TUMOUR DERIVED METABOLIC MODULATORS: WITH PARTICULAR REFERENCE TO MOUSE HEPATIC CITRATE CONTENT

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

September, 1980

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LIST OF ABBREVIATIONS

SN1 = Supernatant 1

SN2 = Supernatant 2

PBS = Phosphate Buffered Saline

LDV = Lactate Dehydrogenase Virus

LDH = Lactate Dehydrogenase (E.C.1.1.1.27)

MDH = Malate Dehydrogenase (E.C.1.1.1.37)

CL = Citrate Lyase (E.C.4.1.3.6)

NAD^+ = Nicotinamide Adenine Dinucleotide

NADH = Nicotinamide Adenine Dinucleotide (reduced form)

r.p.m. = Revolutions per minute

nm = nanometer

um = micrometer

ml = millilitre

BSA = Bovine Serum Albumin

Acknowledgements

My gratitude is expressed to Professor K.C. Calman without whom this work would never have been undertaken, and who advised and encouraged me throughout the entire investigation. I also thank Dr. A. Baxter and Dr. J.P. Durham for their help and encouragement not only in the theoretical but also the practical aspects of this work.

Dr. R.A. McAllister and Mr. E. Campbell, of the University Department of Surgery, were also particularly helpful, and gave freely of their advice and time throughout this investigation. I am also indebted to Dr. D. Sommerville, Department of Virology, Belvedere Hospital, who advised and helped me on the Virology aspects of this work. Mr. J. Parry of the University Department of Virology is also thanked for carrying out electron microscope work presented in this thesis, as is Dr. D. Thompson of the Biochemistry Department, Gartnavel General Hospital, for measuring mouse serum LDH activity.

My gratitude is also expressed to all members of the University Department of Clinical Oncology, not only for their advice and assistance, but for making the Department a very enjoyable place in which to work. I particularly thank several members of the Technical Staff, including Mr. K. Gibson, Mrs. M. Hewitt, Mr. L.T. Richardson and Mr. G. Ross who helped at different stages of this investigation. Animal Technicians, Mrs. A. Millar, Mr. M. Stewart and Miss J.S.C. Glegg are also thanked for their help in looking after experimental animals.

Moreover, Mr. C.T.C. Bowie is thanked for providing a computer programme which was used when carrying out statistical analysis. I am also indebted to the Cancer Research Campaign for providing the funds which made this work possible.

Finally, I wish to thank Dr. N. Wilmott and Mr. R. Nicholson for their advice and encouragement and Mrs M.B. Allison for her careful and patient typing of this thesis.

Declaration

Part of this work has been presented at the 21st Annual General Meeting of the British Association for Cancer Research, and other parts are to be presented in two papers which are currently in print.

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

S U M M A R Y

In recent years much interest has been generated in the concept of tumour agents or "signal factors" (Toporek, 1973) affecting host metabolic processes. It has been shown that tumour growth markedly alters the metabolism of and induces biochemical changes in various tissues and organs of the host (Theologides, 1972). These effects occur at sites remote from the site of tumour growth, therefore the possibility of the secretion of chemical modulators by the tumour cells must be considered.

Previous work (Calman and McAllister, 1975(a), 1975(b)) using a variety of experimental tumours has demonstrated metabolic abnormalities in the livers of tumour-bearing mice. Of the tumour systems studied the TLX-5 lymphoma is of particular interest, since a cell-free ascitic fluid preparation of this tumour, when injected into normal mice, will produce these same liver metabolic changes without subsequent tumour growth occurring (McAllister, Soukop and Calman, 1976). The most significant of these liver metabolite changes produced were an increase in citrate content and a decrease in coenzyme-A levels.

It was postulated, therefore, that a factor or factors present in this tumour cell-free preparation was responsible for producing these metabolic abnormalities, and the work described in this thesis demonstrates the presence of a factor in the ascitic fluid and serum of tumour-bearing mice responsible for increasing hepatic citrate levels. This work also demonstrates the presence of

other factors in ascitic fluid responsible for spleen hypertrophy, thymic atrophy and increases in epididymal fat pad weight.

Since ascitic fluid from mice bearing TLX-5 lymphoma contains a multiplicity of host and tumour derived factors which may be involved in producing the observed metabolic changes, it was necessary to determine whether the factor or factors involved in increasing hepatic citrate levels was of host or tumour origin. It was shown likely to be of tumour origin, since it was present in the cell-free growth medium of TLX-5 cells grown in vitro.

This biologically active factor was also shown to be non-dialysable, heat labile and protein in nature, since treatment of cell-free ascitic fluid with trypsin destroyed its biological activity.

High speed centrifugation of the cell-free ascitic fluid preparation did not affect its biological activity, and resulted in considerable purification of the preparation in terms of protein content, as well as removing any virus particles present. The problem of viral contamination of TLX-5 lymphoma was investigated, and in vivo grown tumour cells were shown to be contaminated with a virus. The factor involved in increasing hepatic citrate levels is, however, unlikely to be viral in nature since it is present in virus free ascitic fluid preparations, and in a preparation of in vitro grown tumour cells which have been shown to be free of virus.

The viral contaminant of the in vivo grown tumour cells may be the Riley virus or lactate dehydrogenase

virus (LDV) since it has been shown to be a widespread contaminant of murine tumours. Electron microscope examination of the in vivo grown cells revealed a virus particle with a similar size and shape to LDV, but this particle could not be detected in the in vitro grown cells.

Injection of the in vivo grown cells into normal mice did produce an elevation of serum lactate dehydrogenase activity in these animals, which is a characteristic effect of LDV. Injection of virus-free ascitic fluid (SN2) into normal animals also produced this effect, therefore it would appear that a factor or factors present in the tumour cell preparation and in the virus-free preparation are responsible for producing this effect.

Gel filtration and gel electrophoresis experiments using SN2 indicate that the factor present in SN2 responsible for increasing hepatic citrate levels has a molecular weight between 6,800-7,600 daltons. Finally, this factor was shown not to be insulin, since injection of normal mice with insulin does not result in an elevation of hepatic citrate levels.

FOREWORD

"Although the path be tortuous and slow,
Although it bristle with a thousand fears,
To hopeful eye of youth it still appears
A lane by which the rose and hawthorn grow."

from (AT GRADUATION 1905) by T.S. Eliot.

