kesper, 1965). We are investigating possible inhibitory mechanisms of this enzyme in livers of tumour-bearing mice.

When the NAD^+ / NADH ratios of mouse liver cytoplasm were calculated, the data showed that significant decreases had occurred in livers of these mice bearing either of the three tumours under investigation. Since the lactate dehydrogenase system is located exclusively in the cytoplasm, these ratios reflect the redox-state of the free adenine dinucleotides in that compartment of the liver cell (Williamson, Lund and Krebs, 1967). Further, since it is well-recognised that glycolysis and gluconeogenesis in liver are

cytoplasmic events, where during glycolysis, hydrogen transfer from glyceraldehyde-3-phosphate to NAD^+ takes place, with the reverse process in gluconeogenesis, the direction of the two, depends on the redox-state of the hydrogen carrier system (Williamson, Lund and Krebs, 1967).

Our data therefore show that the liver of tumour-bearing mice is in a more reduced state than normal, and by analogy with similar change in fasted rats (Williamson, Lund and Krebs, 1967) this might favour gluconeogenesis. This would also support our previous proposal, that the increased citrate content of these livers would favour gluconeogenesis due to citrate-inhibition of phosphofructokinase. However, the proviso must be made that such a change in the NAD^+ / NADH ratio would also tend to suppress the conversion of lactate to pyruvate, and hence decrease gluconeogenesis from this source, as has been shown to occur during ethanol metabolism (Lochner, Wulff, and Madison, 1967). A decrease in the oxaloacetate in the cytoplasm of the liver cell would also indicate increased utilisation for glucose synthesis, as has been reported by Wieland (1968) in rats under gluconeogenic conditions. As discussed later (p. 105) it should however be noted that derivation of the tissue content of these metabolites by calculation require that certain assumptions are necessary, as described in detail by Williamson, Lund and Krebs, (1967) and this necessitates that much caution is used in the interpretation of such data.

Whether a fall in the mitochondrial oxaloacetate content in these livers would decrease the rate of the tricarboxylic acid cycle, since this compound is one of the substrates for citrate synthase, with a concomitant decrease in energy available to the cell, remains to be clarified. It is of interest however that the second substrate for citrate synthase, namely acetyl CoA, is also known to be decreased significantly in these livers, as was the ATP content in livers of mice bearing TLX-5 lymphoma.

As has been discussed in detail by several workers (Wieland, 1968; Tepperman and Tepperman, 1970; Newsholme and Start, 1973), a decreased rate of the tricarboxylic acid cycle necessitates adjustments in order for the cell to obtain energy, and this is achieved under gluconeogenic conditions, by increased oxidation of fatty acids to acetyl CoA with formation of acetoacetate, and liberation of free CoA.

These compensatory mechanisms would therefore appear to be lacking in livers of tumour-bearing mice.

The significant decreases in the hepatic contents of glucose-6-phosphate and fructose-6-phosphate requires verification in other tumour-bearing animals, due to the possible effects of invasion of liver by the lymphoma cells. A fall in the content of these hexose monophosphates may have been due to anorexia in these animals since in starved rats, the contents of both metabolites in liver decrease (Start and Newsholme, 1970). In these animals however it was also shown that this only occurred when the glycogen stores became

depleted. Under these conditions, the maximal activities of phosphofructokinase and fructose diphosphatase do not change, as was also found in liver of mice bearing TLX-5 lymphoma. In this connection it is of interest that in an earlier study (Gutman, Thilo and Biran, 1969) it was reported that the activity of fructose diphosphatase was only marginally increased in liver of rats bearing a non-metastasising sarcoma. Since fructose-6-phosphate is a substrate for phosphofructokinase a decrease in the hepatic contents of this metabolite would decrease the activity of the enzyme, and if as has been proposed by Start and Newsholme (1970) a substrate cycle exists between fructose-6-phosphate and fructose diphosphate, a decrease in the activity of the kinase, would result in an increase in the hydrolysis of fructose diphosphate.

Thus in mice bearing TLX-5 lymphoma, the above findings suggest that the rate of gluconeogenesis is increased. As was previously suggested here, the increase in the hepatic content of citrate in liver of tumour-bearing mice would also inhibit phosphofructokinase in vivo. This suggestion however is not negated by the finding that the activity of this enzyme was not altered in these livers, since the conditions of the assay in vitro would have relieved this inhibition.

On the basis of a previous report (Hornbrook, Burch and Lowry, 1965) that citrate accumulated in liver of rats following the injection of lactate, we examined the blood lactate content of mice bearing Sarcoma 180 or TLX-5 lymphoma. The finding that in the former

model, the lactate content of blood did not alter, whereas in the latter, significant decreases were found, negated this proposal that an increase in blood lactate was involved in the accumulation of citrate in liver of these animals, since as previously described this was a feature common to both.

The significant decreases in the hepatic contents of the three adenine nucleotides in mice bearing Sarcoma 180, and that of ATP and ADP in mice bearing the lymphoma was unexpected, since for example a fall in the content of ATP due to increased utilisation would be expected to result in an increase in AMP. However, a fall in the hepatic contents of ATP and free coenzyme A could be involved in the decreased activity of ATP-citrate lyase which is described later (Chapter 4).

As was shown here, the ATP/ADP and ATP/AMP ratios did not change in liver of mice bearing Sarcoma 180, and only the ratio ATP/AMP showed a significant decrease in mice bearing the lymphoma. A fall in the latter ratio also occurs in liver of starved rats, but under these conditions, the ATP/ADP ratio also decreases (Start and Newsholme, 1968). However under conditions of starvation the energy charge remains constant (Start and Newsholme, 1968, Guynn, Veloso and Veech, 1972). The data presented here showed that the energy status of the cells in liver of tumour-bearing mice was also constant, but until a satisfactory explanation can be obtained for the fall in the hepatic contents of the three adenine nucleotides in these livers, little interpretation of this can be made.

The Problem of Compartmentation

The caution necessary in the use of calculated values for metabolites has been stressed by workers who have been responsible for the development of such methods (Gumma, McLean and Greenbaum, 1971; Williamson, 1969). Thus the use of the method of Williamson, Lund and Krebs (1967) for calculation of the cytosolic oxaloacetate content depends upon near-equilibrium of malate dehydrogenase, and an assumed gradient for the malate distribution. It is now known however that both the cytosolic and mitochondrial malate dehydrogenase are displaced from equilibrium (Tischler, Hecht and Williamson, 1977) and the calculated gradients of oxaloacetate do not agree with those found by measurement. Therefore the data presented here on the cytosolic oxaloacetate content are no longer tenable. Initially in these studies, attempts were made to determine the hepatic contents of oxaloacetate, which are notoriously difficult due mainly to the low content present, and resort had to be made to the calculated values. These however have been retained here, but must therefore be considered only of a preliminary nature offering a lead towards further work since in spite of the above reservations, the values were found to be decreased in liver of tumour-bearing mice.

A major, and obvious defect in the determination of the whole cell content of metabolites is the lack of knowledge on their distribution in the mitochondrial and cytosolic compartments, and this has been a major

problem in this field. Recently however, methods have been developed that permit rapid separation of particulate components and cytoplasm of isolated rat liver cells (Zuurendonk and Tager, 1974; Tischler, Hecht and Williamson, 1977). Some discrepancies however have been found between the different methods, and further work is required before their full potential can be explored.

These methods however have only been applied to rat liver, and since the initial step requires perfusion of the excised liver, it is unlikely that this could be readily achieved in the mouse.

The decrease in the glucose content of blood in mice bearing Sarcoma 180, is in contrast to a previous report (Shapot and Blinov, 1974) of normal values in mice bearing this tumour. These workers have proposed that in these animals, the normoglycaemia was due to an enhancement of gluconeogenesis, that depended upon the gluconeogenic response of the host tissues to glucocorticoids. Stimulation of gluconeogenesis in the host has also been proposed to account for the normoglycaemia in rats bearing a non-metastasising sarcoma (Gutman, Thilo and Biran, 1969) due to an increased activity of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase.

The possibility of an increase in the circulating level of insulin in mice bearing either TLX-5 lymphoma of Sarcoma 180 has not been examined. It is of interest however, that diminished serum glucose levels in rats bearing Walker 256 carcinoma have been reported

(Goodlad, Mitchell, McPhail and Clark, 1975) to be accompanied by marked decreases in serum insulin (and somatomedin). These workers conclude that this fall in serum glucose most probably reflects the general disturbances of energy metabolism in the host associated with tumour growth.

CHAPTER 4

METABOLIC CHANGES IN LIVERS OF TUMOUR-BEARING MICE

Activity of Citrate-Cleavage Enzyme in Liver and the Effect of Fasting on the Hepatic Contents of Acetyl CoA, CoASH, Citrate and Triglycerides

Disturbances of lipid metabolism in tumour-bearing animals have been well-documented. As discussed previously in this thesis, such changes include loss of body fat (Haven and Bloor, 1956; Costa and Holland, 1962), and hyperlipidaemia with alterations in the pattern of circulating lipoproteins (Creinin and Narayan, 1971; Cox and Gokcen, 1975; Brenneman, Mathur and Spector, 1975).

The mechanism involved in the aetiology of these changes in tumour-bearing animals however remains unknown.

The finding that the hepatic content of citrate was increased in tumour-bearing mice (Chapter 2), prompted the tentative suggestion, that this might be due to some disturbance of lipid metabolism in these animals. As mentioned therein, citrate is the main source of carbon for the extra mitochondrial synthesis of fatty acids in mouse and rat liver (Spencer and Lowenstein, 1962; Bhaduri and Srere, 1963; Spencer, Corman and Lowenstein, 1964).

The initial stage of this reaction is cleavage of citrate in the cytosol to acetyl CoA and oxaloacetate by the citrate cleavage enzyme (Spencer and Lowenstein, 1962). Much evidence has

shown that the activity of the enzyme in liver is dependent on the nutritional status. Thus decreases in activity have been reported in starved rats re-fed high fat diets (Kornacker and Lowenstein, 1963; Kornacker and Lowenstein, 1965a). On the other hand, marked increases in activity of the enzyme occur in starved rats, re-fed diets high in glucose (Kornacker and Lowenstein, 1965a). These latter changes are in accordance with the marked increase in fatty acid synthesis that occurswhen starved animals are re-fed diets with a low fat content but high content of carbohydrate (Tepperman and Tepperman, 1958; Masoro, 1962; Spencer, Corman and Lowenstein, 1964).

In view of the accumulation of citrate that was found to occur in tumour-bearing mice, it was of interest to examine the activity of citrate-cleavage enzyme in livers of these animals. In addition, the hepatic contents of acetyl CoA, CoASH, citrate and triglycerides were studied in fasted tumour-bearing mice to see whether there were changes in acetyl CoA associated with tumour-induced lipid mobilisation.

Materials and Methods

Weight-matched CBA mice bearing either TLX-5 lymphoma or Sarcoma 180 were used. Tumours were implanted in these animals exactly as described in Chapter 1.

Other details as to time of fasting animals, and the stage of tumour growth when the observations were

made are detailed in the text. Urine samples from fed normal mice and those bearing the tumour were obtained by placing the animals in small plastic dishes. Samples so obtained were then tested for ketones using a conventional test strip (Multistix, Ames). Where the effects of starvation were studied the animals were placed in clean cages and food but not water withdrawn for 48 hours.

The acetyl CoA, CoASH, citrate, triglyceride content of liver, and activity of the citrate cleavage enzyme were determined by methods already described. The epididymal fat pads were carefully removed and weighed wet.

Results

Changes in the Triglyceride Content of Liver and Weight of Epididymal Fat Pads in Tumour-Bearing Mice

Figure 21 shows the changes in the triglyceride content of liver of mice bearing either TLX-5 lymphoma or Sarcoma 180.

In the former model, the triglyceride content of liver showed no significant change from normal values after seven days of tumour growth.

In contrast, the data show that in mice bearing Sarcoma 180, there were highly significant reductions in the triglyceride content of liver of these animals. In these studies, the determinations were made on animals 12 days following implant, and the mean tumour weight was 4.3 g $\pm$ 0.36 g S.E.M.

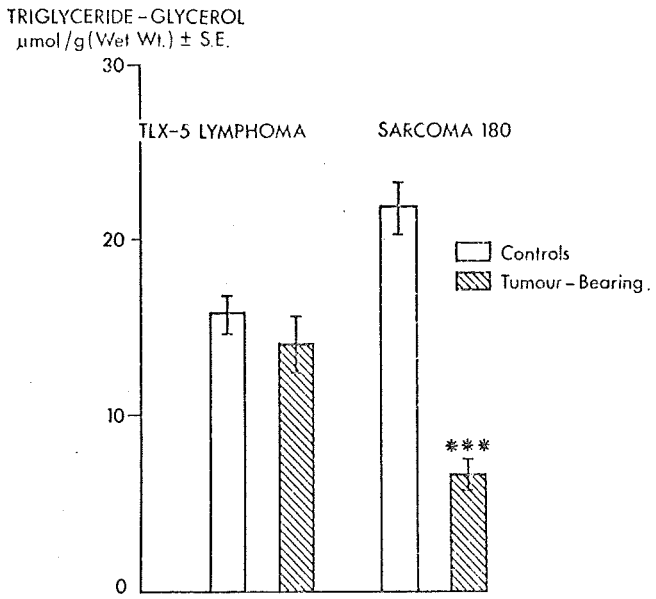


FIGURE 21 The triglyceride-glycerol content of liver of mice bearing either TLX-5 lymphoma after seven days of tumour growth, or Sarcoma 180 after 12 days of tumour growth. Results expressed as means of six animals studied.

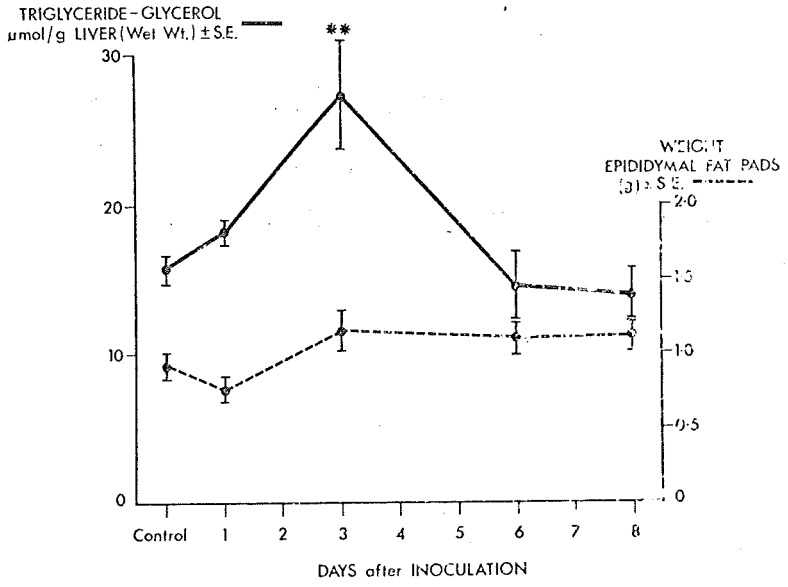


FIGURE 22 Daily changes in the triglyceride-glycerol content of liver, and weight of the epididymal fat pads in mice bearing TLX-5 lymphoma. Six animals were used for each time interval.

The daily changes in the triglyceride content of liver of mice bearing TLX-5 lymphoma were then studied over eight days of tumour growth. The weight of the epididymal fat pads in these animals were also recorded daily.

From the data shown in Figure 22, it will be seen that there was a progressive increase in the triglyceride content of liver, which reached significant values when compared to the control group, on the third day of tumour growth, and which then decreased to normal values by the eighth day. As is also shown, the mean weight of the epididymal fat pads did not alter from normal values over the eight days of tumour growth.

Since it was previously shown in this thesis that in mice bearing TLX-5 lymphoma there was a marked reduction in food intake, particularly on the second day following the injection of the tumour cells, and that a decrease in food intake continued over the next five days, the accumulation of triglycerides in liver on the second to third day was attributed to anorexia.

In contrast to that found in mice bearing TLX-5 lymphoma, the weight of the epididymal fat pads of mice bearing Sarcoma 180 fell significantly when compared to normal controls.

However, as also described previously, mice bearing Sarcoma 180, although presenting anorexia on the first to fourth day following tumour implant, after about the sixth day, these animals continued to eat normally. Therefore, the decreases in the hepatic content of triglycerides could not be related to anorexia in these

animals.

At this stage of the investigation it therefore appeared that the two different tumour systems studied, produced different effects on their host in terms of mobilisation of fat, or its accumulation. The common denominator however, was that as shown previously in both models, significant decreases in the acetyl CoA content of liver occurred, even though in mice bearing Sarcoma 180, the evidence was that fat was being mobilised.

In order to confirm that anorexia was the major factor in the accumulation of triglycerides in liver of mice bearing TLX-5 lymphoma, and since these animals did not present complete cessation of food intake, the effect of starvation for 48 hours on the triglyceride content of liver, and weight of the epididymal fat pads was examined.

In addition, the effect of a 48 hour fast on these parameters in mice bearing Sarcoma 180 was also studied, as were changes in the hepatic contents of acetyl CoA, CoASH and citrate.

The Effect of Fasting on Fat Mobilisation in Tumour-Bearing Mice

The data in Table 16, shows that when tumour-free control mice were fasted for 48 hours, the mean weight of the epididymal fat pads did not alter significantly, as shown in Figure 23 however marked accumulation of triglycerides in liver occurred.

TABLE 16

THE EFFECT OF FASTING FOR 48 HOURS ON LIVER WEIGHT, AND WEIGHT OF THE EPIDIDYMAL FAT PADS OF CONTROL MICE AND MICE BEARING TLX-5 LYMPHOMA

Conditions	Liver Wt. (g)	Wt. of Epididymal Fat Pads	Fat Pads as % of Body Wt.
Fed Controls	1.51 ± 0.06	0.898 ± 0.117	2.93 ± 0.32
Fasted Controls	1.22 ± 0.08	0.870 ± 0.164	2.95 ± 0.41
P	< 0.02	N.S.	N.S.
Fed Tumour-Bearers	1.62 ± 0.072	1.33 ± 0.16	4.16 ± 0.48
Fasted Tumour-Bearers	1.74 ± 0.12	1.35 ± 0.10	4.32 ± 0.26
P	N.S.	N.S.	N.S.

Six animals were studied in each group.

N.S. = Not Significant.

Results expressed as means $\pm$ S.E.M.

Since liver weight is known to decrease in starved animals (Harrison, 1953) as is also demonstrated in Table 17, the triglyceride content of these livers was also calculated in terms of whole liver content (not shown). However this was still significantly increased when compared to the fed group ($P < 0.01$). In contrast, fasting did not alter the weight of the liver in mice bearing TLX-5 lymphoma (Table 16).

The data in Table 16 also shows that the mean weight of the epididymal fat pads in both the fed and fasted tumour-bearing group had increased by about 50 percent compared to the fed or fasted normal animals. When these data were expressed in terms of a percentage of body weight, the fasted normal animals still maintained a value close to that of the fed controls.

The effect of a 48 hour fast on the triglyceride content of liver of mice bearing TLX-5 lymphoma is shown in Figure 23.