I N T R O D U C T I O N

1. TUMOUR-HOST INTERACTION

(i) Cancer Cachexia

The profound effects of tumour growth on host metabolism have been reviewed extensively. Tumour growth markedly alters the metabolism of the host, and induces measurable biochemical changes in various tissues and organs (Theologides 1972). The resulting syndrome is referred to as cachexia, a condition described by Costa (1977) as being "characterized primarily by weakness, anorexia, and the depletion and redistribution of host components". According to Theologides (1972) the clinical features of cachexia are the most obvious "manifestations" of a profound "systemic derangement" of the host metabolism. Shapot (1979) made the observation, however, that cachexia should not be recognized as an obligatory manifestation of the tumour's systemic effect on the host.

Various hypotheses have been put forward in an attempt to explain the pathogenesis of cancer cachexia, and the differences between the metabolism of the tumour-bearing host and the non tumour-bearing animal. Although the mechanism underlying the capacity of malignant cells to grow destructively, infiltrating normal tissues, remains obscure, a popular view (Shapot, 1972) relating to the problem was that proteolytic enzymes or toxic substances liberated by tumours into their surrounding

environment, would specifically destroy normal cells.

There is a great deal of evidence that tumours produce biologically active compounds which affect the metabolism of normal cells (Sibley and Lehninger, 1949; Suda, Tanaka, Sue, Harano and Morimura, 1962; Begg, 1958; Costa and Holland, 1962; Argyris and Argyris, 1962; Rubin, 1970). Considerable support for this theory was generated by the isolation of "toxohormone" from tumour cells by Nakahara and Fukuoka (1949). When this biologically active compound was injected into normal mice, it was shown to stimulate some of the systemic effects of tumours in vivo (Kampschmidt, Adams and McCoy, 1959; Nakahara and Fukuoka, 1958).

Interest in the release of toxic substances by tumour cells was revived due to experiments by Holmberg (1965, 1968), who isolated a polypeptide that exerted a toxic effect on cells in culture. He was able to isolate this polypeptide from (a) ascitic fluid of Ehrlich carcinoma and Landschutz carcinoma: (b) interstitial fluid of solid transplantable tumours and (c) blood plasma of tumour-bearing mice. However, Shapot (1972) argues that Holmberg's data is irrelevant to the problem of destructive tumour growth, since he did not test the effect of this polypeptide on normal cell lines but on tumour cell lines.

On the other hand, Devlin and Costa (1964) considered that an alteration in the oxidative and energy conserving mechanisms of the host, as a result of tumour growth, may

be related to the cachectic syndrome. Devlin and Pruss (1958) detected the presence of a substance in the 25,000 gmax supernatant of a homogenate of the Novikoff hepatoma of the rat. This substance was able to uncouple the oxidative phosphorylation of normal liver mitochondria. Nanni and Casu (1961) were able to demonstrate the presence of such substances in the serum of sarcoma-bearing rats.

Moreover, Theologides (1972) proposed that alterations in the metabolic patterns of the tumour-bearing host, and changes in various metabolic equilibria and concentrations of metabolites, might be the result of allosteric transitions, activations and inactivations of various enzymes in the host tissues. Tumour cells, by producing low molecular weight molecules, particularly polypeptides, would modify the activity of these host enzymes, thus throwing host metabolism into a state of chaos.

The profound alterations of host organs and functions during tumour-growth is considered by Costa (1977) to be a result of multiple interactions between destruction, attempted repair, homeostasis, and the production of biochemical toxins by the tumour. Much attention has been focused on distant metabolic effects produced by tumours, and since the degree of cachexia bears no simple relationship to tumour type or burden, nor can it be correlated to the nutritional status of the host, Costa (1977) considers the postulation of chemical mediators

released by the tumour tissue, inescapable.

Begg (1958) concludes that tumours release substances into the host circulation, which produce structural and functional changes in the tissues of the host, as well as facilitating the concentration of metabolites in the tumour to the detriment of the host.

Examples of the systemic effects of tumours, which are features of the tumour-host relationship (Begg, 1958) may be found in alterations of enzyme activity, metabolism, nutrition, hormone balance, composition of the blood and in the structure of tissues. It becomes clear, therefore, that although cancer cachexia is widely recognized, the mechanisms involved in producing this symptom-complex are still poorly understood.

In a review of tumour and host relationships, Shapot (1979) distinguished between two principle and inter-related groups of manifestations of the systemic effects of tumour growth. One of these groups consists of changes in the metabolism and hormonal balance of the host caused by successful competition of the neoplasm with normal tissues for important metabolites and trophic factors. The other may be defined as the influence on distant tissues manifested by decreasing differentiation, changes in enzymic characteristics, diminution of the sensitivity of target tissues to the hormones that regulate their functions, and disturbances in the negative feedback systems which co-ordinate the activities of central and peripheral endocrine glands. Some of these

tumour-host relationships will be discussed in later sections of this thesis.

Since most studies on tumour-host relationships have used animal model systems, the extent to which these systems are relevant to that of the cancer patient is still obscure. Most animal tumours studied are either induced or transplantable, rapidly growing malignancies, in contrast to the situation of the cancer patient in which the tumour arises spontaneously and may be slow-growing.

Nevertheless, a comparative study of the influence of tumour growth on host metabolism in mice and man has been carried out by Lundholm (1978). He demonstrated that a close similarity exists between metabolic alterations in skeletal muscle and liver tissue from cancer patients and that of mice bearing a transplantable methylcholanthrene-induced, non-metastasizing tumour. These results reinforced the relevance of using experimental animal models in the search for the mechanisms behind the debilitating effects of tumour growth.

A major determinant of cancer cachexia is anorexia. Theologides (1972) described the clinical features of cachexia, characterized by anorexia, as the most "obvious manifestations" of a profound "systemic derangement" of host metabolism. He later proposed (1979) that peptides, oligonucleotides and other small metabolites produced by the tumour cells contributed to the genesis of anorexia

via a peripheral effect on neuroendocrine cells and neuroreceptors, and through a direct effect on the central nervous system. Additional factors, including emotional and psychological reactions to the disease, changes in the senses of taste and smell, would all contribute to progressive emaciation of the host. The pathophysiology of anorexia in the tumour-bearing animal has been difficult to study according to De Wys (1977) because of multiple variables that influence the clinical picture.

Many metabolic and physiological factors influence food intake, and simple starvation results in metabolic changes, some of which depress appetite. De Wys (1979) is of the opinion that the possible elaboration of anorectic peptides by the tumour cells may contribute to the anorectic syndrome in cancer patients.

(ii) Metabolic Aspects of Tumour Growth

(a) Carbohydrate Metabolism

According to Holroyde, Gabuzds and Putnam (1975) many facets of glucose metabolism in the host may be associated with the onset of cancer cachexia, and a study of the role of glucose recycling would only add to an understanding of this common clinical situation. Shapot (1972) is in no doubt that the process of tumour growth acts as a powerful hypoglycaemic factor in the host, and that this tendency to disturb the homeostasis of carbohydrate metabolism may result in the derangement

of host protein and lipid metabolism owing to a forced gluconeogenesis. Studies of gluconeogenesis in the liver and kidney of tumour and non-tumour bearing animals have been carried out. The liver is known to maintain normoglycaemia, altering the rate of gluconeogenesis in correspondence with fluctuations in the composition of the body's "inner medium" (Exton, 1972; Eisenstein, 1973). Cahill (1970) showed that the kidney is also involved in this process, since the inhibition of gluconeogenesis in both kidneys results in hypoglycaemia in the tumour-bearing animal within one hour.

Since gluconeogenesis is under multihormonal control, Shapot (1979) discusses several factors which may be responsible for the stimulation of gluconeogenesis in the host. Insulin would act as an inhibitor, whereas glucagon, epinephrine, growth hormone and glucocorticoids would accelerate endogenous glucose formation. The possibility of the hypersecretion of epinephrine is consistent with the phenomenon of a stimulated lipolysis in adipose tissues and muscle, described in the tumour-bearing host by Shapot (1974, 1979).

Moreover, there are many observations of the elevation of glucocorticoids in cancer patients (Saez, 1971, 1974) and tumour-bearing animals (Samundjan, 1973). According to Herrman and Staib (1969) glucocorticoids induce hyperglycaemia in rats, and raise the activity of key enzymes of gluconeogenesis. Hyperfunction of the adrenal cortex (Weber, 1968; Knox and Sharma, 1968;

Mertvetzov, 1969) may play an important role in the intensification of gluconeogenesis as a response to hypoglycaemic strain of the tumour on the host, and that normoglycaemia is ensured by stimulated gluconeogenesis from non- carbohydrate sources. The impairment of this system would result in hypoglycaemia. On the other hand, Suda, Tanaka, Sue, Harano and Morimura (1966) have reported a predominantly glycolytic pathway in the liver of rats hosting a Walker 256 carcinosarcoma.

Also, McAllister (1978) has shown that the activities of phosphofructokinase and fructose -1,6- diphosphatase are not altered in the livers of mice bearing a variety of experimental tumours, but there are changes in the hepatic content of acetyl-CoA and citrate (Calman et al; 1975, 1976) that are opposite to those found in animals under gluconeogenic conditions. McAllister concedes however that an increased citrate content in liver in vivo would favour gluconeogenesis by an inhibition of phosphofructokinase. He has shown, by measuring NADH/NAD ratios, that the cytoplasm of liver cells in these animals was in a more reduced state than normal, and this would favour gluconeogenesis.

It has also been shown (Engel, 1942; McFadzean and Jeung, 1956; Shonfield, Baggot and Gundersen, 1961; Landau, Wells, Craig and Leonard, 1962; Grusdeira, 1966; Carey, Pretlow, Ezdine and Holland, 1966; Tashiro and Sakurai, 1970) that the liver of tumour-bearers, both

humans and animals, contains relatively small amounts of glycogen. This was thought by Shapot (1972) to be a result of an increased need for glucose owing to the presence of the tumour. Equally probable is an impairment of the enzyme systems of the liver involved either in glycogen synthesis or its mobilisation, as a result of a toxic action of the tumour.

However, Greenfield (1954) believes it important to note that many enzymes in the tissues of the host do not appear to be affected by the presence of a tumour. Begg (1958) considers that in some manner the tumour produces a selective effect at a distance, with a resultant change in the activity of some tissue enzymes, and thus in the metabolism of the host, not wholly attributable to changes in the hormonal or nutritional status of the host, but implicating the secretion of a toxic factor by the tumour cells.

(b) Protein Metabolism

Much attention has been centralized on protein metabolism in the tumour-bearing host. Mider (1951, 1955) described the tumour as a "nitrogen trap", with nitrogen being lost to the host. Mischenko (1940) inferred that tumour growth is at the expense of assimilation of products of host's muscle protein breakdown. He demonstrated that even during starvation the host tissues supply the tumour with amino acids, and that cachexia may be caused by an intensified hydrolysis of host tissues. Begg (1958) believed that a prominent

tumour-host effect is the ability of the tumour to cause mobilisation of host protein for the benefit of the tumour.

The work of Mischenko and Mider was confirmed by Le Page (1952) who demonstrated that after radioactive glycine was incorporated into the tissues of tumour-bearing rats, subsequent starvation led to a decrease in the total activity of the organs of the rats, but to an accumulation of the radio-activity in the tumour. Greenlees and Le Page (1955) extended this study and showed that the tumour was indeed a "nitrogen trap" in the relative sense only, and does to some degree participate in other dynamic exchanges of metabolites within the body, whilst still having a tendency to accumulate amino acids.

Moreover, data derived by Fenninger and Mider (1954) from both spontaneous tumours in man and transplanted tumours in animals, indicate that nitrogen is stored during tumour growth as long as the dietary intake is high enough, and the body fat stores great enough to supply the demands of the host and the tumour for energy. The tumour, however, may continue to grow even in the presence of a negative nitrogen balance. The work of Lundholm (1975, 1976) supports the view of Shapot (1979) that the tumour somehow enhances protein catabolism of the host tissues, and may at the same time hinder protein synthesis in them. The possibility of the secretion of toxic factors by the tumour tissue cannot be excluded

from an investigation of the mechanisms involved.

However, Mullen, Buzby, Gertner, Stein, Hargrove, Oram-Smith and Rosato (1980) have shown a positive correlation between tumour protein synthesis rate and protein synthesis rate in adjacent tissue, from the organ of tumour origin, which they consider indicates that the rates of protein synthesis of tumour cells are primarily determined by the inherent nature of the tumour, rather than by other environmental or remote factors.