In these animals, fasting induced a significant increase ($P < 0.001$) in the triglyceride content of liver when compared to the fed tumour-bearers. It will be noted however, that in this experiment, the triglyceride content of liver of the fed tumour-bearing animals was decreased when compared to the normal control group. We have no satisfactory explanation for this, but this does not negate the observation that fasting of tumour-bearing animals induces an increase in the triglyceride content of liver. This increase however is by no means comparable to the very marked accumulation of triglycerides that

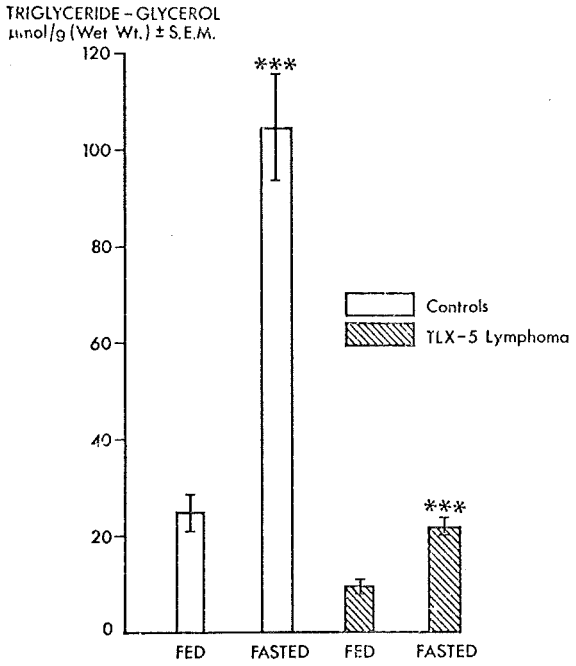


FIGURE 23 The effect of fasting for 48 hours on the triglyceride-glycerol content of liver of normal mice, and mice bearing TLX-5 lymphoma. Six animals were studied in each group.

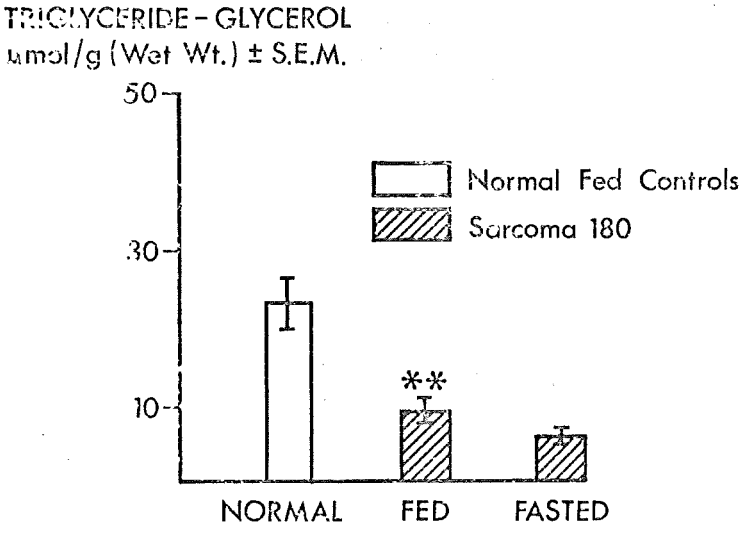


FIGURE 24 The effect of fasting for 48 hours on the triglyceride-glycerol content of liver of mice bearing Sarcoma 180. Six animals were studied in each group.

occurred when control mice were fasted for 48 hours (Figure 23).

When similar studies were made on mice bearing Sarcoma 180, different effects of fasting of these animals were found.

Thus as shown in Table 17, the mean weight of the epididymal fat pads in fed mice bearing Sarcoma 180 was decreased compared to fed controls. This decrease was found to be statistically significant ($P < 0.01$). Although starvation of controls did not produce a significant decrease in the weight of the epididymal fat pads, a further significant decrease occurred ($P < 0.01$) in the starved tumour-bearing group. When the mean weight of the fat pads were expressed as a percentage of body weight, these changes were also significant.

As is also shown in Table 17, there were no significant differences in the mean tumour weight in the fed or fasted mice, and fasting did not significantly alter liver weight in these animals.

The data in Figure 24 show the effect of a 48 hour fast on the triglyceride content of liver of these animals. In agreement with the previous observation, the growth of this tumour induced a significant decrease in the triglycerides content of liver of fed mice.

A further decrease occurred as a result of fasting but this was not significant when compared to the fed tumour-bearing group.

TABLE 17

THE EFFECT OF FASTING FOR 48 HOURS ON LIVER WEIGHT, TUMOUR WEIGHT, AND WEIGHT OF THE EPIDIDYMAL FAT PADS OF NORMAL MICE AND MICE BEARING SARCOMA 180

Conditions	Liver Wt. (g)	Tumour Wt. (g)	Wt. of Fat Pads (g)	Fat Pads as % of Body Wt.
Fed Controls	1.46 ± 0.073	-	0.62 ± 0.077	2.25 ± 0.203
Fasted Controls	1.21 ± 0.061	-	0.41 ± 0.068	1.73 ± 0.231
P	< 0.05	-	N.S.	N.S.
Fed Tumour Bearers	1.42 ± 0.024	4.31 ± 0.36	0.26 ± 0.031	1.00 ± 0.12
Fasted Tumour Bearers	1.29 ± 0.050	3.88 ± 0.21	0.10 ± 0.019	0.41 ± 0.08
P	N.S.	N.S.	< 0.01	< 0.01

Six animals were studied in each group. Tumours were implanted 12 days prior to the experiment.

N.S. = Not Significant. Values given are means $\pm$ S.E.M.

The Effect of Fasting on the Hepatic Content of Acetyl CoA, Free CoA and Citrate in Tumour-Bearing Mice

Acetyl Coenzyme A and CoASH

Figure 25 shows the effect of fasting for 48 hours on the 'total' CoA, CoASH and acetyl CoA content of liver of control CBA mice.

These animals had received 2×10^6 normal spleen cells i.p. from CBA mice seven days prior to the study.

The data clearly shows that fasting induced significant increases in the acetyl CoA content of liver, whereas the free coenzyme A content had decreased significantly ($P < 0.001$).

The 'total' CoA values are also included in Figure 25, but as already noted, in the assay method used, this represents the sum of the acetyl CoA and free CoA content and are included for comparison of the distribution of the two forms of the coenzyme.

Also shown in Figure 25, is the effect of TLX-5 lymphoma on the content of these metabolites in liver, where it will be seen that in agreement with previous studies (Chapter 2), marked decreases in the acetyl CoA and free coenzyme had occurred.

However in fasted mice bearing TLX-5 lymphoma, although the acetyl CoA content of liver increased slightly, and that of the free coenzyme showed a slight decrease, neither of these changes were significant

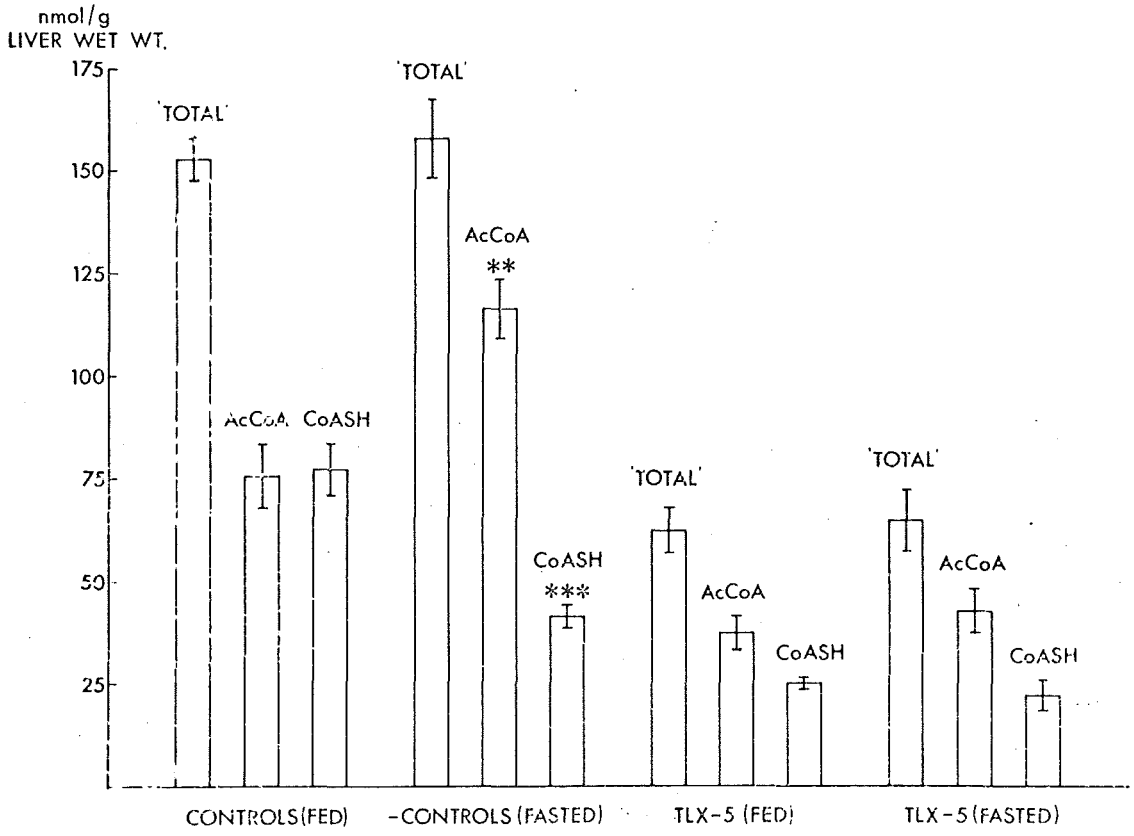


FIGURE 25 The effect of fasting for 48 hours on the hepatic contents of 'Total' CoA, Acetyl CoA and CoASH of normal mice and mice bearing TLX-5 lymphoma. Results are expressed as means $\pm$ S.E.M. Six animals were studied in each group.

when compared to the fed tumour-bearing group.

Fasting of mice bearing TLX-5 lymphoma did not therefore induce an increase in the acetyl CoA content of liver.

Results of a similar study on mice bearing Sarcoma 180 are shown in Figure 26.

These data show that in fed mice bearing Sarcoma 180, there are significant decreases in the acetyl CoA and free coenzyme A content of liver of these animals, when compared to a normal group.

However when mice bearing Sarcoma 180 were fasted for 48 hours prior to these determinations, the acetyl CoA content of liver increased slightly, but not significantly so when compared to the fed tumour-bearing group. Fasting however induced a significant decrease in the free coenzyme A content of liver.

Citrate

Changes in the citrate content of liver of animals used in the above studies are presented in Figures 27 and 28.

The data presented in Figure 27 show the significant fall in the hepatic content of citrate following a 48 hour fast in control mice that had received 2×10^6 spleen cells intraperitoneally. This fall is in agreement with previous reports of the effect of fasting on the citrate content of normal rat liver (Start and Newsholme, 1968; Herrera and Freinkel, 1968). Also shown (Figure 27) is the marked increase in citrate

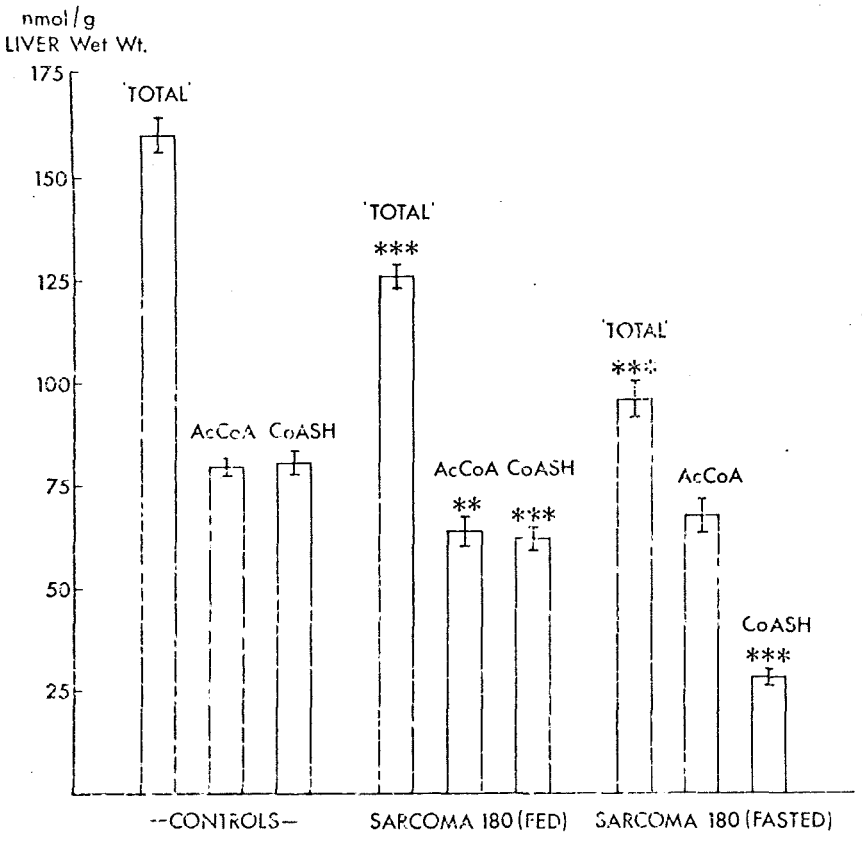


FIGURE 26 The effect of fasting for 48 hours on the hepatic contents of 'Total' CoA, Acetyl CoA and CoASH in mice bearing Sarcoma 180. Values are means $\pm$ S.E.M. Six animals were studied in each group.

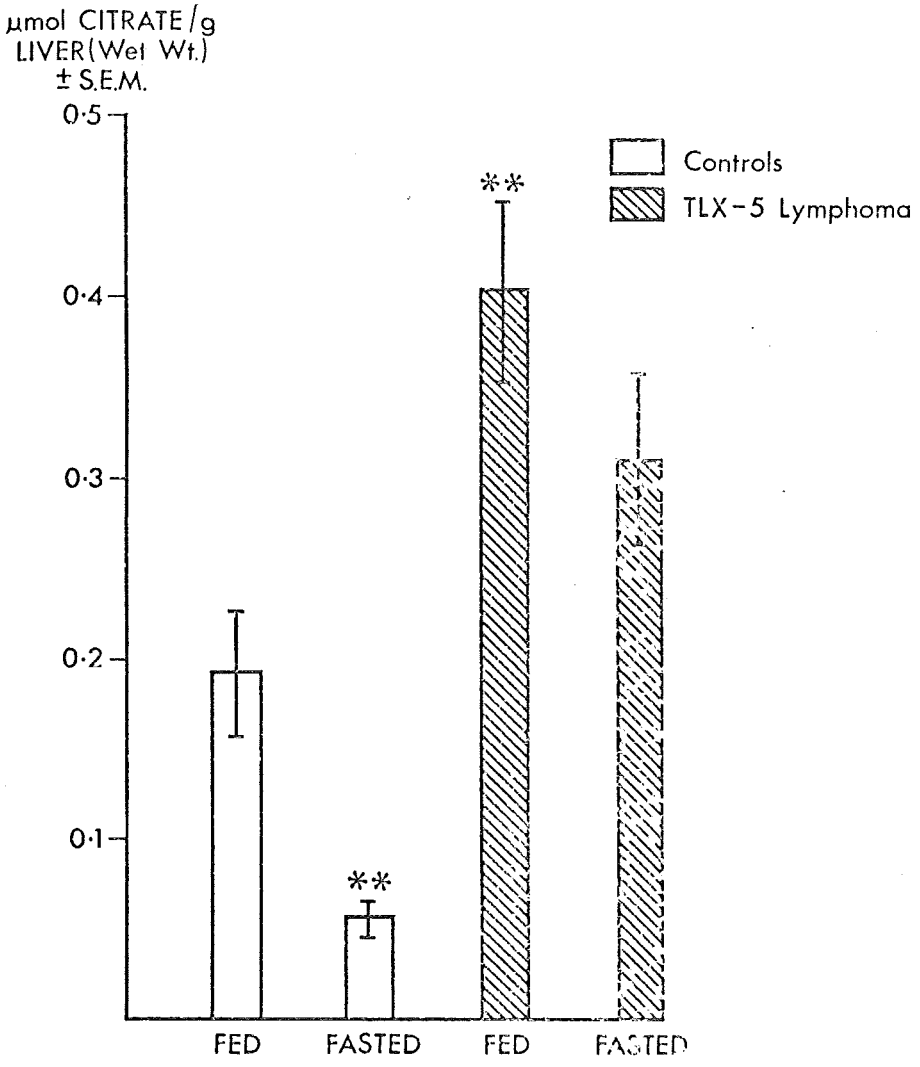


Figure 27 The effect of fasting for 48 hours on the hepatic content of citrate in normal mice and mice bearing TLX-5 lymphoma. Results are the means of six animals in each group.

that occurred in fed mice bearing TLX-5 lymphoma, when compared to fed controls.

However, when mice bearing TLX-5 lymphoma were fasted for 48 hours prior to the determination, although the citrate content of liver decreased compared to the fed tumour-bearing animals, this still remained at a high level but was not statistically significant when compared to the fed tumour bearers.

In contrast, although fed mice bearing Sarcoma 180 showed also an increase in the hepatic content of citrate, in agreement with our previous observations (Chapter 2, Table 5), fasting of a similar group bearing this tumour further increased the citrate content of liver of these animals (Figure 28).

Activity of Citrate-Cleavage Enzyme in Livers of Normal, Fasted and Tumour-Bearing Mice

The activity of the citrate cleavage enzyme in livers of normal mice, and mice that had been starved for 48 hours is shown in Table 18. These data are in agreement with the decreased activity of the enzyme that has been reported (Kornacker and Lowenstein, 1965a) to occur in normal rats starved for the same period.