(c) Lipid Metabolism

Haven, Bloor and Randall (1949) showed that in rats bearing Walker 256 carcinosarcoma, carcass lipids varied inversely with the size of the tumour, and blood lipids were increased, in most cases to a very great extent. Mider (1948) concluded that the loss of lipid in the tumour bearer suggested that the growth of the Walker tumour imposes an increased energy requirement on its host.

Histological techniques employed by Dalton and Peters (1944) showed a decrease in stainable lipid in the adrenals of tumour-bearing mice, and Aoki (1938) reported a decrease in the concentration of adrenal fatty acids, cholesterol, cholesterol esters and phospholipids in rats bearing a hepatoma. Hyperplasia of the adrenals was

also characteristic of rats bearing large tumours in a study conducted by Haven (1949) who emphasized that the possibility of a hormonal explanation for the lipemia observed should be kept in mind.

Shapot (1979) suggested that the mobilized lipids are assimilated to a certain extent by the tumour, as precursors of its membrane lipids, but mainly by the host tissues which "switch over" to the oxidation of fatty acids as an energy source, owing to the deficiency of glucose intercepted by the tumour. Weber (1966) considered hyperlipidemia capable of limiting the utilisation of glucose by muscle tissue, whilst at the same time inducing the synthesis of enzymes involved in gluconeogenesis.

Moreover, Mider (1955) made the observation that tumour-bearing rats with lyophilised Walker 256 carcinosarcoma included in their diet maintained their appetite and body weight. Haven and Bloor (1956) demonstrated that the active principle involved in this phenomenon was in a phospholipid fraction extracted from the tumour. The administration of the phospholipid to cachectic tumour-bearers led to a gain in body weight and activity. Begg (1958) considered this to be the first demonstration of a tumour factor that counteracts tumour-host effects. He believed it possible that such effects are produced both by a "toxohormone" given off from the tumour, and by substances such as the phospholipid, concentrated in the tumour, and denied to the host.

(iii) Effect of Tumour Growth on the Morphology of Host Tissues

Many striking morphological and biochemical changes have been observed in tumour-bearing organisms. Some of these morphological changes will now be discussed.

(a) Liver

Ghadially and Parry (1965) undertook a study of the morphological changes in the livers of tumour-bearing mice in an attempt to establish a morphological basis for some of the observed biochemical differences between the liver of the normal and the tumour-bearing animal. A constant phenomenon associated with tumour growth is an increase in liver weight (Annau, Manginelli and Roth, 1951; Medigreceanu, 1910). McEwan and Haven (1941 have shown that a part of this increase is due to an increase in liver water content, and this is borne out by Ghadially and Parry (1965).

It is now clear that the increase in liver weight is also due to an increase in liver nitrogen content (Sherman, Morton and Mider, 1950; Yeakel and Tobias, 1951). Kay (1961) was of the opinion that protein is deviated not only to the growing tumour cells, but also to the liver of the cachectic host.

The reasons for these changes is unclear, but Harrison (1953) showed that malnutrition was not responsible, since in starved animals liver weight decreases. Maintenance of food intake did not alter the enlargement of liver (and spleen) observed by Stewart and

Begg (1953), that occurs in tumour-bearing rats. Also, Toporek (1973) has shown a decrease in the synthesis of serum proteins and an increase in the synthesis of liver proteins in the livers of tumour-bearing rats. These changes according to Toporek (1973) may involve a humoral factor secreted by the tumour-cells.

An interesting observation made by Ghadially and Parry (1965) was a marked increase in the number of lysosomes in the livers of tumour-bearing rats. They suggested that an increase in these organelles may be involved in the production of cachexia, since the lysosomal enzymes may be released and act at sites distant from their origin (De Duve, 1963). The liberated hydrolytic enzymes may then be regarded as a mechanism whereby normal tissues are broken down and metabolites mobilised to meet the metabolic needs of the growing tumour (Wiseman and Ghadially, 1958).

(b) Spleen

Hypertrophy of the spleen of tumour-bearing hosts has been frequently observed (Stewart and Begg, 1953; Konda and Smith, 1973; McAllister, Soukop and Calman, 1976) and is associated with histological changes.

Rapid enlargement of the spleen has also been observed in normal animals following the injection of various tumour extracts, in particular "toxohormone" (Nakahara and Fukuoka, 1958; Kampschmidt, Adams and McCoy, 1959). McAllister, Soukop and Calman (1977) also showed that the injection of a cell-free extract of TLX-5

lymphoma tumour into normal mice induced spleen hypertrophy. It was thought that these observations supported the view of Konda and Smith (1973) that hypertrophy of spleen is due to the immune response, and also suggest that tumour cell products may be involved in producing this effect, a phenomenon investigated in this study, which will be discussed later.

(c) Thymus

Atrophy of the thymus is a frequent finding in tumour-bearers. Konda and Smith (1973) observed a striking decrease in thymus cell numbers over the course of tumour growth. Although tempted to conclude that this was due to simple thymus involution similar to that observed following short-term cortisol treatment, they decided that these changes were not identical. They also presented indirect evidence that the functional, immunologically competent cells of the thymus, as in the spleen, were increased during tumour-bearing.

Ertl (1973) showed that in rats bearing the Walker 256 carcinosarcoma, the commencement of thymic atrophy could be related to the onset of progressive cachexia. These observations were confirmed by McAllister, Soukop and Calman (1977).

(d) Adrenals

Shapot (1972) considered that the adrenal cortex system of the tumour-bearer would be functioning under great strain due to hypersecretion of glucocorticoids which may be involved in the enhancement of gluconeogenesis

in these organisms.

According to several authors (Mizukami, Kozako and Nishio, 1969; Kawai, Tamura, Tanimoto, Honna and Kuzyya, 1969; Evgenieva, 1970) there is hyperplasia of the adrenal cortex and hypersecretion of glucocorticoids during the early phase of tumour growth. The later stages of tumour development are reported to be characterized by atrophy of the gland and depletion of the glucorticoid reserves. On the other hand, Nakata, Suematsa, Nakata, Matsumoto and Sakomoto (1964) have described increased hypophyseo-adrenal function and increases in plasma corticosterone levels in the advanced stages of tumour growth in rats.