Also shown are the values for the activity of the enzyme in liver of mice bearing TLX-5 lymphoma. It will be seen that in these animals there was a much greater decrease in activity than that found in starved normal mice. Significant reductions in activity of citrate cleavage enzyme were also found to occur in mice

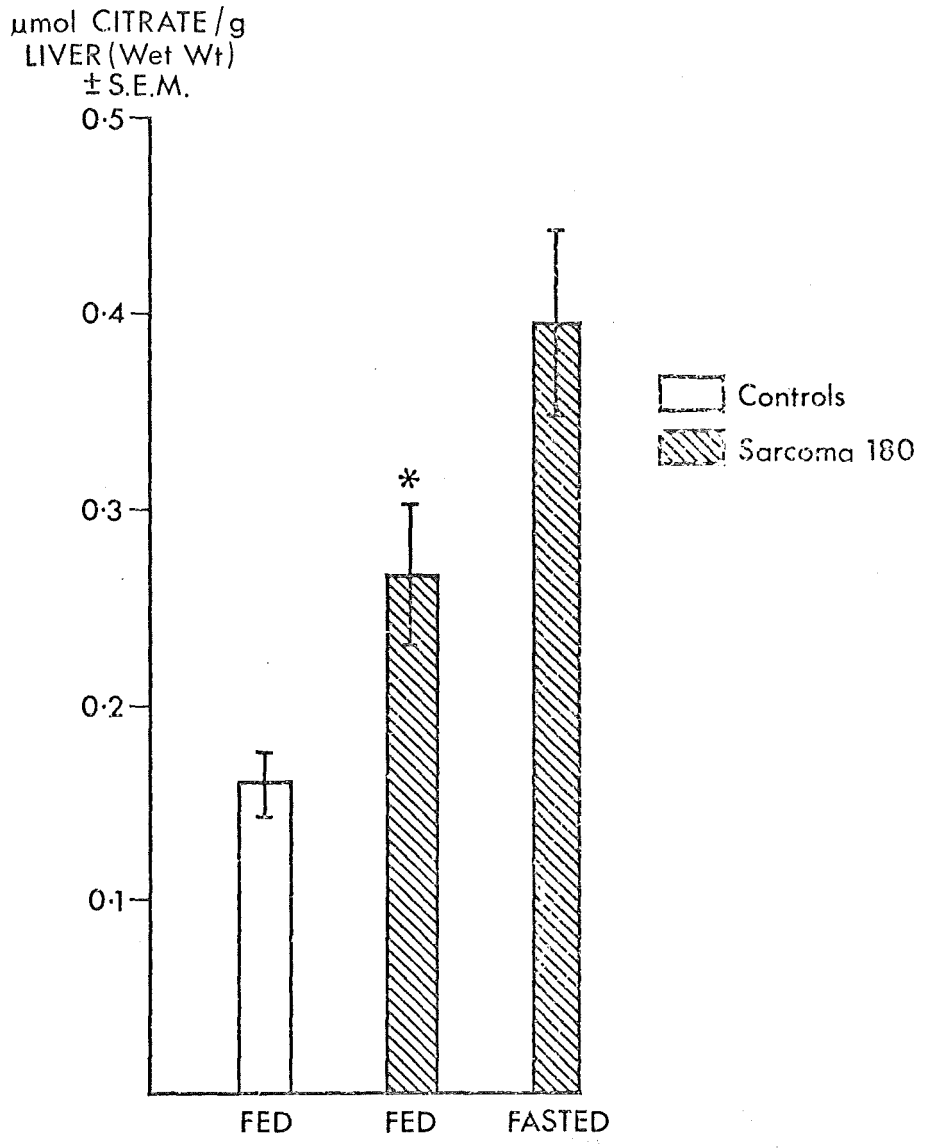


Figure 28 The effect of fasting for 48 hours on the hepatic content of citrate in mice bearing Sarcoma 180. Results are the means of six animals in each group.

TABLE 18

ACTIVITY OF CITRATE CLEAVAGE ENZYME IN THE EXTRAMITOCHONDRIAL SOLUBLE FRACTION OF MOUSE LIVER AT pH 7.5 AND 37°C

Conditions	Enzyme Activity (umol/mg Protein/Hr.)	P
Fed Normals	0.619±0.017 (6)	-
Starved Normals (24 Hr.)	0.451±0.015 (6)	$< 0.001^a$
TLX-5 Lymphoma	0.172±0.021 (6)	$< 0.001^b$
Controls	0.486±0.024 (6)	< 0.01
Sarcoma 180	0.373±0.021 (6)	

Animals bearing TLX-5 lymphoma were studied seven days after tumour implant and those bearing Sarcoma 180, 12 days after tumour implant.

a and b = Statistical comparison with fed normal mice.

Values given are the mean ± S.E.M. Numbers in parenthesis are numbers of mice in each group.

bearing Sarcoma 180.

Ketone Bodies in Urine of Normal and Tumour-Bearing Mice

Fed normal mice were found to contain a trace of ketone bodies in urine, as were fed mice bearing TLX-5 lymphoma or Sarcoma 180, after seven and 12 days of tumour growth respectively.

The Acetyl CoA/CoASH Ratios

As shown in Table 19, the acetyl CoA/CoASH ratio in liver of fed mice bearing Sarcoma 180 was very similar to that found in normal fed mice. Fasting of either normal or mice bearing the Sarcoma increased the ratios significantly. In contrast, fed mice bearing TLX-5 lymphoma showed a significant increase in the acetyl CoA/CoASH ratio when compared to control mice that had received lymphocytes i.p., but fasting of these tumour-bearing animals did not induce a significant increase.

Discussion

Starvation for a period of 48 hours induced 'fatty' livers in normal mice but did not cause significant depletion of epididymal adipose tissue in these animals. These effects are opposite to those found in starved rats, where decreases in the hepatic content of triglycerides (Lippel, 1972) and decreases in the weight of the epididymal fat pads (Herrera and Freinkel, 1968; Ryan, Blackburn and Clowes, 1974) have been reported.

TABLE 19

THE ACETYL CoA/CoASH RATIOS IN LIVER OF FED AND FASTED NORMAL AND TUMOUR-BEARING MICE

Conditions	Acetyl CoA/CoASH
Fed Normal	1.00 ± 0.06 (6)
Fasted	2.11 ± 0.21 (6)[a]
Sarcoma 180 (Fed)	1.04 ± 0.08 (6)[b]
Sarcoma 180 (Fasted)	2.44 ± 0.22 (6)[c]
Fed Controls (L)	1.02 ± 0.16 (6)
Fasted Controls (L)	2.83 ± 0.12 (6)[d]
TLX-5 Lymphoma (Fed)	1.47 ± 0.08 (6)[e]
TLX-5 Lymphoma (Fasted)	2.07 ± 0.31 (6)[f]

a = Significant change ($P < 0.001$) when compared to normal.

b = Not significant when compared to normal fed mice.

c = Significant change ($P < 0.001$) when compared to fed tumour-bearing mice.

d = Significant change ($P < 0.001$) when compared to fed normals that had received lymphocytes i.p. (L).

e = Significant change ($P < 0.05$) when compared to fed normals that had received lymphocytes i.p. (L).

f = Not significant when compared to fed mice bearing TLX-5 lymphoma.

These differences in response to starvation between the two species, may be due to the fact that lipid metabolism is easily perturbed in the mouse. For example, Estller (1974) has shown that 'fatty' livers can be induced in mice in as short a time as two hours following the injection of ethanol. But as has recently been discussed by Hems (1975), little is known of the pathogenesis of 'fatty' livers, although nutritional factors e.g. dietary deficiencies are known to be involved (Tucker and Eckstein, 1937; Williams, Cardle and Meader, 1959; Porta and Hartcroft, 1970).

In an earlier investigation, Adams (1950) studied the effect of starvation on the total lipid content of liver of mice bearing a transplantable lymphosarcoma. He found that starvation induced 'fatty' livers in these animals. In contrast, starvation did not induce the accumulation of lipids in livers of normal mice. Adams (1950) however determined total lipids from the nitrogen and water content of these livers, and his data may therefore be suspect.

In the present studies, there were some discrepancies when the triglyceride content of liver of mice bearing TLX-5 lymphoma were studied, since in one experiment, this decreased significantly whereas in another no changes occurred. We have no explanation for this, but the fact that this tumour invades liver cannot be excluded from the aetiology of these changes.

When mice bearing the lymphoma were starved the hepatic content of triglycerides increased significantly, but only to normal values, whereas in mice bearing

Sarcoma 180 in which the triglyceride content of liver was already decreased starvation induced further decreases.

Data from the present studies also showed that in mice bearing Sarcoma 180, the weight of the epididymal fat pads had decreased significantly, whereas in mice bearing the lymphoma, an almost two-fold increase occurred. When these tumour-bearing mice were starved, this had no effect on the weights of the epididymal fat pads in mice bearing TLX-5 lymphoma, but in mice bearing the Sarcoma, further decreases occurred.

These observations suggested that the two tumour systems studied had different effects on lipid metabolism in the host, a fact that has been previously observed in animals hosting different tumours, where different effects on serum total lipids, and the pattern of lipoproteins have been observed (Liebelt, Liebelt and Johnston, 1971; Cox and Gokcen, 1975).

The reason for the increase in the weight of the epididymal fat pads in mice bearing TLX-5 lymphoma is unclear. The possibility exists that this tumour invades adipose tissue, as has been described in detail in mice bearing MT 890 'ascites' tumour by Siegler and Koprowska (1962). Also hormonal disturbances in these animals may be involved since it is recognised that triglyceride lipase in adipose tissue is regulated by several hormones, both lipolytic and antilipolytic (Siddle and Hales, 1975; Newsholme and Start, 1972; Newsholme and Start, 1973).

Thus stimulation of triglyceride lipase in

adipose tissue by adrenaline, glucagon or growth hormone (Exton et al. 1972) would explain the decrease in triglycerides in liver in mice bearing Sarcoma 180, as well as loss of fat from epididymal adipose tissue. On the other hand, hyperinsulinism could offer an explanation for the increase in weight of the epididymal fat pads in mice bearing TLX-5 lymphoma due to stimulation of triglyceride synthesis (Topping and Mayes, 1972). As was previously reported however (Chapter 3), significant decreases in blood glucose levels were found in mice bearing either the lymphoma or Sarcoma 180, and it is difficult to correlate these observations. As discussed later however, an increase in the level of circulating insulin could offer an explanation for the increase in the hepatic content of citrate in these animals.

A report by Ryan, Blackburn and Clowes (1974) that starvation of rats with experimental peritonitis reverted the depletion of epididymal adipose tissue to normal values, due to increases in serum insulin levels, prompted the question of whether mice bearing TLX-5 lymphoma had become septic, but this was negated by bacteriological examination of the ascitic fluid from these animals.

Changes in Activity of the Citrate-Cleavage Enzyme

The increase in the hepatic content of citrate in mice bearing TLX-5 lymphoma or Sarcoma 180, raised the question as to whether this involved a decrease in citrate cleavage enzyme activity in these livers. As

shown here however, in both models, significant decreases in activity had occurred. Decreases in activity of the enzyme in normal mice following 48 hours starvation were also found, and similar changes have been reported in starved rats (Kornacker and Lowenstein, 1965a), but under these conditions, the hepatic content of citrate decreases (Herrera and Freinkel, 1968; Start and Newsholme, 1968). However, as is well recognised, under conditions of increased fatty acid synthesis, e.g. following re-feeding of starved rats with glucose (Kornacker and Lowenstein, 1965a; Lowenstein, 1968), the activity of the citrate-cleavage enzyme increases (Kornacker and Lowenstein, 1965a), as does the hepatic content of citrate (Newsholme and Start, 1972). But as has been shown by Foster and Srere (1968), the enzyme is not rate-limiting in fatty acid synthesis, since during the first 12 hours following re-feeding of starved rats with glucose, the marked increase in fatty acid synthesis was not accompanied during that period by an increase in the activity of the citrate cleavage enzyme.

Whether the decreased activity of this enzyme in liver of tumour-bearing mice, can be related to the increase in the citrate content remains to be clarified. It is of considerable interest however, that fasting of mice bearing Sarcoma 180 induced a further increase in the hepatic content of this metabolite, and in mice bearing TLX-5 lymphoma, although a decrease occurred under these conditions, this was not significant when compared to fed tumour-bearing animals. Thus fasting of tumour-bearing mice failed to decrease the hepatic

content of this metabolite, as occurs in starved normal mice, and rats under which conditions in normal animals fatty acid synthesis is decreased.

Possible Effect of the Increase in the Hepatic Content of Citrate

Irrespective of the cause of the increase in the hepatic content of citrate in these mice, the possible significance of this increase may be derived from the part played by this metabolite in the regulation of fatty acid synthesis in liver, since it is an activator of acetyl CoA carboxylase (Numa, Ringelman and Lynen, 1965; Goodridge, 1972). As has been shown recently by Newsholme and Start (1972), increases in the hepatic content of citrate occur in starved rats refed either glucose, fructose, casein or leucine, all of which elicit insulin release. These workers have proposed that the physiological significance of the increase in citrate in response to insulin can be explained by the regulation of fatty acid synthesis by this metabolite. Such a proposal would be in accordance with the dependence of fatty acid synthesis on high levels of circulating insulin (Fain, Scow, Urgoiti and Chernick, 1965).

In Table 20 we have compared the data of Newsholme and Start (1972) with the findings here reported on the hepatic content of citrate in fed and fasted tumour-bearing mice. As can be seen, the hepatic content of this metabolite in fed and fasted tumour-bearing mice are in the main very close to those found by Newsholme and Start (1972) in fasted rats refed the different diets.

TABLE 20

CHANGES IN THE HEPATIC CONTENT OF CITRATE IN ANIMALS UNDER DIFFERENT METABOLIC CONDITIONS

Conditions	Hepatic Content µmol/g Liver Wt. Weight	Source
Rat (Fed)	0.262±0.020 (6)	Start and Newsholme (1968)
Rat (Starved 48 hrs)	0.136±0.008 (4)	
Mouse (Fed)	0.160±0.017 (6)	Figure 9
Mouse (Fed)	0.191±0.034 (6)	Figure 27
Mouse (Starved 48 hrs)	0.055±0.009 (6)	Figure 27
Rat (Starved 48 hrs)	0.144±0.006 (13)	Newsholme and Start (1972)
Rat (Starved and refed fructose for 60 minutes)	0.365±0.039 (5)	
Rat (Starved and refed glucose for 60 minutes)	0.390±0.023	
Rat (Starved and refed leucine)	0.370± -	
Fed Mouse (TLX-5 lymphoma)	0.401±0.049 (6)	Figure 27
Mouse (TLX-5 lymphoma) fasted 48 hrs.	0.309±0.047 (6)	
Fed Mouse (Sarcoma 180)	0.267±0.036 (5)	Figure 28
Mouse (Sarcoma 180) fasted for 48 hrs.	0.397±0.04 (5)	

Figures in parentheses are numbers of animals used.

Results are expressed as Means ± S.E.M.

Thus in tumour-bearing mice, the increase in the hepatic content of citrate would appear to be of sufficient magnitude to stimulate fatty acid synthesis in liver. In these animals however, this mechanism would appear to be continuing in an uncontrolled manner, since in contrast to the situation in normal starved animals, the hepatic content of citrate does not fall significantly, and in fact as was shown here in mice bearing Sarcoma 180, further increases occurred under conditions of starvation.

Effect of Fasting on the Hepatic Contents of Acetyl CoA, and CoASH in Tumour-Bearing Mice

A rather similar situation occurred when tumour-bearing mice were starved, in that the hepatic content of acetyl CoA did not increase in the direction expected. However in mice bearing Sarcoma 180, starvation induced a further decrease in the hepatic content of the free coenzyme. This was not found in mice bearing the lymphoma, under conditions of fasting. Although in mice bearing Sarcoma 180 there was evidence that fat was being mobilised, and that further loss of fat from the epididymal adipose tissue occurred when the animals were fasted, this did not induce an increase in the hepatic contents of acetyl CoA, which would be expected, providing of course that the increased flux of fatty acids was shunted into the oxidative pathway. As discussed below however, we found no evidence of ketonuria in fed mice bearing the sarcoma.

Other workers have studied the effect of starvation

on the control of fatty acid synthesis liver of rats hosting Hepatoma 7777 (Halperin, Taylor, Cheema-Dhadli, Morris and Fritz, 1975). These studies showed that starvation induced increases in the hepatic content of long-chain acyl CoA, as is also found in normal rats under conditions of starvation (Tubbs and Garland, 1964). The rate of lipogenesis in these tumour-bearing rats was also decreased, as was the percentage of pyruvate dehydrogenase present in the active form. Thus the response of host liver in these animals to starvation was similar to that found in starved tumour-free animals (Tepperman and Tepperman, 1970; Wieland, Patzelt and Loffler, 1972).

Unfortunately we have no information on the hepatic contents of long-chain acyl CoA compounds in livers of tumour-bearing mice, and whether these would increase under conditions of starvation.

Changes in the Acetyl CoA/CoASH Ratios

The acetyl CoA/CoASH ratios in liver of fed mice bearing Sarcoma180 were not significantly altered when compared to normal fed mice. Starvation of these tumour-bearing animals increased the ratio to that found in starved normal mice. In contrast, in fed mice, the acetyl CoA/CoASH ratio was slightly increased compared to normal ($P \leq 0.05$), but when these animals were starved significant changes in the ratio did not occur.

It should be noted however, that in contrast to that found in starved tumour-free animals, in which

the increased ratio is due to increases in acetyl CoA and a fall in the free coenzyme A content, in tumour-bearing mice, changes in the ratio are largely dependent on a fall in CoASH, since the hepatic contents of acetyl CoA in these animals did not increase significantly under conditions of fasting. Thus the use of these ratios in attempts to interpret possible effects on metabolism in these livers, requires that these reservations are taken into consideration. Thus, although an increased ratio, as was found in fed mice bearing TLX-5 lymphoma would be expected to indicate increased fatty acid oxidation in liver, we found no evidence of ketonuria in these animals. It is of interest that according to a previous report (Haven, Bloor and Randall, 1951), there was no evidence of ketosis in rats bearing Walker 256 carcinoma even though rapid mobilisation of body fat was in evidence. However it has been shown that free fatty acids can be elevated in vivo without increases in ketone synthesis (Seyffert and Madison, 1967). Thus an increased flux of fatty acids to liver is not sufficient in itself to induce ketosis (For review see McGarry and Foster, 1972). Clearly much further information is required as to the levels of free fatty acids in blood of these tumour-bearing mice.

CHAPTER 5

THE EFFECT OF CURATIVE RESECTION OF A SUBCUTANEOUS MAMMARY TUMOUR ON THE HEPATIC CONTENT OF ACETYL CoA, CITRATE, PYRUVATE α-OXOGLUTARATE AND CoASH IN THE MOUSE

Introduction

In view of the changes that were found in the hepatic contents of acetyl CoA, CoASH and citrate in livers of mice bearing a subcutaneous C_3H mammary tumour curative resection of this tumour was studied in order to establish whether this reverted the changes in these metabolites to normal values.

Part of this study has been presented elsewhere (McAllister, Soukop and Calman, 1977).

Methods

Weight-matched C_3H male mice were used throughout. These were three to four months old at the start of the experiments.

Five groups of mice were studied, these were:

(1) Normal C_3H male mice.

(2) Mice bearing the C_3H mammary tumour, implanted subcutaneously as described previously (Chapter 1).

(3) Tumour-bearing mice in which a curative resection had been carried out.

(4) Sham-operated group of normal mice.

(5) Mice in which the tumour had recurred following their operation.

Details

It was considered essential that the studies were carried out at the same period of tumour growth, and that the post-operative period was long enough to allow for recurrence of the tumour.

The studies were therefore carried out as follows:

Tumour-bearing mice were sacrificed 17 days after tumour implant, at which stage the mean weight of the tumour was 0.55 g $\pm$ S.E.M. 0.11 g. Curative resection was carried out on another group in which the tumour had also been implanted 17 days earlier. After a further period of 17 days, these animals were sacrificed and the estimations carried out on freeze-clamped livers as described previously. In the operated group, the recurrence rate of tumour growth was about 50 percent, and this was attributed to tumour cells being left in the needle-track at the time of inoculation. Another group of normal mice had a sham-operation, and then were sacrificed 17 days later.

Surgical Resection of Tumours

Mice were anaesthetised with ether and the tumours resected by Professor K C Calman. The wound was then sutured and sprayed with Nobecutane. The same procedure was carried out on the sham-operated group of normal mice.

Estimations

The acetyl CoA, CoASH, citrate, pyruvate and α-oxoglutarate were determined on freeze-clamped livers.

The mean tumour weights, spleen and thymus weights were also recorded.

At autopsy on tumour-bearing mice that had had surgical resection of their tumours, a careful examination was made to ascertain whether the tumours had recurred.

Results

The mean weight of thymus in the tumour-bearing group did not alter significantly when compared to normal controls, (Table 21) but spleen weights showed significant increases ($P < 0.01$). As is also shown in Table 21, a curative resection of the tumour reverted spleen weights to normal values. It will also be seen however that where the tumour recurred following resection, increases in the mean weight of spleen again occurred, and this was statistically significant ($P < 0.001$) when compared to the group in which the surgical resection was successful.