Also, Shapot (1972) considers it possible that an elevated level of gluconeogenesis in the tumour-bearer is caused by the hypersecretion not only of glucocorticoids, but of epinephrine (Rinard, Kuno and Haynes, 1969) or glucagon (Salter, Ezrin, Laidlow and Cornell, 1960).

(iv) <u>The Effect of Tumour Growth on Biochemical Characteristics of Host Tissues; with particular reference to liver tissue</u>.

The liver plays a fundamental role in the metabolism of carbohydrate, fat and amino acids (Newsholme and Start, 1974) and it is in this tissue the metabolic pathways for these metabolites are closely integrated and regulated. It is for this reason that the following discussion will concentrate mainly on the effect of tumour growth on

several of the biochemical characteristics of liver tissue.

(a) Liver Catalase

Studies of liver catalase activity have been important in attempts to understand the malignant process (Kampschmidt, 1965). Since the work of Greenstein (1941, 1942) it has been known that the activity of catalase in the host liver is suppressed, and returns to normal after surgical removal of the tumour. Greenstein (1941, 1942) postulated the secretion of toxic substances by tumour cells in an attempt to explain the depression of liver catalase activity in tumour-bearing animals, but was unable to demonstrate the presence of these toxins in later experiments (1943).

In a later study, however, Nakahara and Fukuoka (1948, 1949, 1960, 1958) injected concentrated tumour extracts into normal mice and were able to influence liver catalase activity in these mice. This material was named "toxohormone", and has been the subject of extensive reviews (Busch, 1962; Nakagaura, 1952; Nakahara and Fukuoka, 1958).

However, Riley (1959) obtained a depression of liver catalase activity in mice by injecting homogenates of normal liver, kidney and spleen. Day, Gabrielson and Lipkind (1959) also found that spleen homogenates depressed liver catalase activity to an extent comparable with tumour tissue.

Toxohormone was also found to produce effects other than the depression of liver catalase. Fukuoka and

Nakahara (1952) observed that injections of toxohormone produced thymus involution. Kampschmidt, Adams and McCoy (1959) also observed a slight anaemia which develops 24-48 hours after a single injection of toxohormone, becoming more severe after multiple injections. Plasma iron was very susceptible to change with small doses of toxohormone (Fujii, Kawachi, Okuda, Haga and Yamamura, 1960) as were increases in the weights of liver, adrenals and spleen (Jakubovič, Keilová and Šorm, 1961; Kampschmidt, Adams and McCoy, 1959). Among many other effects, toxohormone also decreased liver coenzyme A levels (Kuzin, Sharoukhova and Chudinova, 1955). Most of these changes have been shown to be similar to those which occur in the tumour-bearer.

Toxic materials (Kampschmidt, 1965) still seem to offer the best explanation for the marked lowering of liver catalase activity, and the various effects mentioned above. Kampschmidt (1963) considered it unlikely, however, that the tumour tissue itself is the source of the toxin, and other possible sources could be bacterial or viral contaminants or reactions of the host animal to the presence of the tumour. The possibility that viral contamination of the tumour may be responsible for or involved in producing some of the observed effects of tumour growth, will be discussed in more detail in a later section of this thesis.

(b) LIVER COENZYME A

Various workers have investigated the levels of coenzyme A in animal tissue, particularly rat tissue,

following gluconeogenic stimuli such as starvation and the induction of diabetes (Start and Newsholme, 1968; Herrera and Frienkel, 1968) and following treatment with various hormones (Greenbaum Gumaa and McLean, 1972; Exton and Harper, 1972). Hornbrook, Buch and Lowry (1965) demonstrated changes in the levels of liver coenzyme A during glycogen synthesis induced by lactate and hydrocortisone.

However, there is little reference in the literature to the content of this regulatory intermediate in the livers of tumour-bearing animals. Shils, Friedland and Shapiro (1956) did measure the amount of coenzyme A and various vitamins in the liver and other organs of tumour-bearing mice, but did not investigate in non tumour-bearers. Earlier work by Higgins, Miller, Price and Strong (1951) measured the levels of coenzyme A in rat livers and tumours, and found that coenzyme A is present in appreciably lower amounts in rat liver tumours than in normal liver, but no detailed analysis of the levels of this metabolite in tumour and various other tissues had appeared before this work.

Since Rapp used a non-specific assay method for determining coenzyme A (Handschumacher, Muller and Strong, 1951) it is not possible to interpret his data in terms of the actual form of the coenzyme present, since this method also measures acetyl-coenzyme A, acyl thiol esters of coenzyme A and various precursors in the biosynthesis of the coenzyme (Cavallini, Mondovi, De Marco and Ferro-Luzzi, 1959). Rapp (1973) did suggest that the phenomenon he

observed, however, might be reponsible for some of the debilitating systemic effects observed during tumour growth.

Studies carried out by Calman and McAllister (1975a, 1975b) and McAllister, Soukop and Calman (1976) investigated in great detail the acetyl-coenzyme A and free coenzyme A content of liver in tumour and non tumour-bearing mice. These studies showed that in mice bearing the TLX-5 lymphoma, there were significant reductions in the hepatic contents of acetyl-coenzyme A, and free coenzyme A which could be detected as early as 24 hours after tumour implant. Total coenzyme A, which is the sum of acetyl-coenzyme A, oxidised coenzyme A and reduced coenzyme A, remained at significantly low levels throughout the period of the tumour growth. Two days after tumour implant acetyl-coenzyme A levels returned to normal. These early changes in coenzyme A levels occurred before visible signs of cachexia became evident, suggesting that cachexia was not a prerequisite for these metabolic abnormalities.

McAllister, Soukop and Calman (1976) also studied the levels of these metabolites in the livers of mice bearing Sarcoma-180 and C3H-mammary tumours, and found a similar pattern of changes occurring. The food and water intake of these animals was monitored and the overall situation found to be one of anorexia. Mice injected with a cell-free preparation of the TLX-5 lymphoma also showed decreases in hepatic coenzyme A levels, and in mice bearing the C3H-mammary tumour, these changes reverted to normal

after curative resection of the tumour.

(c) Liver Citrate

Dickens (1941) showed that a high concentration of citric acid was characteristic of rapidly growing tissue, including a variety of animal and human tumours. A study by Haven, Randall and Bloor (1949) showed that in rats bearing Walker-256 carcinosarcoma, the citrate content of liver, kidney and spleen was higher than that found in corresponding organs of control animals. It was suggested that the presence of neoplasm in the animal may have caused the various organs to assume "chemically neoplastic qualities" resulting in an increased formation or decreased utilisation of citrate.

The hepatic citrate content of tumour bearing mice has also been studied extensively by Calman and McAllister (1974a, 1975b) and McAllister, Soukop and Calman (1976), who observed significant increases in the hepatic citrate content of mice bearing the TLX-5 lymphoma, Sarcoma-180 and C3H-mammary tumours. In the case of the TLX-5 lymphoma-bearing mice, this change could be detected 24 hours after tumour implant. A similar pattern of changes was observed in mice bearing the other experimental tumours, and these changes were shown to return to normal after curative resection of the tumours of mice bearing C3H-mammary tumours.

The direction of changes in both citrate and coenzyme A levels described by these workers is opposite to that found in normal animals under conditions of forced

gluconeogenesis (Wieland, 1966; Start and Newsholme, 1968). McAllister, Soukop and Calman (1976) are of the opinion that a factor or factors released from the tumour cells cannot be excluded from the aetiology of these changes. The evidence they present for this is, as previously mentioned, the ability of a cell-free preparation of TLX-5 lymphoma cells to bring about these liver metabolite changes, and the reversion of these changes to normal after the curative resection of tumour in mice bearing the C3H-mammary tumour.

It is of interest that McAllister, Soukop and Calman (1976,1977) also examined the influence of TLX-5 lymphoma on mouse spleen, thymus and epididymal fat pad weight. Their results show that thymus weight decreases significantly 24 hours after tumour implant and continues to do so over the period of tumour growth. Spleen weights were shown to increase at about two days of tumour growth, with a concomittant increase in epididymal fat pad weight.

(V) Tumour-Derived Metabolic Modulators

In previous sections of this Introduction, a great deal of reference has been made to the concept of tumour-derived toxins or chemical mediators (Kampschmidt, Adams and McCoy, 1959; Okudu, Ikagami and Fujii, 1972; Olivares, 1970; Ohashi and Ono, 1959; Nakahara and Fukuoka, 1958, 1949; Greenstein, Jenrette and White, 1941; Shapot, 1972, 1979; Costa, 1979). Costa (1977) believed that the unambiguous demonstration of the existence of the tumour toxins, and the definition of their role might be a

significant breakthrough in oncology. In consequence, a great deal of work has been carried out in attempts to elucidate and characterize substances of tumour origin which are responsible for modifying the tumour environment in order to facilitate its growth. 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, 1977). Perhaps the most well documented tumour toxin or metabolic modulator is "toxohormone", which has been discussed, previously.

Recently another phenomenon has been elucidated, that of tumour angiogenesis. Experiments by Greenblatt and Shubik, (1978) suggest that tumours stimulate neovascularization by releasing a "diffusible" material. This material can be isolated from both the solid and ascites forms of tumours (Folkmann, Merler, Abernathy and Williams, 1971) and is known as tumour angiogenesis factor (TAF). The presence of TAF in tumour cell cytoplasm and nucleus, and in tumour cell cultures has been demonstrated (Tuan, Smith, Folkmann and Merler, 1973; Folkmann, 1974). TAF has been purified from tumour cell nuclei (Folkmann, Merler, Abernathy and Williams, 1971) and found to be a non-histone protein. Tuan, Smith, Folkmann and Merler, (1973) then showed this protein to be capable of producing neovascularization in vivo, and in later work, Weiss, Brown, Kuman and Phillips (1979) isolated a low molecular weight, non-protein component from rat Walker-256 tumours, which was highly active in inducing

angiogenesis in vitro, and capable of stimulating the growth of endothelial cells in culture. It is thought that once the chemical nature of this angiogenic factor is established, it may have both prophylactic and diagnostic implications in human malignant disease (Weiss, Brown, Kumar and Phillips, 1979).

There are many other reports in the literature concerning the elucidation of tumour-derived toxins. Ting, Tsai and Rogers, (1977) reported the presence of a humoral factor in the ascitic fluid of mice bearing an intraperitoneal tumour, and in the pleural effusions of cancer patients. This factor was found to promote the growth of a murine tumour and to suppress cell-mediated immunity. It was also shown that host animals who recovered from the immunosuppressive state produced a serum factor that could neutralize the immunosuppressive effect of the humoral factor, and this counter-factor may play an important role in the control of tumour growth.

Moreover, Henriksen and Law, (1977) presented data suggesting that mKSA and L1210 cell-free ascitic fluids contain a component which is able to prolong the "immunologically paralysed state" of tumour-bearing animals, and involves a continuous interaction between tumour cells and host. This component has been partially purified, and is described as a low molecular weight, neutral compound. The authors suggest that if all oncogenic tumours secrete, or induce the host to manufacture tumour growth-promoting substances, then the

isolation and chemical characterisation of these substances could be of great diagnostic and therapeutic importance.

More recent work carried out by Cheung, Cantarrow and Sundharadas, (1979) described another low molecular weight factor produced by various mouse tumours and by mKSA and LLC cells in tissue culture. This is a macrophage modulating factor which affects closely related macrophage properties such as spreading and migration. It also inhibits lipopolysaccharide-induced tumoricidal activity of macrophages, and is thought to be of tumour origin. The point is made, though, that tumour tissue is known to contain host cells, bacteria and in some cases, viral contamination. Degradative events may be occurring in the vicinity of the tumour cells, hence it may be argued that this factor may not be a product of tumour cells. Some mouse tumours are known to contain lactate dehydrogenase virus, (Riley, 1960) and the appearance of this macrophage modulating factor may possibly be related to the presence of this virus. This possibility was ruled out by the observation that LLC and mKSA cells cultured <u>in vitro</u> for five passages did not contain the virus, but contained macrophage-modulating factor (Cheung, Cantarrow and Sandharadas, 1979).

Other examples of tumour-derived chemical mediators include the isolation by Nair and De Omo (1973) of a soluble fraction from solid, spontaneous, primary mouse mammary tumours which stimulated the growth of density-inhibited mouse embryo cells in monolayer culture. The

possibility of this stimulatory effect being due to the presence of a virus was excluded. Nepom, Hellström and Hellström, (1979) were able to purify and characterise a tumour specific blocking factor from the sera of mice with growing, chemically-induced sarcomas, and Hellström, and Hellström (1979) demonstrated the enhancement of tumour growth by tumour-associated blocking factors. Elleman and Eldinger, (1977) found suppressive factors in the ascitic fluid and sera of mice bearing several types of ascites tumour. Their results suggest the existence of a wide-ranging series of tumour and host factors that influence both tumour growth, and immunological responses in vitro.