In Figure 29 are shown the hepatic content of acetyl CoA of normal mice, those bearing the mammary tumour, and of mice in which the tumour had been successfully removed or where the tumour had recurred. Values are also shown for the sham-operated group. The hepatic content of this metabolite was significantly

TABLE 21

EFFECT OF CURATIVE RESECTION OF A C_3H MAMMARY TUMOUR ON SPLEEN AND THYMUS WEIGHTS IN THE MOUSE

Group	Thymus Wt. (mg)	Spleen Wt. (mg)
Normal Controls	30.4 ± 2.16 (6)	124 ± 10.99 (6)
Tumour Bearers	28.51 ± 3.25 (6)[a]	240.45 ± 28.77 (6)[b]
Curative Resection	25.13 ± 0.44 (6)[c]	142.26 ± 7.64 (6)[d]
Unsuccessful Resection	19.80 ± 2.73 (6)[e]	210.51 ± 12.76 (6)[f]
Sham-Operation	23.56 ± 1.13 (6)[g]	141.11 ± 4.32 (6)[h]

a = Not significant when compared to normal controls.

b = Significant ($P < 0.01$) when compared to normal controls.

c = Not significant when compared to tumour-bearers.

d = Significant fall in spleen weight when compared to tumour-bearers.

e = Not significant when compared to curative resection group.

f = Significant increase in spleen weight when compared to curative resection group.

g = Significant decrease when compared to normal controls.

h = Not significant when compared to normal controls.

Figures in parentheses are numbers of animals in each group.

Values in terms of means $\pm$ S.E.M.

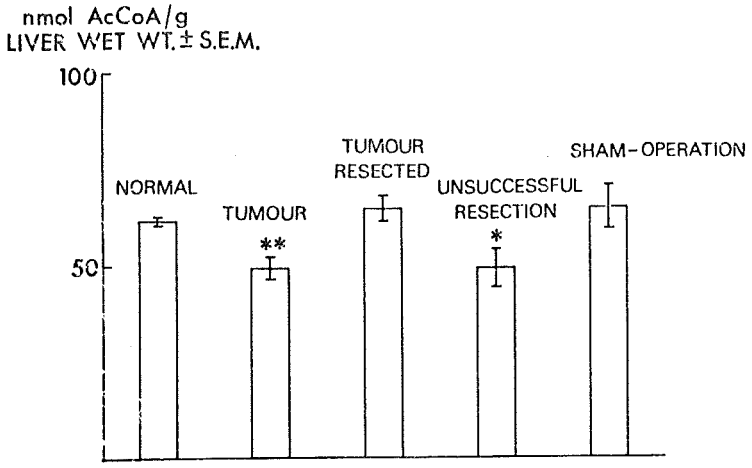


FIGURE 29 The effect of curative resection of a C_3H transplantable mammary tumour on the hepatic contents of acetyl CoA in the mouse. Results are expressed as means. Six animals were studied in each group.

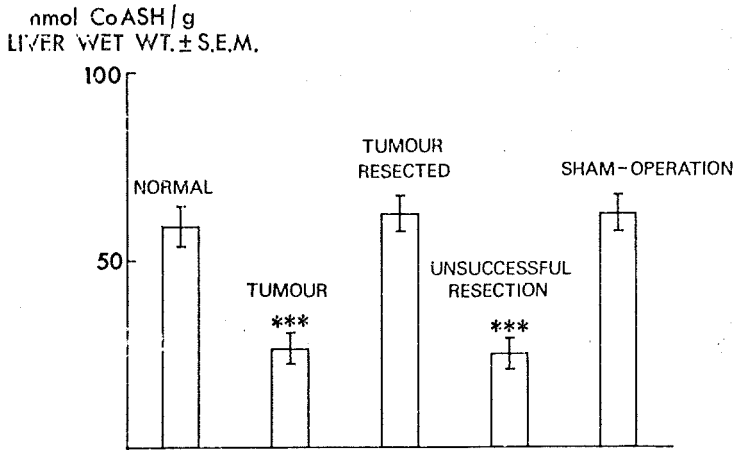


FIGURE 30 The effect of curative resection of the tumour on the hepatic content of CoASH. Results are expressed as means. Six animals were studied in each group.

depressed in mice bearing the mammary tumour (unoperated group) when compared to normals, confirming the previous observation.

Curative resection of the tumour however had reverted the hepatic content of acetyl CoA to normal values 17 days after the operation. Where however the tumour had recurred, the hepatic content of this metabolite again decreased significantly. It will also be seen that 17 days after a sham-operation on normal mice, the acetyl CoA content of liver was found to be at normal levels.

A similar picture is seen in Figure 30 where the hepatic contents of free coenzyme A were studied in the five groups of mice. Again tumour growth induced a significant fall in CoASH, whereas curative resection reverted this to normal values. The hepatic content of the free coenzyme however again fell significantly in those mice in which the tumour recurred. A sham operation on normal mice had no effect on the content of the free coenzyme in liver.

Although it was shown previously that starvation in normal mice induced significant decreases in the hepatic content of free coenzyme A in liver, the decreases in this metabolite reported here could not be attributed to anorexia, since none of the groups of mice studied showed any ill effects of either their operation, or even in those bearing tumours, and food intake remained normal.

Table 22 shows the hepatic content of citrate, pyruvate and α-oxoglutarate in the five groups of

TABLE 22

THE HEPATIC CONTENTS OF CITRATE PYRUVATE AND α-OXOGLUTARATE OF MICE FOLLOWING CURATIVE RESECTION OF A C_3H MAMMARY TUMOUR

Group	Citrate μmol/g Liver	Pyruvate μmol/g Liver	α-Oxoglutarate μmol/g Liver
Normal Controls	0.313±0.028 (6)	0.128±0.024 (6)	0.096±0.020 (6)
Tumour-Bearers	0.329±0.032 (6)	0.117±0.014 (6)	0.097±0.016 (6)
Curative Resection	0.189±0.016 (6)[a]	0.144±0.024 (6)	0.128±0.014 (6)
Unsuccessful Resection	0.264±0.015 (6)	0.101±0.006 (6)	0.091±0.012 (6)
Sham-Operation	0.138±0.013 (6)[a]	0.160±0.016 (6)	0.129±0.015 (6)

Values given are the mean ± S.E.M. Figures in parentheses are the number of animals in each group.

a = Significant fall in citrate ($P < 0.01$) when compared to normal controls.

mice studied. As will be seen, the hepatic content of citrate of mice bearing the tumour did not alter significantly from normal values, nor did the level of pyruvate or α-oxoglutarate. Previously it was shown that content of the latter two metabolites in liver of mice bearing this tumour did not alter, but it was also shown in an earlier Chapter in this thesis, that the citrate content increased significantly. It was stressed therein however, that this was found to occur in mice bearing very small C_3H mammary tumours, and that it appeared that this effect, as well as causing decreases in the acetyl CoA content of liver appeared to tail off as the tumour became larger. In the present studies, the mean weight of this tumour in the unoperated group was 0.55 g $\pm$ S.E.M. 0.11 g and 0.51 g $\pm$ S.E.M. 0.03 g in those in which curative resection was carried out. In the group in which the tumour had recurred 17 days following the operation, the mean weight was very close to those in the other groups, being 0.53 g $\pm$ S.E.M. 0.017 g.

As is also shown in Table 22, curative resection in fact decreased the citrate content of liver significantly ($P<0.01$) when compared to normal controls, but this procedure did not alter either the pyruvate or α-oxoglutarate content. It is of interest that the sham-operated group also showed significant decreases in the citrate content of liver, but again the pyruvate or α-oxoglutarate did not change. We have no adequate explanation for these changes in citrate content which would appear from

the evidence to be due to some effect of the operation.

In Figure 31 are shown the mean weight of the epididymal fat pads in these animals. In the tumour-bearing group, and in those in which the tumour had recurred significant increases were found ($P < 0.02$ and < 0.05 respectively). Successful resection of the tumour reverted this effect of the tumour to normal values, and a sham-operation on normal mice did not alter the mean weight of the fat pads from normal values.

DISCUSSION

The data presented show that the changes previously found in the hepatic contents of acetyl CoA, and CoASH in mice bearing a transplantable mammary tumour can revert to normal following curative resection. These changes again occurred when the resection was unsuccessful, adding further support to the concept that the changes induced by the tumour in the content of these metabolites in liver are effected by the release of some product or products from the tumour cells.

Other reports also show that some of the systemic effects of tumour growth in small animals can be reverted to normal following curative resection. Thus Sibley and Lehninger (1949) have shown that the increased activity of aldolase in serum of rats bearing Sarcoma 256, decreases to normal values following removal of the tumour. More recently Shirasaka and Fujii (1975) have shown that the activity

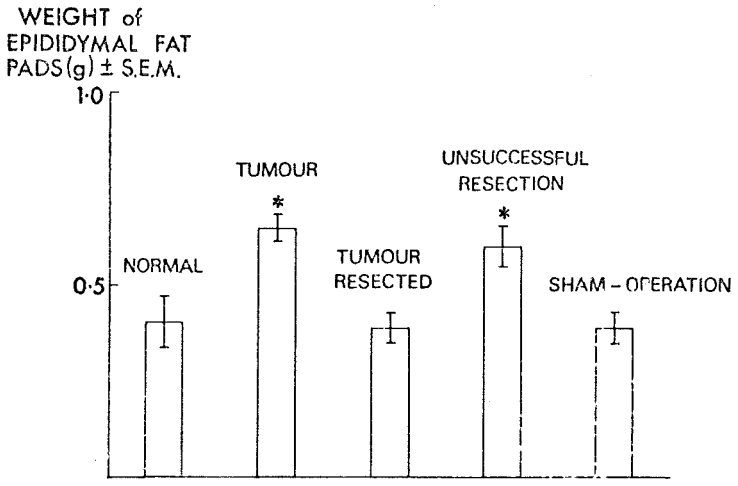


FIGURE 31 The effect of curative resection of the tumour on the weight of the epididymal fat pads in the mouse. Results are expressed as means. Six animals were studied in each group.

of thymidine kinase is increased in liver and serum of rats bearing Yoshida sarcoma (solid type). They also showed that these changes rapidly reverted to normal when the tumour was removed, with a decrease in about half the activity occurring seven hours following the resection.

It has also been reported that the leukocytosis of mice bearing a transplantable mammary tumour regresses following removal of the tumour (Liebelt, Gehring, Delmonte, Schuster and Liebelt, 1974).

In an effort to establish whether the changes in the hepatic contents of different metabolites in liver that were found in tumour-bearing mice were in fact due to products released from tumour cells, an investigation working on these lines was next carried out, and this is the subject of the next Chapter.

CHAPTER 6

METABOLIC CHANGES IN LIVER OF NORMAL MICE INDUCED BY INJECTION OF A CELL-FREE PREPARATION OF TLX-5 LYMPHOMA CELLS OR NON-VIABLE TUMOUR CELLS

Introduction

The previous observations that decreases in the hepatic contents of acetyl CoA, and CoASH, and increases in the weight of the epididymal fat pads and spleen in mice bearing a C_3H mammary tumour could be reverted to normal following curative resection of the tumour, suggested that a product or products of the tumour cells might be involved in the aetiology of these changes.

This chapter presents evidence that changes in the hepatic contents of acetyl CoA free coenzyme A and citrate, similar to those found in tumour-bearing mice, can be induced in normal mice following the injection of a cell-free preparation of TLX-5 lymphoma cells, and in some instances by the injection of the non-viable tumour cells.

Part of these studies have been presented elsewhere (McAllister, Soukop and Calman, 1977).

Background

Much attention has been paid to the possibility that products of tumour cells may be involved in some of the systemic effects of a tumour on its host, but

the existence of such factors in vivo has been difficult to prove.

The best known product that has been implicated in some of these effects is the so-called toxohormone, that was first isolated from human cancers by Nakahara and Fukuoka in 1948.

Toxohormone

Interest in this product arose from the observation of Nakahara and Fukuoka (1949), that injection of normal mice with toxohormone, caused marked decreases in the catalase activity in liver of these animals, and therefore simulated an effect that had previously been reported to occur in liver of tumour-bearing animals (Greenstein, Jenrette and White, 1941).

Later it was shown that treatment of normal rats with toxohormone, also induced depression in plasma iron, thymic atrophy, and increases in spleen, liver and adrenal weights in these animals (Kampschmidt, Adams and McCoy, 1959).

Various workers have shown that toxohormone can be extracted from several different tumours in animals (Nakahara and Fukuoka, 1958; Okuda, Ikegami and Fujii, 1972). It has also been purified and its chemical composition determined (Ohashi and Ono, 1959; Olivares, 1970; Okuda, Ikegami and Fujii, 1972). These reports suggest that the material is a peptide of low molecular weight.*

However according to Okuda, Ikegami and Fujii

* The polypeptide is made up of 30 to 40 amino acids with a high content of glutamate, glycine and aspartate (Olivares, 1970).

(1972), toxohormone is not a single substance, but a mixture consisting of various biologically active factors that include a catalase-depressing factor as well as one that depresses serum iron *in vivo*. They showed that toxohormone obtained from Rhodamine sarcoma in the rat would depress the plasma iron levels in normal rats, but not the activity of catalase in liver of these animals.

Several doubts as to whether toxohormone is a tumour-specific product arose by the finding that this material could be extracted in small amounts from various normal tissues (Greenfield and Meister, 1951; Day, Gabrielson and Lipkind, 1954) or from autolysates of normal tissues (Olivares, Callao and Montoya, 1967). According to Olivares (1970), however, the small amount of toxohormone that is found in normal tissues may be due to autolysis *in vivo*.

Kampschmidt and Schultz (1963) consider that toxohormone is not produced by cancer cells, but more probably from bacteria present in many tumours. This view has been refuted by Nixon and Zinman (1966), and more recently by Olivares (1970).

The major objection to the concept of toxohormone being the active factor in causing depressions of plasma iron, and catalase activity in liver is that these changes are not maintained following repeated injection of the material into normal animals which apparently develop a tolerance to it (Kampschmidt, Adams and McCoy, 1954; Fukuda, Okada, Akikawa, Matsuda and Urushizaki, 1966).

Also although catalase activity is well-recognised to be depressed in livers of tumour-bearing animals (Thompson and Klipfel, 1958; Kampschmidt, 1965), the biological significance of this effect is obscure, and further it is not specific to the presence of a tumour, since the activity of the enzyme is also decreased in starved tumour-free animals (Miller, 1948) as well as following the injection of some drugs (Mochizuki, Itabashi and Tsukada, 1972) into normal animals.

There is however still current interest in the toxohormone problem (Olivares, 1970; Kampschmidt, 1970; Ikuda, Ikegami and Fujii, 1972; Goodlad and Raymond 1973), and some authorities consider that the presence of a circulating toxin may still offer the best explanation for some of the systemic effects of a growing tumour (Kampschmidt, 1970).

However products of tumour necrosis (Bondy, 1972) or products of autolysis, either from tumour cells or those of the surrounding area, cannot be excluded from the aetiology of some of these effects (Olivares, 1970).

There is also considerable evidence that tumours secrete other biologically active products. These include immunosuppressive material that are found in the body fluids of cancer patients (Field and Caspary, 1972; Glasgow et al. 1974), as well as tumour-bearing animals (Chan and Sinclair, 1973; Yamazaki, Nitta and Umezawa, 1973), as well as material that show biological activity against a variety of cell types (Smith,

Bausher and Adler, 1970; Round, 1970).

Experimental Approach

The experiments were designed to examine whether the changes previously observed in the hepatic contents of metabolites in liver of tumour-bearing mice, could be due to factors either in tumour cells *per se*, or released from tumour cells *in vivo*.

In order however to avoid the possibility of the formation of artefacts that might have arisen during the preparation of extracts of solid tumours such as Sarcoma 180, it was decided to use the ascitic fluid from mice bearing TLX-5 lymphoma, since this offered the closest approximation to conditions *in vivo*.

Materials and Methods

Weight-matched three to four month old, male CBA mice were used throughout the study.

(a) Preparation of Cell-Free Supernatants of TLX-5 Lymphoma

TLX-5 lymphoma cells were harvested from mice that had been injected intraperitoneally with 2×10^6 TLX-5 lymphoma cells seven days earlier.

Aliquots of the ascitic fluid were then well-mixed and the number of tumour cells counted as described previously, then further diluted with Hank's Balanced Salt Medium (Flow Laboratories) to give 2×10^6 tumour cells / 0.5 cm^3.

The cell suspension was then subjected to high speed centrifugation for two hours at 100,000 g at a temperature of $4^{o}C$. This gave a preparation from which all tumour cells and sub-cellular organelles had been removed.

The supernatant was then carefully removed and 0.5 cm^3 injected into groups of mice.

(b) Non-Viable Tumour Cell Suspensions

TLX-5 lymphoma cells were harvested from mice as described above.

The ascitic fluid containing the tumour cells was then mixed with about 5 cm^3 of Hank's Balanced Salt Medium, then centrifuged at 2,700 g for 15 minutes. The supernatant was then removed, the cells resuspended in the same volume of the medium, then recentrifuged under the same conditions. This washing process was carried out for a total of three times.

The washed lymphoma cells were then diluted with the same medium, and the number of cells counted as described previously, then diluted to give a cell population of 2×10^6 / 0.5 cm^3 of Hank's Balanced Salt Medium.

The preparation was then well mixed, and frozen in liquid nitrogen for a few minutes. It was then thawed out, and refrozen.

The freezing and thawing process was conducted for a total of 10 times, then after bringing it to

room temperature, 0.5 cm^3 was injected intraperitoneally into groups of mice.

(c) Control Groups

Three different control groups were used:

1. Normal CBA mouse spleen cells were separated as described previously then counted and diluted with Hank's Balanced Salt Medium to give a cell population of 2 x 10^6 / 0.5 cm^3 of the medium. This was subjected to high speed centrifugation exactly as described above, then 0.5 cm^3 of the supernatant injected intraperitoneally into mice.

2. Normal mice received 2 x 10^6 TLX-5 lymphoma cells according to the standard procedure, that is the cells were harvested from mice bearing the tumour, counted and diluted without further prior treatment.

3. TLX-5 lymphoma cells were harvested as before, then mixed with approximately 5 cm^3 of Hank's Balanced Salt Medium. After centrifugation at 2,700 g for 10 minutes, the supernatant was removed, and the cells resuspended in about 5 cm^3 of the medium. After again centrifuging, the supernatant was removed, and the washing process repeated. The supernatant was again removed and discarded, then the cells were counted and diluted to give a cell population of 2 x 10^6 / 0.5 cm^3 and this was injected i.p. into mice.

In summary therefore, five different groups of

animals were studied. These had received:

1. High-speed supernatants from TLX-5 lymphoma cells.
2. Non-viable washed TLX-5 lymphoma cells.
3. Washed live TLX-5 lymphoma cells.
4. High-speed supernatants of normal spleen cells.
5. Untreated viable TLX-5 lymphoma cells.

Metabolites Studied

The hepatic contents of acetyl CoA, CoASH and citrate were determined on freeze-clamped livers as described previously.

During the freeze-clamping procedure, a small piece of liver was left *in situ*, and after the main portion had been clamped, this remaining portion was used for the determination of triglycerides.

Other Determinations

The liver, spleen, and thymus weights were also recorded.

RESULTS

Notes

1. For purposes of clarity, the statistical significance of the data presented in the composite graphs could not be included. However, these are given in Tables adjacent to the Figures.
2. The injection of normal mice with the high-speed supernatant of normal CBA mice spleen cells had no

effect on any of the parameters studied, with the exception that thymus weights increased significantly on the first and third day following the injection. These data are therefore not included.

Mice that had received either the cell-free preparation of TLX-5 lymphoma cells, or the non-viable tumour cells showed no ill-effects of this treatment, and no changes were found in the weight of liver of these animals. In both groups, observations were carried out for a period of 21 days following the injections in order to allow sufficient time for growth of the tumour to occur due to possible contamination of the preparation with viable tumour cells. At the end of this time, the animals were examined carefully at autopsy for any evidence of tumour growth.

The data presented in Figure 32 show the daily changes in the hepatic contents of triglycerides following the i.p. injection of either TLX-5 lymphoma cells, or the cell-free preparation of the tumour cells. In both groups, the triglyceride content in liver increased in parallel, reaching a peak on the second day following the injections, then fell again in both models on the third day. There was a slight increase again in those bearing the tumour, as well as in those that had received the cell-free preparation, on the third to fifth day. The latter group however showed a further marked increase, which reached a peak on the sixth day, and thereafter decreased (day 10). This was followed by an increase on the 15th day following the injection.

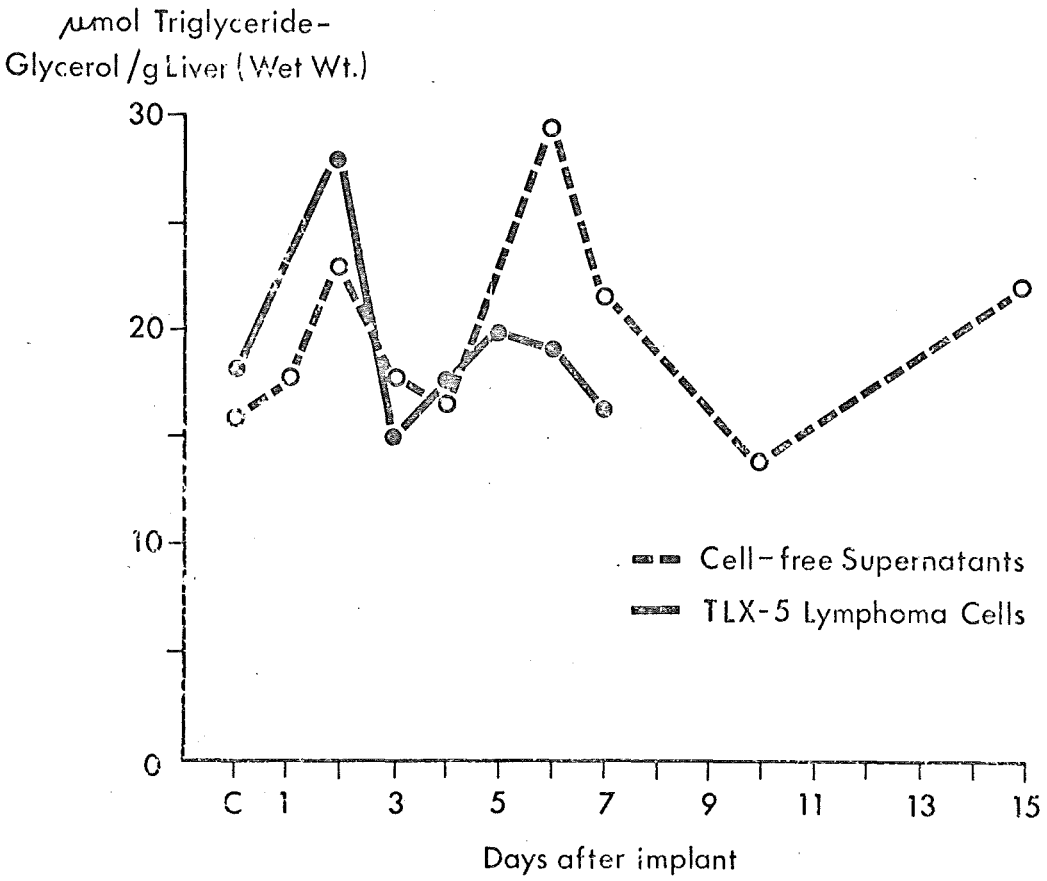


FIGURE 32 Daily changes in the triglyceride content of liver of mice following the i.p. injection of TLX-5 lymphoma cells, or the cell-free preparation of the lymphoma cells. Results are expressed as means. Six animals were used for each time interval.

Statistical evaluation of the results however showed that changes in the hepatic content of triglycerides only reached significance ($P < .02$) on the sixth day following the injection of the cell-free preparation. No significant changes occurred at any other time, nor in those bearing the tumour.

Changes in Thymus Weights (Figure 33)

The composite graph shown in Figure 33 presents the changes in the mean thymus weights of the four models studied. In each of these, thymus weights showed increases on the first day following the injection, thereafter progressive decreases occurred which paralleled each other until the sixth or seventh day, at which period the experiments on mice bearing the tumour were terminated.

After the fifth day following the injection of dead tumour cells, thymus weights in these animals increased and by the 21st day had returned to normal values. Mice that had received the cell-free tumour cell preparation also showed an increase in thymus weight from the sixth to the 10th day, but in contrast to those that had received the dead tumour cells, thymus weights in this group thereafter showed a further fall as determined on the 21st day.

Changes in Spleen Weights (Figure 34)

Spleen weights in mice that had received either the dead tumour cells, or the cell-free preparation of the tumour cells increased in parallel with those

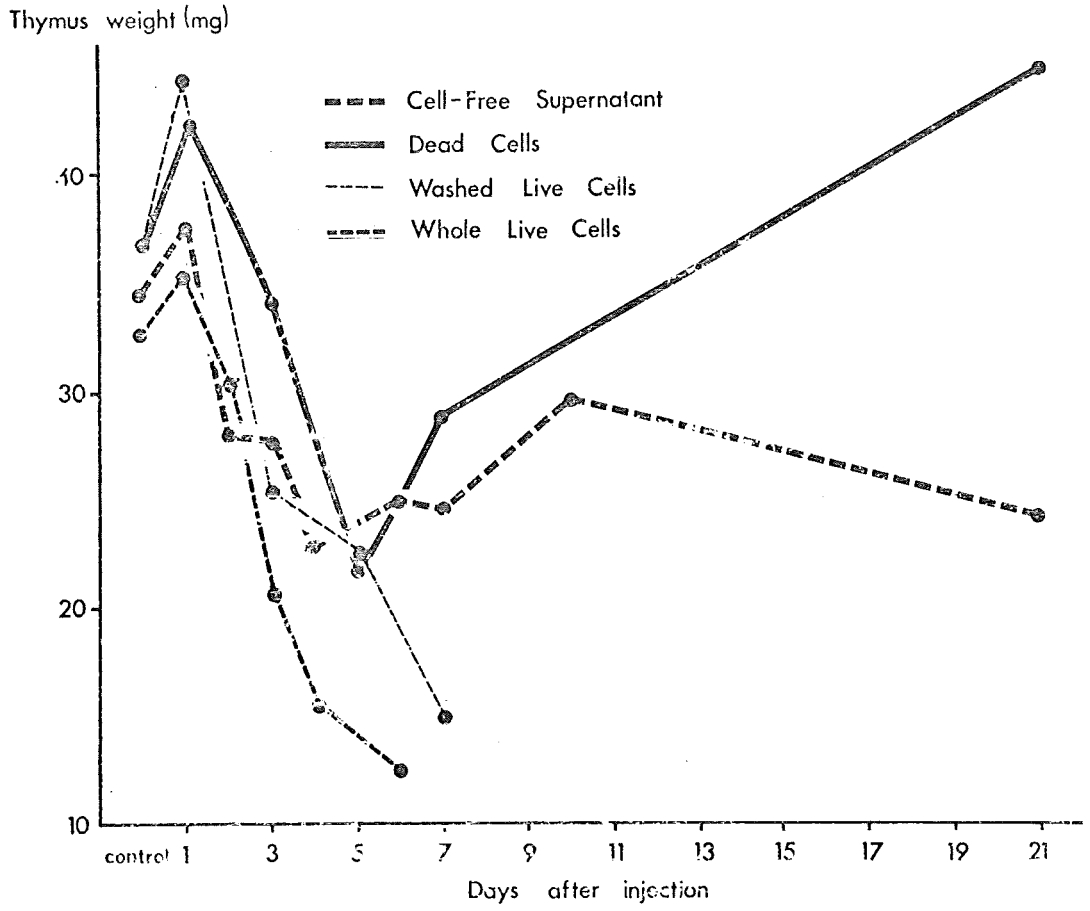


FIGURE 33 Daily changes in thymus weight in the four models studied. Results are expressed as means. Six animals were used for each time interval.

TABLE 23

STATISTICAL EVALUATION OF DATA PRESENTED IN FIGURE 33

EFFECT ON THYMUS WEIGHTS

Day	Cell-free Supernatant	Dead Cells	Washed Live Cells	'Whole' Live Cells
	P	P	P	P
1	N.S.	N.S.	N.S.	N.S.
2	N.S.	-	-	N.S.
3	N.S.	N.S.	< 0.001	N.S.
4	< 0.02	-	-	< 0.05
5	-	< 0.001	< 0.01	-
6	< 0.05	-	-	< 0.01
7	< 0.01	N.S.	< 0.001	-
10	N.S.	-	-	-
21	< 0.02	N.S.	-	-

N.S. = Not significant when compared to control group.

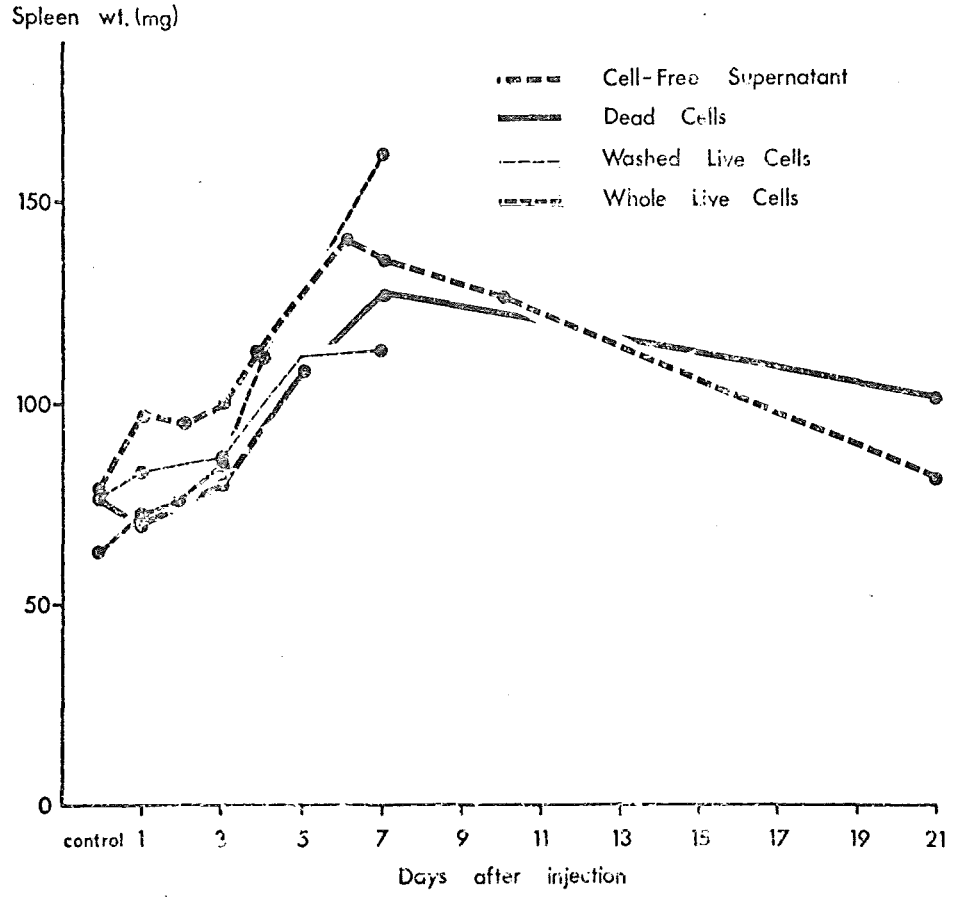


FIGURE 34 Daily changes in spleen weight in the four models studied. Results are expressed as means. Six animals were used for each time interval.

TABLE 24

STATISTICAL EVALUATION OF DATA PRESENTED IN FIGURE 34

EFFECT ON SPLEEN WEIGHTS

Day	Cell-free Supernatant	Dead Cells	Washed Live Cells	'Whole' Live Cells
	P	P	P	P
1	< 0.02	N.S.	N.S.	N.S.
2	< 0.01	-	-	< 0.02
3	< 0.02	N.S.	N.S.	< 0.01
4	< 0.001	-	-	< 0.001
5	-	< 0.01	< 0.001	-
6	< 0.001	-	-	-
7	< 0.001	< 0.001	< 0.001	< 0.001
10	< 0.001	-	-	-
21	N.S.	< 0.001	-	-

N.S. = Not significant when compared to control group.

from mice growing the tumour. Thereafter, spleen weights of those that had received either the dead tumour cells, or the cell-free preparation showed a progressive fall that had returned to normal values on the 21st day following the inoculations. Mice that had received the 'whole' live tumour cells however showed a greater increase in spleen weight on the seventh day when the experiment was terminated, than in those that had received the washed tumour cells.

Changes in the Hepatic Content of Citrate (Figure 35)

Injection of the cell-free preparation into normal mice induced significant increases in the hepatic content of citrate on the first and second day, these changes were paralleled in mice that had received the 'whole' live tumour cells, with increases in the hepatic content of this metabolite occurring on the first, second and third day. In these animals, the citrate content then decreased to normal values on the fourth day, but then rose to highly significant values ($P \leq 0.001$) on the seventh day, when the experiment was terminated. The cell-free preparation of the tumour cells however did not induce any further significant changes in the hepatic content of citrate in the recipients.

In contrast, in mice that had received the non-viable tumour cells the citrate content of liver decreased significantly on the first day, and no significant changes occurred until the 21st day, when the hepatic content then increased significantly.

Washing of the live tumour cells prior to their

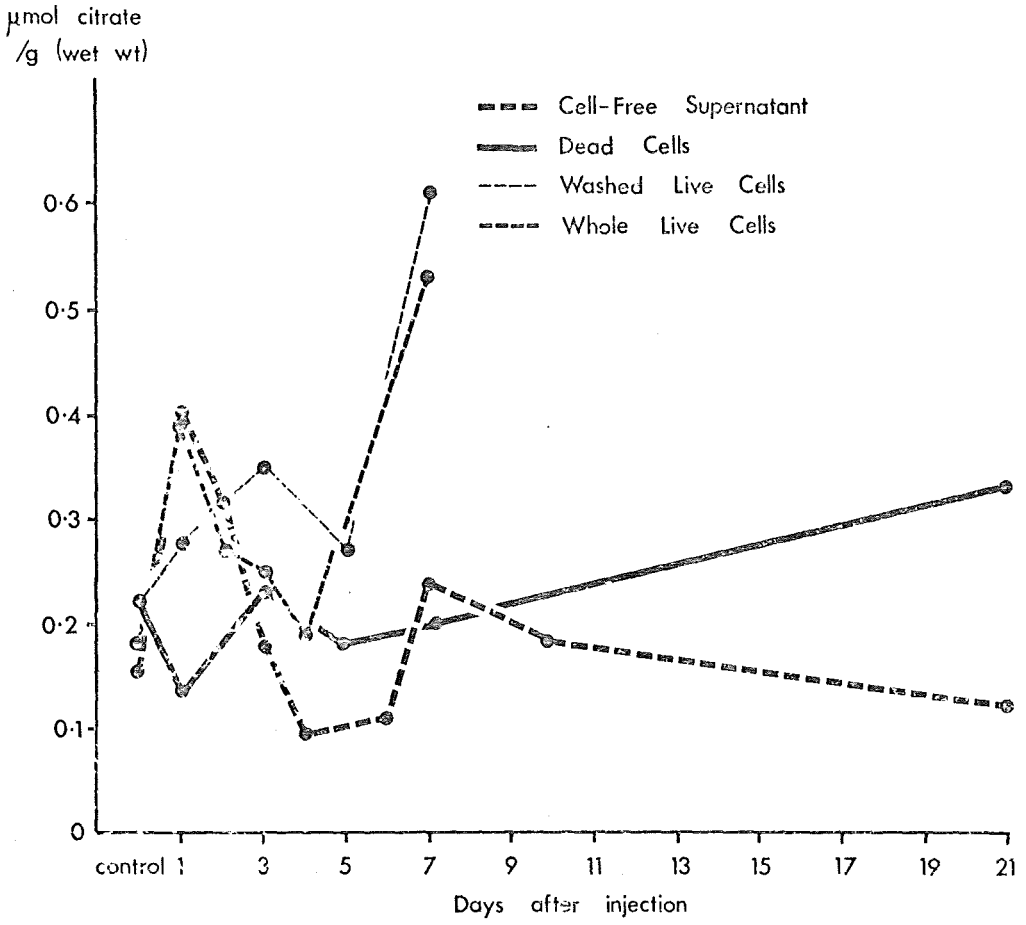


FIGURE 35 Daily changes in the hepatic content of citrate in the four models studied. Results are expressed as means. Six animals were used for each time interval.

TABLE 25

STATISTICAL EVALUATION OF DATA PRESENTED IN FIGURE 35

EFFECT ON THE HEPATIC CONTENT OF CITRATE

Day	Cell-free Supernatant	Dead Cells	Washed Live Cells	'Whole' Live Cells
	P	P	P	P
1	< 0.01	< 0.05	N.S.	< 0.01
2	< 0.02	-	-	< 0.05
3	N.S.	N.S.	< 0.02	< 0.02
4	N.S.	-	-	N.S.
5	-	N.S.	N.S.	-
6	N.S.	-	-	-
7	N.S.	N.S.	< 0.001	< 0.001
10	N.S.	-	-	-
21	N.S.	< 0.05	-	-

N.S. = Not significant when compared to control group.

injection into normal mice, appeared to delay the initial changes in the citrate content of liver, which only showed a significant increase on the third day. However, a highly significant increase had occurred on the seventh day, in parallel with those that had received the 'whole' live tumour cells.

Changes in the Hepatic Content of 'Total' CoA (Figure 36)

Mice that had received the 'whole' live tumour cells showed significant decreases in the hepatic content of 'total' CoA over the whole six days following the injection. These changes were paralleled closely with those that had received the cell-free preparation of the tumour cells, with significant decreases also occurring from the first to the sixth day. In these animals, the decreased content of 'total' CoA was maintained up to the 10th day, but had returned to normal on the 21st day.