Most of the recent work studying tumour-derived chemical mediators has concentrated on the isolation of substances which affect measurable immunological parameters. On the other hand, Vaage and Agarwall (1977) used the production of ornithine decarboxylase as an indicator of mitotic activity in neoplastic cells. Their results show that the level of production of ornithine decarboxylase in these cells can be used to detect, in serum and malignant effusion fluid, factors which are associated with progressive neoplastic growth, and which may influence the replication of neoplastic cells.

Moreover, McAllister, Soukop and Calman (1976,1977) were able to demonstrate the existence of factor(s) in the cell-free ascitic fluid from TLX-5 lymphoma-bearing mice which was able to influence liver metabolism in normal mice, as measured by increases in hepatic citrate content

and decreases in hepatic coenzyme A levels. An attempt was therefore made in this study to isolate and characterise the factor or factors present in this cell-free tumour preparation responsible for increasing hepatic citrate levels in normal mice, to determine whether it was a host or a tumour product, and whether or not the observed effects were caused by viral contamination of the tumour cells. Experimental procedures and results will be discussed in later chapters of this thesis.

The TLX-5 Lymphoma tumour model was chosen for this study since ascitic fluid from mice bearing this tumour contains a multiplicity of host and tumour factors which may be involved in producing the observed metabolic abnormalities. Large volumes of ascitic fluid can be readily obtained from relatively few tumour-bearing mice. The tumour is also fast-growing since it will kill mice injected intraperitoneally with 2×10^6 viable cells within 7-8 days.

2. VIRAL CONTAMINATION OF EXPERIMENTAL TUMOURS

In search of a simple diagnostic test for the early detection of malignant disease, many investigators have looked for differences in the activity of various serum or plasma enzymes in the tumour and non tumour-bearing host. Hill and Levi (1954) found that the enzyme lactate dehydrogenase (LDH) was elevated in the serum of a number of patients with malignant disease. Hsieh, Suntzeff and Cowdry (1955) reported similar findings in tumour-bearing mice.

While studying the relationship between enzyme elevation and tumour growth, Riley (1960) found that when plasma or cell-free extracts from the tissues of the tumour-bearing mice were injected into normal mice, the recipients developed a significant increase in serum LDH activity within 48 hours. Subsequent studies showed that the LDH agent was a virus (Bladen, 1969; Notkins, 1965). This lactate dehydrogenase virus (LDV) has been shown to be carried as a contaminant by a number of serially transplanted mouse tumours (Notkins, 1965).

In addition to increasing serum LDH levels, a number of other enzymes were shown to be elevated in the serum of mice infected with LDV, in particular isocitrate dehydrogenase (Notkins, 1965). Because of the increase in glycolytic enzyme activity in the serum of infected mice, Riley (1963) studied the glycolysis of infected and non-infected tumours. He found that the anaerobic glycolysis of a methylcholanthrene-induced tumour which had been infected with LDV was higher than its non-infected counterpart, but the reasons behind this are unknown.

There are several ways in which tumours can be freed from LDV contamination. One example is by passaging the tumour through rats (Notkins, Berry, Moloney and Greenfield, 1962). Another method is by passaging the tumour through cell lines which do not support LDV replication, or by several passages through tissue culture (Plagemann, Watanabe and Swim, 1962).

Later work by Mergenhagen, Notkins and Dougherty

(1967) has shown that the infection of adult mice with LDV enhances the antibody response of the mice to intraperitoneal injections of human gamma-globulin, showing that the virus is acting as an immunological adjuvant. On the other hand, a report by Riley and Speckman (1976) found the LDV to function as an immunosupressant and tumour-enhancing agent through its adverse effects on T and B lymphocytes. The destruction of T lymphocytes and host tissue by the virus is apparently caused indirectly through a typical stress response that includes an elevation in plasma corticosterone, which is known to "attack" lymphoid cells and tissues (Santisteban and Riley, 1973; Spackman and Riley, 1976).

All of these findings emphasize the need to screen for the presence of this ubiquitous virus, especially in experimental systems in which tumour lines are maintained by passage in mice, and where there is a high risk of cross-infection, as after surgical manipulation.

However, this LDV agent has not been identified in species other than mice (Notkins, 1965), and it is interesting to note that Hill and Levi (1954) found elevated serum LDH levels in patients with malignancies. Bodansky and Scholler (1956) confirmed the increase in serum LDH activity in rats, and Begg (1958) considered that these observed increases in serum LDH were a result of the release of enzyme from the tumour, but this has not been confirmed.

Since this study involved working with a murine tumour, the TLX-5 lymphoma, and various cell-free preparations of this tumour, it was thought necessary to screen for the presence of the LDV in the tumour cells. The first method of detection involved electron microscope examination of the tumour cells. The second method involved measuring LDH activity in the serum of mice injected with tumour cells or tumour cell-free preparations, and comparing these levels with those of normal mice.

In order to eliminate the presence of this agent from the tumour cells, the cells were passaged through tissue culture for at least five passages to ensure they were free from contamination. Cell-free tumour preparations were ultracentrifuged at 240,000g<u>max.</u> for 24 hours to eliminate any viral particles present. Results of this work will be discussed in later chapters.

OUTLINE OF PRESENT STUDY

This present study looked at the factor or factors present in the ascitic fluid of mice bearing the TLX-5 lymphoma, responsible for increasing hepatic citrate levels, as described by Calman and McAllister (1975a, 1975b), and McAllister, Soukop and Calman (1976, 1977). It was not known whether this biologically active factor(s) was of host or tumour origin, and the possibility of viral contamination of the tumour being involved in producing the observed metabolic effects (Notkins, 1965) could not be discounted. Tumour cells grown both *in vivo* and *in vitro* were examined using electron microscopy to determine whether or not a virus was present. Ultracentrifugation of a tumour cell-free preparation was carried out to remove any viral particles present, without removing or in any way affecting the biological activity of the preparation. The biological activity was determined by measuring hepatic citrate levels in mice injected with the preparation and comparing these levels with those of mice given a placebo injection.