As was seen in the delay that occurred before the onset of changes in the hepatic content of citrate in mice that had received the washed live tumour cells, the 'total' CoA in livers of these animals did not change significantly on the first day following the injection, and had not altered significantly on the third day, but on the seventh day, when the experiment was terminated, highly significant reductions in the hepatic content of 'total' CoA had occurred. The initial changes in animals that had received the washed live tumour cells in fact paralleled those that had received the non-viable tumour cells, with increases

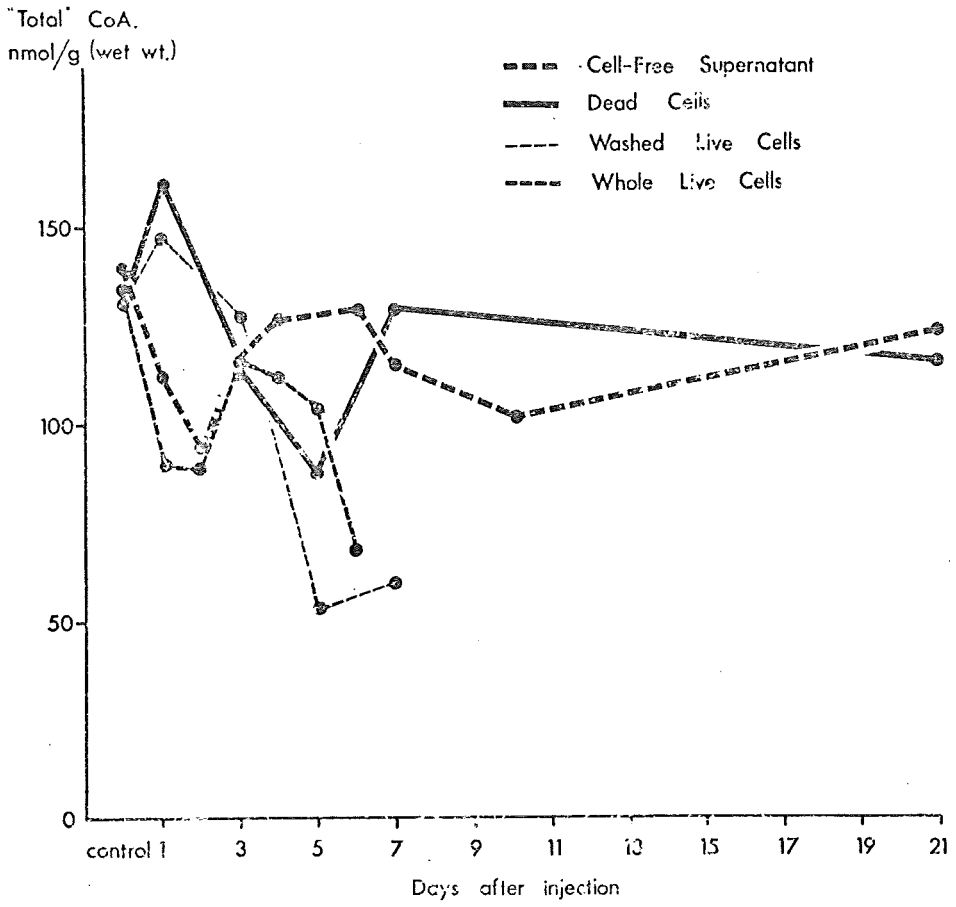


FIGURE 36 The 'total' CoA content of liver of mice in the four models studied. Results are expressed as means. Six animals were used for each time interval.

TABLE 26

STATISTICAL EVALUATION OF DATA PRESENTED IN FIGURE 36

EFFECT ON THE HEPATIC CONTENT OF 'TOTAL' CoA

Day	Cell-free Supernatant	Dead Cells	Washed Live Cells	'Whole' Live Cells
	P	P	P	P
1	< 0.02	< 0.001	N.S.	< 0.001
2	< 0.001	-	-	< 0.01
3	< 0.001	N.S.	N.S.	< 0.02
4	< 0.02	-	-	< 0.05
5	-	< 0.001	< 0.001	< 0.001
6	< 0.05	-	-	< 0.001
7	< 0.001	N.S.	< 0.001	-
10	< 0.001	-	-	-
21	N.S.	N.S.	-	-

N.S. = Not significant when compared to control group.

occurring on the first day, which in the case of the latter model this change was highly significant ($P<0.001$). The 'total' CoA in liver of animals that had received the non-viable tumour cells then fell in parallel with those growing the tumour, with a significant decrease being found on the fifth day.

Changes in the Hepatic Contents of Acetyl CoA and CoASH (Figures 37 and 38)

Mice that had received the cell-free preparation of the tumour cells, showed a significant fall in the hepatic contents of acetyl CoA on the second and third day following the injection (Figure 37). Thereafter, no significant changes were found, until the seventh and 10th day, when marked decreases occurred ($P<0.001$). On the 21st day, however, the hepatic contents of this metabolite had returned to normal values. In contrast, in animals that had received the 'whole' live tumour cells, there were significant decreases in the hepatic content of acetyl CoA on the first day with no further changes until the fifth and sixth day when significant decreases again occurred ($P<0.05$ and <0.001 respectively).

As shown in Figure 38, significant changes in the hepatic content of CoASH occurred in normal mice on the first, second and third day following the injection of the cell-free preparation of the tumour cells, and in this respect paralleled closely similar changes occurring at that period in mice growing the tumour. However in the latter group the hepatic content of CoASH continued at significantly low levels over the

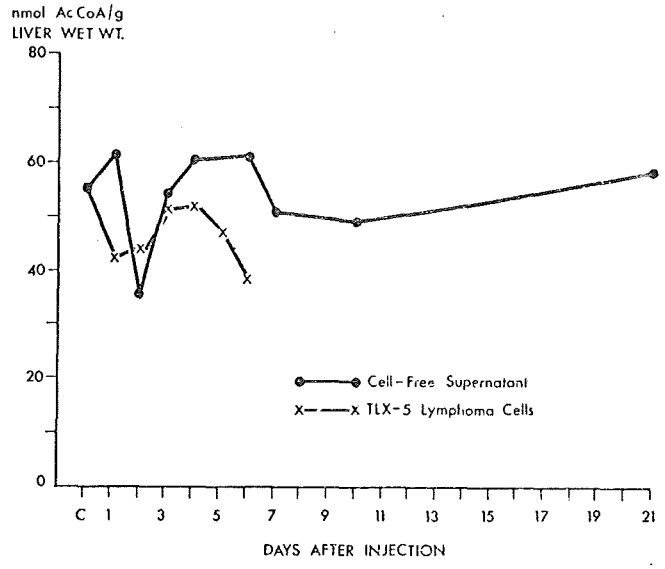


FIGURE 37 Daily changes in the hepatic content of acetyl CoA in mice following the injection of TLX-5 lymphoma cells, or a cell-free preparation of the cells. Results are expressed as means. Six animals were used for each time interval.

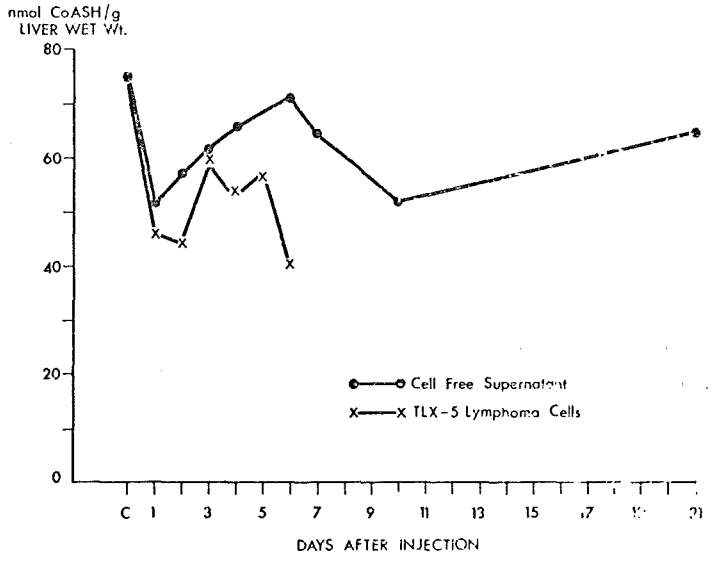


FIGURE 38 Daily changes in the hepatic content of CoASH in mice following the injection of TLX-5 lymphoma cells or a cell-free preparation of the cells. Results are expressed as means. Six animals were used for each time interval.

six days of tumour growth, whereas in those that had received the cell-free preparation the hepatic content of this metabolite again decreased significantly on the seventh and 10th day.

In contrast, in mice that had received the non-viable tumour cells (Figure 39), there was a significant increase in the hepatic content of acetyl CoA and CoASH on the first day following the injection. On the fifth day however, significant decreases occured in the level of both metabolites.

When the TLX-5 lymphoma cells were washed prior to their injection into normal mice, the hepatic content of acetyl CoA decreased significantly ($P < 0.05$) on the first day (data not shown), with no further significant changes until the fifth and seventh day, when decreases ($P < 0.001$ and < 0.01 respectively) occurred. These changes were similar to those found at these periods in mice following the injection of the 'whole' live tumour cells.

However in contrast, it was found that the injection of the washed tumour cells into mice did not induce significant decreases in the hepatic content of CoASH (data not shown) until the fifth day following the injection. This was in marked contrast to those animals described above that had received the 'whole' live tumour cells.

Catalase Activity in Liver

Normal mice that had received the cell-free preparation of TLX-5 lymphoma cells or non-viable

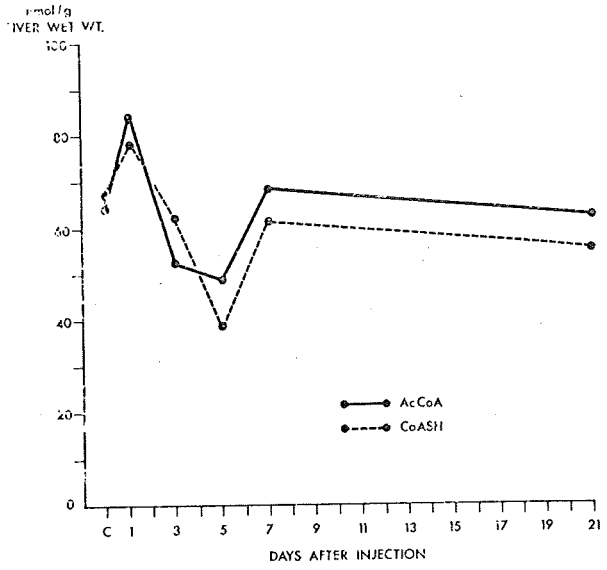


FIGURE 39 Daily changes in the hepatic content of acetyl CoA and CoASH in mice following the i.p. injection of non-viable TLX-5 lymphoma cells. Results are expressed as means. Six animals were used for each time interval.

TABLE 27

STATISTICAL EVALUATION OF DATA PRESENTED IN FIGURES 37, 38 AND 39

EFFECT ON THE HEPATIC CONTENT OF ACETYL CoA

Day	Cell-free Supernatant	Dead Cells	'Whole' Live Cells
	P	P	P
1	N.S.	< 0.001	< 0.02
2	< 0.001	-	N.S.
3	< 0.01	N.S.	N.S.
4	N.S.	-	N.S.
5	-	< 0.02	< 0.05
6	N.S.	-	< 0.001
7	< 0.001	N.S.	-
10	< 0.001	-	-
21	N.S.	N.S.	-

N.S. = Not significant when compared to control group.

TABLE 28

STATISTICAL EVALUATION OF DATA PRESENTED IN FIGURES 37, 38 AND 39

EFFECT ON THE HEPATIC CONTENT OF CoASH

Day	Cell-free Supernatant	Dead Cells	'Whole' Live Cells
	P	P	P
1	< 0.05	< 0.05	< 0.01
2	< 0.01	-	< 0.01
3	< 0.02	N.S.	< 0.001
4	N.S.	-	< 0.05
5	-	< 0.001	< 0.001
6	N.S.	-	< 0.001
7	< 0.05	N.S.	-
10	< 0.01	-	-
21	N.S.	N.S.	-

N.S. = Not significant when compared to control group.

tumour cells showed no changes in the catalase activity of liver (data not shown), although as shown in Figure 40 in mice growing the tumour, a significant decrease in activity of the enzyme occurred from the first day following inoculation with the tumour cells, until the seventh day, when the experiment was terminated.

The lack of response in the activity of catalase in liver of mice that have received either the cell-free preparation or the non-viable tumours, therefore differ in this respect to mice growing the tumour. This may have been due to the low concentration of the material injected. It in no way however implies that toxohormone is present in these preparations, which as discussed previously produces decreases in the activity of this enzyme in normal rat liver. This is however a non-specific effect and is also found in normal starved rats (Miller, 1948) and as shown in Figure 41 also in starved mice.

DISCUSSION

Measurement of the daily changes in the hepatic contents of acetyl CoA, CoASH and citrate in mice following the i.p. injection of a cell-free preparation of TLX-5 lymphoma cells showed that the changes produced followed to a large extent, those found in mice that had received viable TLX-5 lymphoma cells. These findings suggested that a product or products of the tumour cells are involved in the aetiology of these changes. They also support the previous

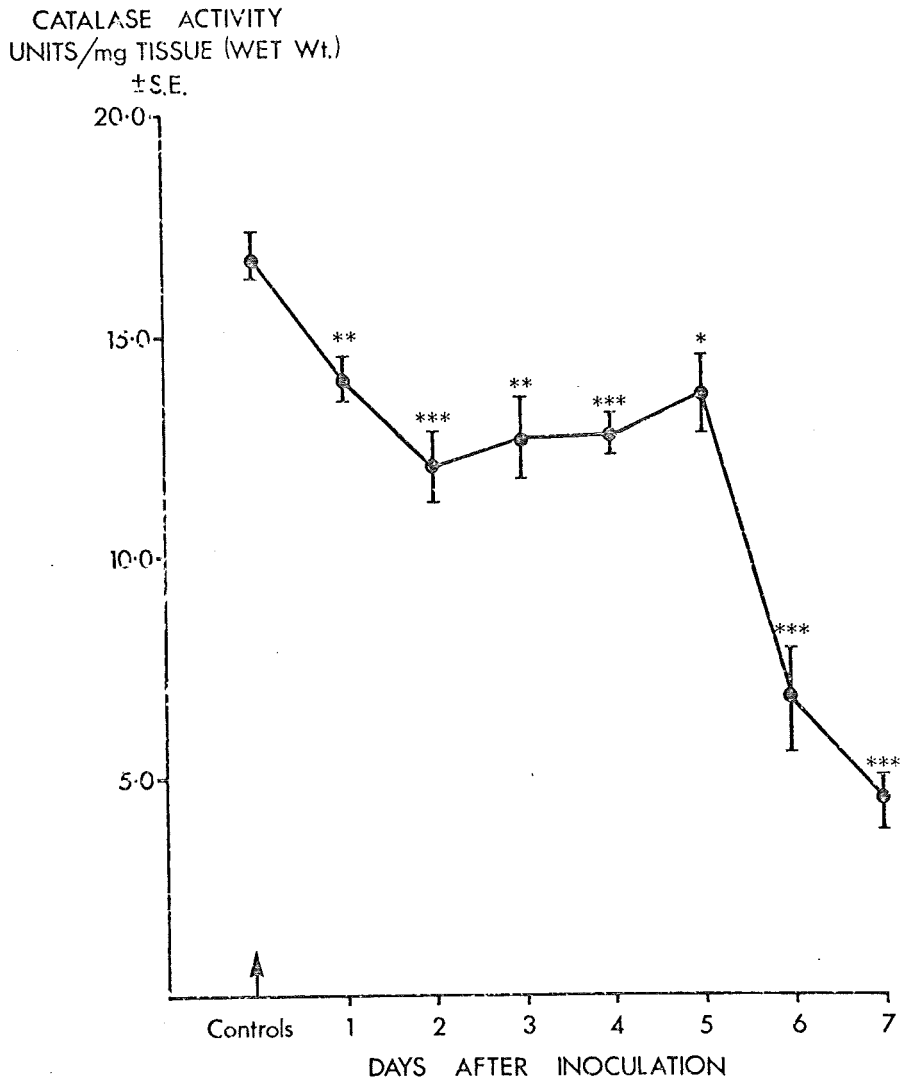


FIGURE 40 Daily changes in the catalase activity of liver of mice bearing TLX-5 lymphoma. Results are expressed as means ± S.E.M. Six animals were used for each time interval.

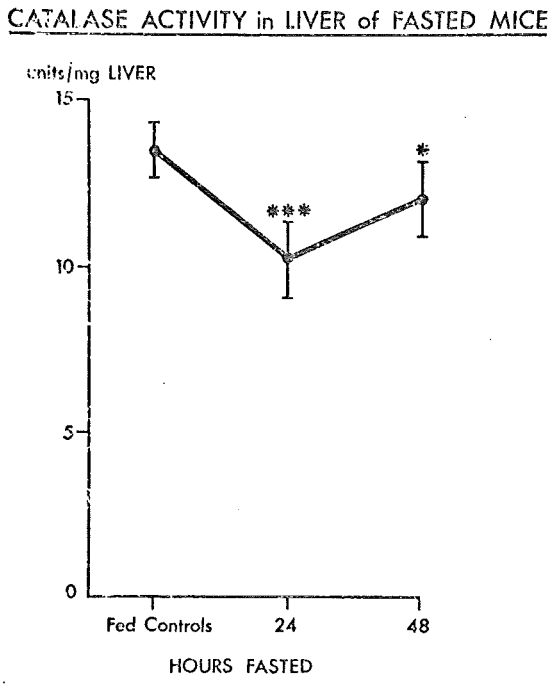


FIGURE 41 Catalase activity of liver of mice after fasting for 48 hours. Results are means $\pm$ S.E.M. Six animals were studied in each group.

observation, that similar changes in the hepatic contents of these metabolites in mice bearing a transplantable C_3H mammary tumour can revert to normal values, following curative resection of the tumour.

A notable feature was that these changes occurred 24 hours after injection of the cell-free preparation, or the live tumour cells (cf. also Figure 8 Chapter 2). In fact, the original observation that viable tumour cells induced these early changes, was the first indication that products present in the ascitic fluid might be responsible.

The cell-free preparation also induced a rapid intracellular response in immunologically associated tissue such as spleen. The onset of thymic atrophy in these animals, also paralleled these changes in thymus of mice bearing the tumour, but as discussed earlier thymic atrophy in tumour-bearing animals requires clarification.

Although it was thought that washing of viable TLX-5 lymphoma cells might induce a delay in the onset of changes in the hepatic content of these metabolites, as well as in spleen and thymus weights, due to removal of factors responsible from the surface of the cells, the results were inconclusive. The possibility also exists that the washing process in fact killed some of the cells.

When the non-viable tumour cells were injected into normal mice, the hepatic contents of acetyl CoA and CoASH increased on the day following the injection, with a concomitant fall in citrate at that time. As discussed previously, these changes in the direction of acetyl CoA and citrate were similar to those found in liver of starved normal rats (Start and Newsholme, 1968; Herrera and Freinkel, 1968; McGarry and Foster, 1972).

Later however, a significant decrease in acetyl CoA and CoASH occurred on the fifth day in liver of these mice, but without a change in the citrate content at that time. The hepatic content of citrate in these livers however showed a significant increase on the 21st day. We have no adequate explanation for the changes in the direction of these metabolites on the first day following the injection of the non-viable tumour cells. Observation of these animals showed however that they suffered no ill-effect of the injection and as far as could be judged food intake remained normal.

In a previous section of this thesis (Chapter 4) it was postulated that a fall in the hepatic content of acetyl CoA, and increase in citrate might be due to some disturbance of lipid metabolism in these animals. It is of interest therefore in the present connection, that in studies on the rapid triphasic loss of body fat in mice bearing Krebs-2 carcinoma, Costa and Holland (1962) have demonstrated that the injection of the frozen and thawed tumour cells into normal mice

also induced loss of body fat and increases in the specific activity of fat from acetate-1-^{14}C. They concluded however that this effect may have been due to an associated virus in their preparation.

It has also been postulated (Liebelt, Gehring, Delmonte, Schuster and Liebelt, 1974) that the triad of fat loss, suppression of appetite and hyperlipaemia in mice bearing a stomach tumour might involve the release of a humoral factor from the tumour cells.

Such products have not however been characterised, although there is evidence that factors are present in the blood of tumour-bearing rats that are responsible not only for stimulation of liver protein synthesis and inhibition of serum protein synthesis (Toporek, 1973), but also for the inhibition of NAD-synthesising enzymes in liver of rats (Waravdekar and Powers, 1957). These latter workers also showed that these effects can be reversed following removal of the tumour.

Although the studies presented here showed that products of tumour cell origin may well be involved in changes in the hepatic contents of acetyl CoA, CoASH and citrate in liver of tumour-bearing mice, much further work is necessary to define the nature of these products.

However, since Parry and Ghadially (1970) have shown that treatment of normal rats with 'toxohormone' will induce ultrastructural changes in the hepatocytes of these animals, we next examined whether such changes, particularly in mitochondria were present in liver of tumour-bearing mice, and whether these could be

mimicked in normal mice, following the injection of cell-free preparations of TLX-5 lymphoma.

CHAPTER 7

A NOTE ON ULTRASTRUCTURAL CHANGES IN LIVER OF MICE FOLLOWING THE INJECTION OF A CELL-FREE PREPARATION OF TLX-5 LYMPHOMA

In recent years, several workers have described ultrastructural changes in livers of animals bearing different transplanted or induced tumours (Ghadially and Parry, 1965; Parry and Ghadially, 1966, 1967, 1969; Bhawan, Friedell and Jacobs, 1975; Khandekar, Dardachte, Garg, Tuchweber and Kovacs, 1972).

These changes include increases in the number of lysosomes with swelling and rupture of these organelles (Ghadially and Parry, 1965; Parry and Ghadially, 1966), pleomorphism and swelling of mitochondria (Ghadially and Parry, 1965; Parry and Ghadially, 1966) with loss of cristae (Bhawan, Friedell and Jacobs, 1975). Increases in the number of microbodies have also been reported (Bhawan et al. 1975).

However, a consistent pattern in these changes has not been observed in animals bearing different types of tumours, and it has been suggested (Khandekar et al. 1972), that differences in the strain of animal used, the type of tumour, and the condition of the animal at the time the observations are made, may well be responsible for these differences.

It is not the object of this presentation to discuss these ultrastructural changes in detail, but rather to focuss attention on two areas in this field,

that were felt to be pertinent to the work in the present thesis. First, in view of our previous observations of alterations in the hepatic contents of several tricarboxylic acid cycle intermediates and adenine nucleotides in tumour-bearing mice, it was of interest to examine whether there were ultrastructural changes in mitochondria in these livers. Second, since it was previously shown that the administration of a cell-free preparation of TLX-5 lymphoma to normal mice, could also induce similar changes in the hepatic contents of some of these metabolites, we also examined whether this treatment would induce ultrastructural changes in liver.

PROCEDURES

Three groups of animals were studied, (a) normal mice, (b) mice bearing Sarcoma 180 and (c) mice that had received 0.5 cm^3 i.p. of the cell-free preparation of TLX-5 lymphoma, details of which have already been given (Chapter 6). The choice of mice bearing Sarcoma 180 was dictated by the fact that TLX-5 lymphoma invades liver.

Sarcoma 180 was implanted subcutaneously in the subscapular position under light ether anaesthesia as previously described. After 12 days of tumour growth, at which period the animals showed no apparent ill-effects of their tumours, and continued to eat normally, they were sacrificed by cervical dislocation.

As described previously, mice that had received

the cell-free preparation of TLX-5 lymphoma showed no ill-effects of this treatment. These were sacrificed two days after injection. In order to check that the cell-free preparation was not contaminated with the tumour cells, another group of mice that had received the same preparation were observed over a period of three weeks. No evidence of tumour growth could be detected in these animals.

Immediately after sacrifice, small pieces of liver were removed from the animals, as well as [illegible] from normal mice of similar age and the tissue processed for electron microscopy by the method already noted.

RESULTS

The electron micrographs of liver of mice bearing Sarcoma 180 showed swelling of mitochondria with prominent vacuolation (Figure 42), loss of cristae was also observed (Figure 43). These changes contrast markedly with mitochondria in the hepatocyte of normal mice (Figures 44 and 45). Other changes seen are the presence of a paracrystalline inclusion, and dilatation of the smooth endoplasmic reticulum.

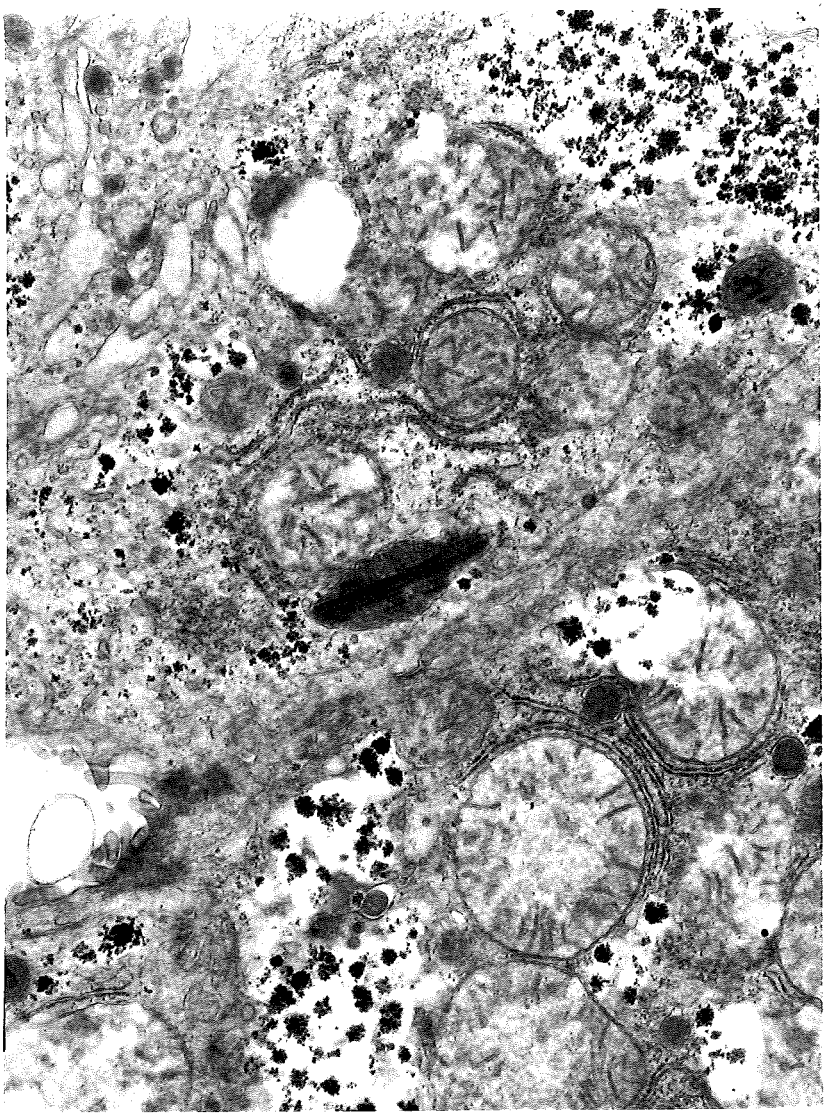
As shown in Figure 46 mitochondria in liver of mice that had received the cell-free preparation of TLX-5 lymphoma, also show swelling with prominent vacuolation, and in this respect mimicked those found in mice bearing Sarcoma 180. Other changes seen in those that had received the cell-free preparation are an increase in the number of microbodies which contain

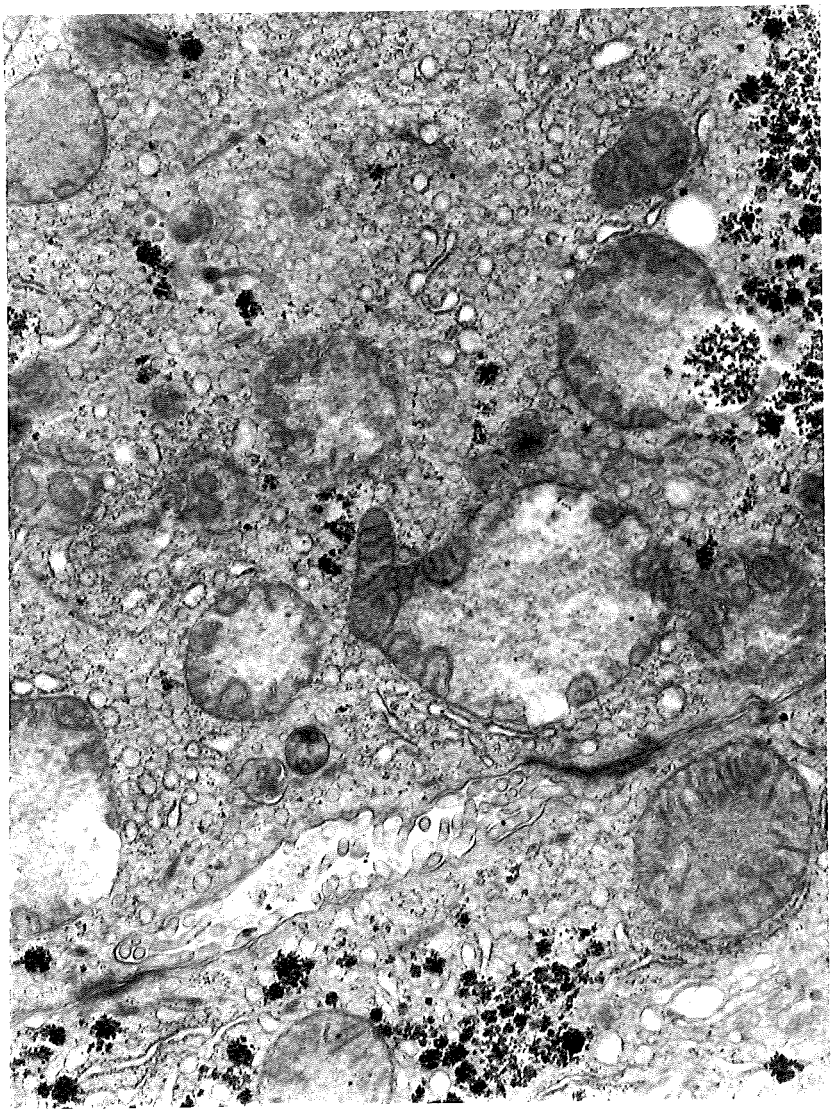
paracrystalline inclusions (Figure 46) and, as shown in Figure 47 a Kupffer cell containing much phagocytosed material.

DISCUSSION

Changes in the structure of mitochondria in liver of mice bearing Sarcoma 180 reported here have also been observed in rats bearing a subcutaneous sarcoma induced by DMBA (Parry and Ghadially, 1966), as well as in rats bearing a subcutaneous mammary tumour (Bhawan, Friedell and Jacobs, 1975). These latter workers have also described increases in the number of microbodies in liver of these animals, as was also found in the present study.

The reason for ultrastructural changes in liver mitochondria of tumour-bearing animals is unclear, and have also been observed in states of hypoxia and hyperoxia (Schaffner, 1970) as well as in iron deficiencies (Dallman and Goodman, 1971). According to other workers (Khandekar, Dardachte, Tuchweber and Kovacs, 1972) alterations in the ultrastructure of mitochondria in liver of tumour-bearing rats may reflect a direct toxic effect of the tumour on the liver. In this connection it is of interest that ultrastructural changes in rat hepatocytes have been reported by Parry and Ghadially (1970) following the administration of toxohormone to these animals. These changes were similar to those found in tumour-bearing animals, and included increases in the number of hepatocellular lysosomes, and focal cytoplasmic





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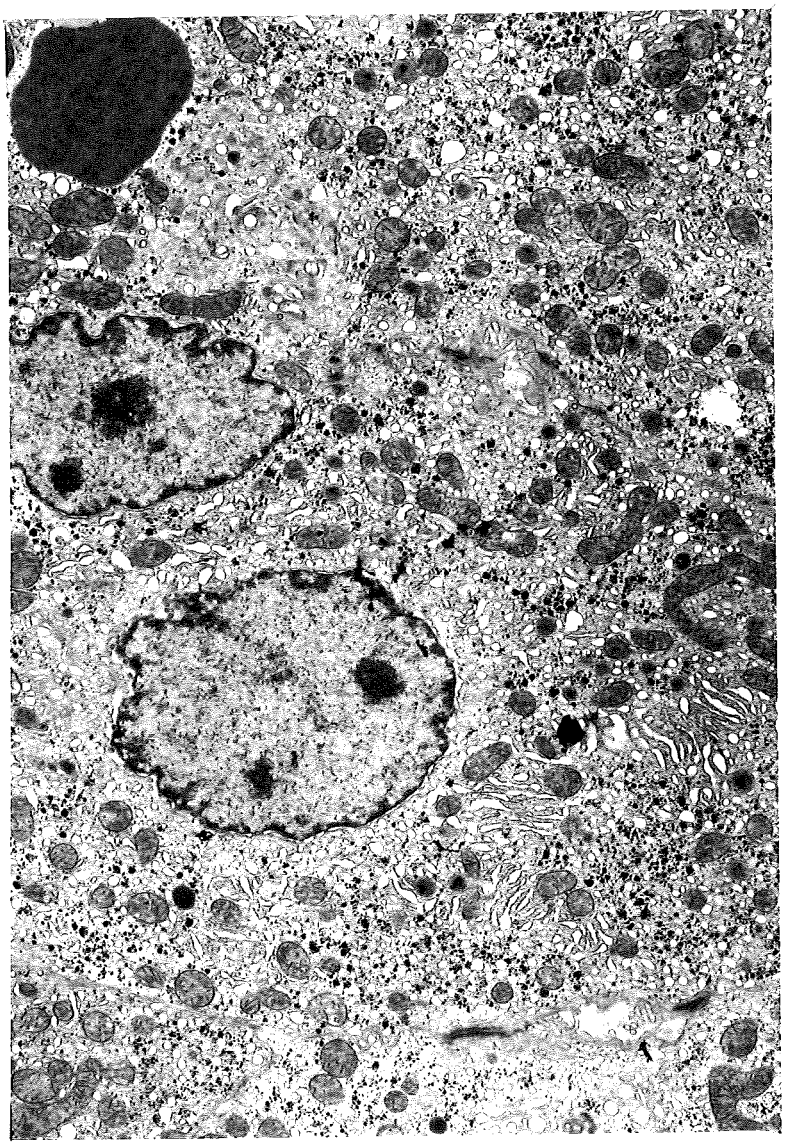

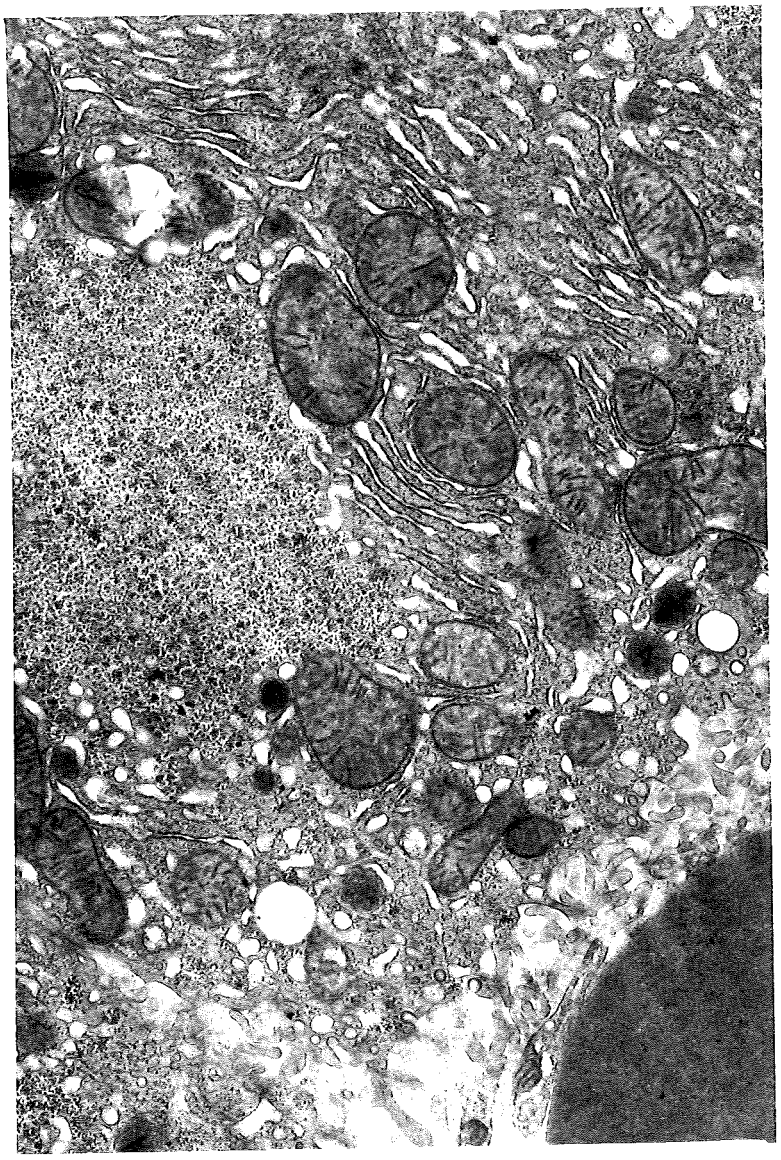

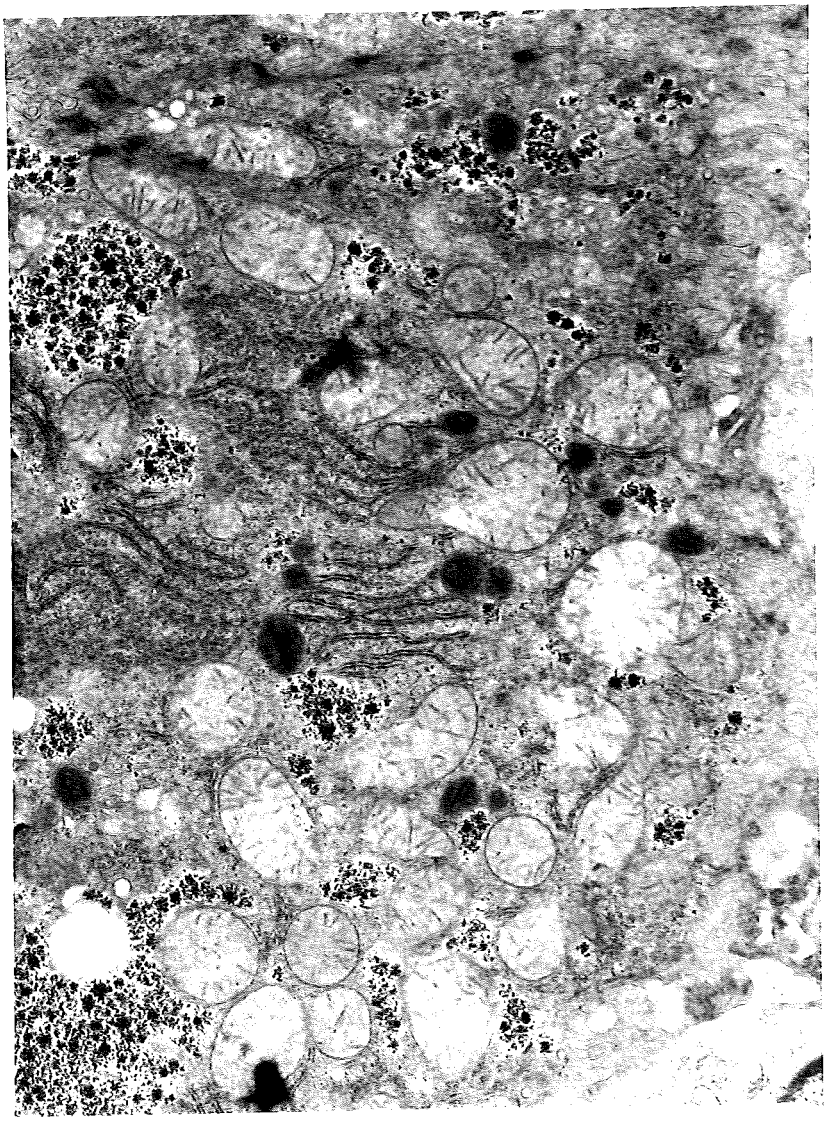

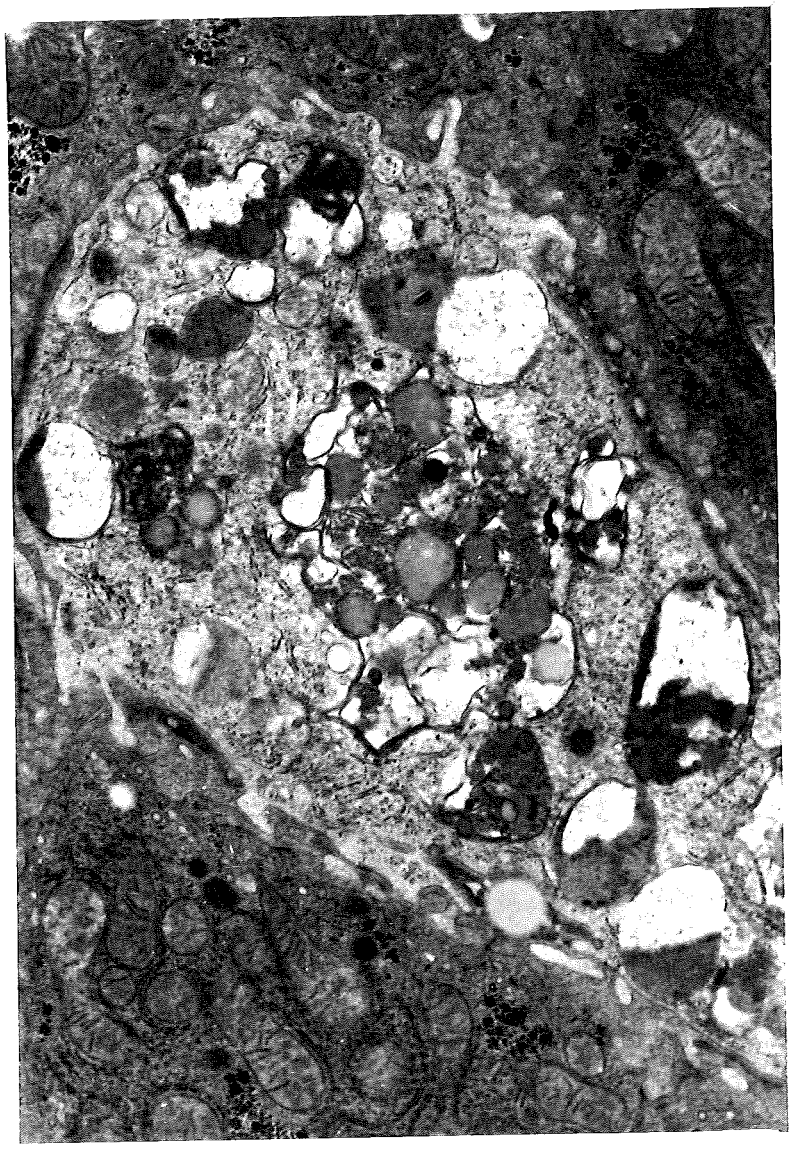

degeneration. However changes in the structure of mitochondria were not observed in these studies, and as previously reported (Parry and Ghadially, 1969) the administration of necrotic tumour tissue to normal rats failed to induce increases in the number of lysosomes, and other ultrastructural changes.

In the studies cited above (Parry and Ghadially, 1970), the animals were given about 100 mg of toxohormone daily for a period of 10 days, prior to examination, whereas in the present investigation, the material injected was derived from 2×10^6 tumour cells. These changes in mitochondria induced by this treatment were observed two days following the injection, and therefore occurred, as was previously shown at a period when changes in the hepatic contents of acetyl CoA, CoASH and citrate were also found.

The present studies therefore suggest that the ultrastructural changes in liver of mice bearing Sarcoma 180 may well involve the release of factors from the tumour cells. It is of particular interest that the changes observed in the mitochondria of mice bearing the sarcoma, can also be mimicked in mice following the injection of a preparation from a different type of tumour.

To suggest however that injury to mitochondria in liver of tumour-bearing mice are responsible for the changes in the hepatic contents of metabolites studied here would at the present stage of knowledge be speculative.

However, swelling of mitochondria is usually indicative of an uncoupling of oxidative phosphorylation

(Packer and Utsumi, 1969), but according to Greene (1960), mitochondria from liver of rats bearing Walker 256 carcinoma did not show a decrease in oxidative phosphorylation. Later, other workers (Baldwin, George and Cunningham, 1975) studied respiratory control in mitochondria from liver of rats bearing Walker 256 carcinoma, using more refined techniques. They showed that there was no significant difference in the rate of loss of respiratory control of liver mitochondria in these animals, when compared to normal controls. These observations were made on animals at a period when they presented no ill-effects of their tumours, although tumour growth was well advanced. However, when animals bearing this tumour were studied in the agonal phase, mitochondria from livers could not catalyse ATP synthesis. They conclude from these studies that the ultrastructural changes in mitochondria reported by Parry and Ghadially (1966) in rats bearing subcutaneous sarcomas induced by DMBA and which were observed during the terminal stage of life, were due to the degenerative process of dying, and not as a result of any direct effect of the tumour on liver.

Clearly the present studies showing that swelling of liver mitochondria occur in apparently healthy animals bearing Sarcoma 180, and that these changes also occur in tumour-free animals that had received a cell-free preparation of TLX-5 lymphoma, refute the above suggestion.

CHAPTER 8

A NOTE ON THE RELATIONSHIP BETWEEN THYMIC ATROPHY AND LOSS OF BODY WEIGHT IN TUMOUR-BEARING MICE

Although as discussed previously thymic atrophy is a frequent finding in tumour-bearing animals, there is still doubt as to the mechanisms involved.

Some studies however have indicated that thymic atrophy in these animals may be associated with other systemic effects of a tumour. Thus Siegler and Koprowska (1962) noted that the beginning of thymic atrophy in mice bearing an undifferentiated C_3H tumour in ascites form, correlated in time with the commencement of hypertrophy of spleen, and associated fatty changes in liver of these animals.

More recently, Ertl (1973) in an extensive study on rats bearing the Walker 256 carcinoma, has shown that the beginning of thymic atrophy in these animals could be correlated with the commencement of loss of body weight and progressive cachexia.

These data suggested that in tumour-bearing animals, there appeared to be a close physiological relationship between thymic atrophy and some of the systemic effects of a tumour on its host.

In view of the importance of these observations, we have confirmed these findings in another species of animal, and in one bearing a different tumour by examining the relationship between thymic atrophy

and loss of body weight in mice bearing Sarcoma 180.

Materials and Methods

Weight-matched three to four month old male CBA mice were used throughout. Sarcoma 180 was implanted subcutaneously in the subscapular position as described in Chapter I.

Twenty-four hours after tumour implant six animals were taken, weighed individually, then sacrificed by cervical dislocation. Thymus glands were carefully removed and weighed rapidly.

Thereafter, the same procedure was carried out at daily for up to 15 days. When tumour growth in these mice became apparent, the tumour was also carefully dissected free from connective tissue and weighed.

Results

As shown in Figure 48, the net body weight (body weight - tumour weight) decreased on the second day following tumour implant, and this corresponded with an increase in thymus weight.

The initial decrease in body weight was most likely due to the trauma of tumour implant, which we have observed previously.

The initial increase in thymus weight on the second day following tumour implant is in agreement with Ertl (1973), but in his study, this occurred at the stage when the tumour became palpable.

The data presented here (Figure 48), show that

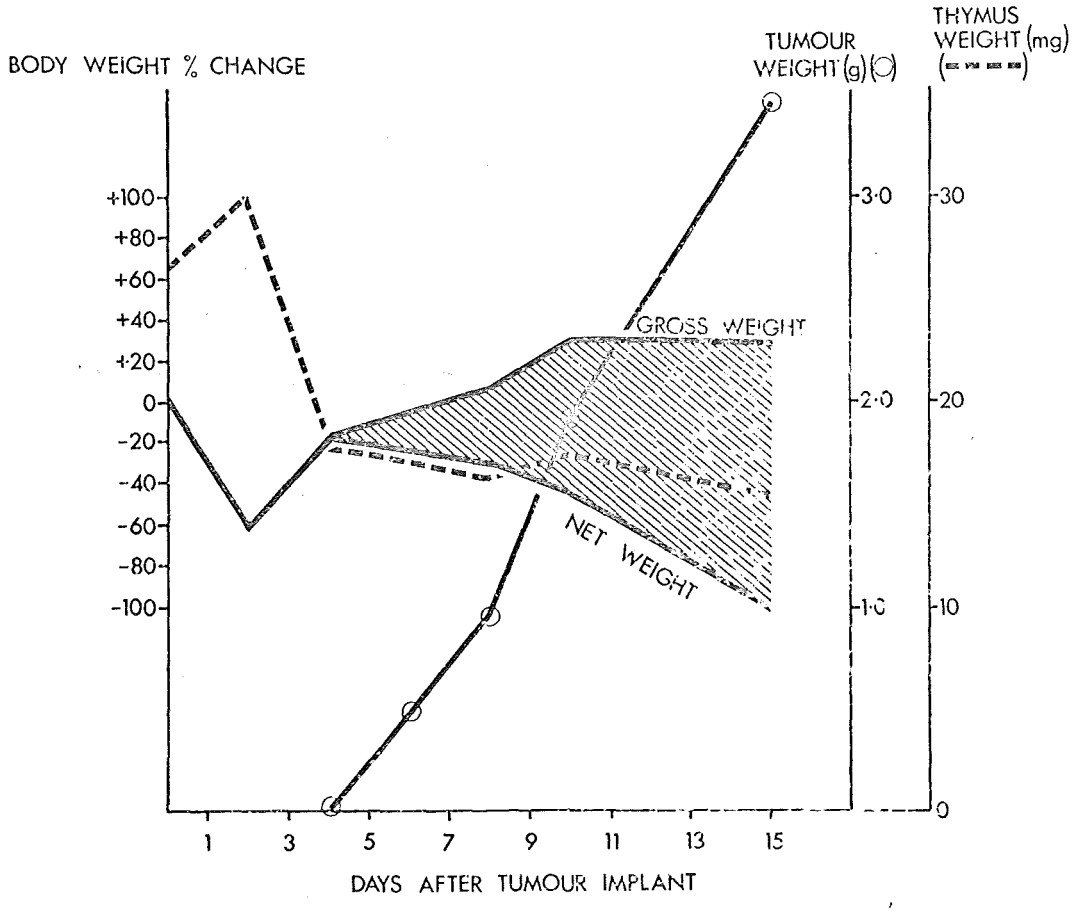

FIGURE 48. The relationship between the beginning of thymic atrophy, loss of net weight (body weight minus tumour weight) and commencement of tumour growth in mice bearing Sarcoma 180.

a marked decrease in thymus weight occurred from the second to the fourth day following tumour implant, and this continued, although to a lesser degree until the 15th day when the experiment was terminated.

It will also be seen that on the fourth day decreases in the mean thymus weight corresponded with the stage when the tumour weight became measurable, and when the net body weight (body weight - tumour weight) began to decrease.

Discussion

The data presented here is in close agreement with the findings of Ertl (1973) who studied these changes in rats bearing the Walker 256 carcinoma.

They differ only in the fact that in his study, an initial increase in thymus weight occurred at the stage when the tumour became palpable. This he attributed to the immune response of the host, but in studies already made in mice bearing TLX-5 lymphoma, it was shown that the weight of thymus progressively decreased following tumour implant (Chapter 2). Therefore the initial increase in thymus weights reported here in mice bearing Sarcoma 180, may depend on the type of tumour used.

On the basis of studying the stage of thymic atrophy in relation to changes in other non-involved organs in rats hosting the Walker 256 carcinoma, Ertl (1973) proposed that these can be correlated with one another. He has suggested an arbitrary classification in terms of pre- and post-involution of thymus in these

animals. Thus histological studies of adrenals showed the beginning of hyperplasia and hyperfunction at the pre-involution stage, whereas in the post-involution stage, this progressed to exhaustive insufficiency. It was also demonstrated (Ertl, 1973) that at the pre-involution stage, liver showed the beginning of hypertrophy, and in the post-involution stage, increases in liver weight occurred. The exact significance of these relationships must however await further investigation.

CONCLUSIONS

This thesis concludes with a short discussion on further approaches considered necessary to elucidate some of the metabolic changes described in liver of tumour-bearing mice.

First, the cause of some of these changes.

Good evidence was presented showing that some of the changes in the hepatic content of metabolites such as acetyl CoA, and citrate were mediated by products of tumour cell origin, and that such products are also involved in some of the ultrastructural changes found in liver of tumour-bearing mice. Much work is now necessary to define the nature of these products, and this is being actively pursued at the present time. The immunological effects of these products are also being investigated by Mr John Rancewicz of the Department of Surgery, and by Professor Kenneth C. Calman's group in the Department of Clinical Oncology.

It was noted at various stages in this presentation that any further biochemical approaches, aimed at defining the mechanisms involved in some of these metabolic abnormalities, will require much more information on the hepatic contents of other metabolic intermediates, as well as on the activities of several enzymes. However, as was stressed previously, the problem of the distribution of these metabolites in the cytoplasmic and mitochondrial compartments remains, and must await further assessment by other workers,

of the methods currently available for rapid cell fractionation. What may be a major problem in the application of such techniques to livers of tumour-bearing animals are the structural abnormalities present in the mitochondria.

However, until these problems of methodology are resolved, several experimental approaches suggest themselves, which were not possible to explore in the time available for the completion of this thesis.

The mechanisms involved in the accumulation of citrate in livers of these mice, requires clarification, and here information is required on the activity of isocitrate dehydrogenase, as well as the reason why tumour-growth induces marked decreases in the activity of the citrate cleavage enzyme.

The important finding of Newsholme and Start (1972) that insulin release induces marked increases in the hepatic content of citrate in tumour-free rats prompts an investigation of the level of circulating insulin in these tumour-bearing mice. In keeping with the above authors we proposed that the tumour-induced increase in the hepatic content of citrate would stimulate fatty acid synthesis, but here the anomalous situation arose when it was found that starvation in tumour-bearing mice induced a further increase in citrate, and this lead to the suggestion that in these livers, the regulation of fatty acid synthesis appeared to be out of control. Clearly a major study of fat metabolism in these animals is indicated.

The finding that the decrease in the hepatic content of free coenzyme A could not be normalised by the administration of pantothenate to these animals suggested that there may be a block in the biosynthesis of the coenzyme. The deleterious effect that this treatment had on the tumour-bearing host defies a satisfactory explanation at this stage, but clearly this may be a very good model for the study of the problem of weight loss induced by a tumour.

Concerning a possible mechanism for the fall in the hepatic contents of free coenzyme in livers of tumour-bearing mice, the question arises as to whether this reflects an increase in the rate of dissimulation. Firstly, it is only recently that the degradation of coenzyme A in living systems has been examined (for review see Abiko, 1975). These studies have shown that the initial step in the dissimulation of the coenzyme in normal rat liver involves its conversion to dephospho CoA by lysosomal acid phosphatase (Bremer, Wojtczak and Skrede, 1972). It is of interest therefore that marked increases in the number of lysosomes, with rupture of these organelles, has been reported to occur in liver of tumour-bearing rats, as shown by electron microscopy (Ghadially and Parry, 1965; Parry and Ghadially, 1966), and increases in the number of lysosomes have also been reported in normal rats following the injection of toxohormone (Parry and Ghadially, 1970). Unfortunately in our own studies on ultrastructural changes in livers of tumour-bearing mice, a detailed study of lysosomes was not carried out, and clearly this is called for.

However, in support of these observations, other workers have reported increased activity of lysosomal enzymes in non-involved liver of patients with cancer (Schersten, Wahlqvist and Johannsen, 1969; Schersten, Walqvist and Barbaro-Jilderos, 1971), although in the former study, it was reported that the activity of lysosomal acid phosphatase only showed a tendency to increase.

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ADDENDUM

Here it is proposed to consider some of the possible effects of these metabolic changes in liver on the condition of the animal, and to consider the possibility of extending some of these studies to cancer patients.

It was noted early in this thesis, that changes in the hepatic contents of metabolites such as acetyl CoA could not be related to anorexia or cachexia in tumour-bearing mice. In this connection the observations of Wieland (1966) are of interest. He found that in cachectic rats with untreated alloxan diabetes in which the fat stores were completely exhausted, the acetyl CoA content of liver remained at normal levels. In fact a significant decrease in the hepatic content of this metabolite is an unusual finding, and has only been previously reported in fasted rats following the inhibition of lupolysis by the administration of nicotinamide (Talke et al. 1973, European Journal of Clinical Investigation 3, 407-474). Of importance too was the observation that fasting of tumour-bearing mice failed to induce increases in the acetyl CoA content of liver, with subsequent ketosis. This in fact means that ketone body utilisation by brain and myocardium as an alternative energy source in starvation, would appear to be unavailable to the anorexic tumour-bearing mouse.

Another important question is whether the decreased content of both free CoA and acetyl CoA reflect a

subclinical deficiency of pantothenate. In recent studies we examined this in pantothenate deficient mice, and found significant reductions in the levels of both of these metabolites. However, as was noted previously we also require information on the levels of precursors in the biosynthesis of the coenzyme in these livers, but here it may be recalled that Rapp (1973) working with a non-specific method of assay that also includes some of these precursors, also found significant decreases in various organs of tumour-bearing animals. Although however, the question of a deficiency of pantothenate is not completely answered, it is of some interest that systemic effects of tumour growth such as thymic atrophy, hypertrophy of spleen and defects in the immune response have also been found in pantothenate-deficient rats and mice. We feel that this offers a good working hypothesis.

Finally, the question arises as to the application of some of these studies to cancer patients. Little is known of the coenzyme A content of blood (Abiko, 1975), but citrate has been reported to be present in blood and urine of humans, and it would be of interest to know whether abnormalities in the level of this metabolite or in its excretion can be detected in patients.

The pantothenate status of such patients would also clearly be of interest. Providing too that biopsies of liver could be obtained it would be of considerable interest to know if the changes reported here in the hepatic contents of metabolites as well

as ultrastructural changes in the hepatocyte could be also detected.