Biological activity was also detected in the "conditioned" cell-free growth medium from *in vitro* grown tumour cells, indicating that the factor or factors involved is primarily of tumour cell origin.

Characterisation of the active factor(s) present in the cell-free ascitic fluid from tumour bearing animals involved heat-treatment, dialysis, trypsin treatment and ammonium sulphate fractionation of the preparation. This was to determine if the factor(s) was protein in nature.

Ammonium sulphate precipitation of the cell-free preparation resulted in a considerable purification in terms of protein content, as did ultracentrifugation.

It was then necessary to obtain a relatively homogeneous sample of this biologically active factor(s) in order to determine molecular weight. Gel filtration is a valuable technique for the isolation and purification of substances of biological origin, particularly proteins (Andrews, 1970), and can also be applied to the determination of molecular weights of unknown compounds. Sephadex is particularly useful for the chromotography of biological substances, as there is no irreversible retention of small amounts of substances on the column. It is therefore useful for analytical purposes. This method was used to fractionate virus-free ascitic fluid, to attempt to isolate the biologically active component(s).

Another method which can be used to determine the molecular weights of unknown compounds, particularly proteins, is gel electrophoresis in polyacrylamide gels. The homogeneity of samples during purification procedures can also be determined using this technique. The method has the advantage of rapidly separating complex mixtures of proteins with high resolution (Brewer, Pesce and Ashworth, 1974; Zahler, 1974) and requires only small amounts of protein. For these reasons this method was employed to determine the homogeneity of fractions obtained from gel filtration experiments, which had demonstrable biological activity. The molecular weight

of proteins present in these biologically active fractions was also estimated.

There were therefore 3 main areas of study involved in this work, and these are summarized below:-

(1) The effects of TLX-5 lymphoma cells and cell-free extracts on mouse hepatic citrate levels, and characterization of the factor (s), present in ascitic fluid from mice bearing TLX-5 lymphoma, responsible for increasing hepatic citrate levels.

(2) The fractionation and isolation of this factor (s) from ascitic fluid and the determination of its molecular weight.

(3) An investigation of the possibility that this metabolic effect of cell-free extracts of TLX-5 lymphoma was due to contamination of the tumour by viruses.

CHAPTER TWO

MATERIALS AND METHODS

1. TUMOUR AND ANIMALS USED

(i) Animals

In all studies reported, 3-4 month old, male CBA mice were used. These mice were from an inbred strain obtained from the Animal House of the University Department of Surgery, and maintained in the Animal House of the University Department of Clinical Oncology by conventional inbred mating systems, on a CRM diet (Labsure).

All mice were kept on a 12 hour light-dark cycle, and given water and food ad libitum. Prior to the beginning of each experiment mice weighing 25-35 grammes were selected and allowed to adapt to their new environment for at least 2 days, to prevent undue stress occuring which might adversely affect experimental results.

(ii) Tumour

The tumour used was the TLX-5 lymphoma. This tumour was first induced by Dr. D.I. Connell at the Institute of Cancer Research, London, by whole body X-irradiation of CBA mice. The enlarged thymuses were subsequently transplanted into CBA mice (McAllister, 1978). Male CBA mice bearing an ascitic form of this tumour were obtained from the Animal House of the University Department of Surgery, and the tumour was routinely passaged every 7 days thereafter.

Histological examination of the tumour cells revealed

that the tumour is extremely invasive, poorly differentiated and uncapsulated (Willmott, *et al* 1980). Histological examination of the livers of mice bearing this tumour (Willmott, *et al* 1980) revealed liver metastasis.

(iii) Routine Tumour Passage

The tumour was passaged routinely every 7 days. Mice bearing the ascitic form of the tumour were sacrificed and the carcasses swabbed with absolute alcohol. Ascitic fluid was removed from the peritoneal cavity using a sterile needle and syringe, and the number of tumour cells counted using an Improved Nebauer Counting Chamber. The number of viable cells were determined using Trypan-blue (Paul, 1970), and the cells diluted to a concentration of $2x10^7$ viable cells per ml, with sterile phosphate-buffered saline (PBS: 8.0g NaCl, 1.21g K_2HPO_4, 0.34g KH_2PO_4 per litre distilled H_2O). Normal mice were then injected with 0.1ml of this cell suspension, intraperitoneally (i.p.). These animals were sacrificed 7 days later and the procedure repeated.

(iv) Control Animals

All animals used in control experiments were injected i.p. with 0.1ml of sterile PBS, unless otherwise stated.

(v) Preparation of Tumour Extracts

(a) Cell-free ascitic fluid (Supernatant-1, SN1)

Tumour cells were harvested as previously described. Ascitic fluid from several mice was mixed and the number of viable tumour cells counted. The ascitic fluid was then diluted with sterile PBS to give a viable cell concentration of $2x10^7$ cells per ml, unless otherwise indicated.

* Visking dialysis tubing was used (Macfarlane Robson Ltd.). The tubing was rehumidified before use by placing it in boiling water for 5 minutes.

The ascitic fluid was then subjected to high speed centrifugation at 4°C in a Beckman L5-50 Ultracentrifuge. This was carried out for 4 hours at 100,000gmax. and produced a supernatant from which all the sub-cellular organelles, as well as the tumour cells, had been removed (Figure 1, SN1). The supernatant was then carefully removed and stored at -70°C, if not immediately required. To ensure that SN1 was completely free of tumour cells, at least 2 mice were inoculated with the preparation and observed for 21 days to find out if tumour growth occurred.

(b) Virus-free ascitic fluid (Supernatant-2, SN2)

SN1 was prepared and subjected to high speed centrifugation at 4°C in a Beckman L5-50 Ultracentrifuge, for 24 hours at 240,000gmax. This produced a supernatant from which all viruses had been removed (Notkins, 1965). The supernatant was then removed and stored as for SN1.

(c) Heat-treated SN1 and SN2

10ml aliquots of SN1 and SN2 were heated to 100°C, with stirring, for 5 minutes, then cooled rapidly on ice. Precipitates were then removed by filtration.

(d) Dialysis of SN1 and SN2

5ml aliquots of SN1 and SN2 were dialysed for 24 hours at 4°C, with gentle stirring, against 1 litre of sterile PBS.*

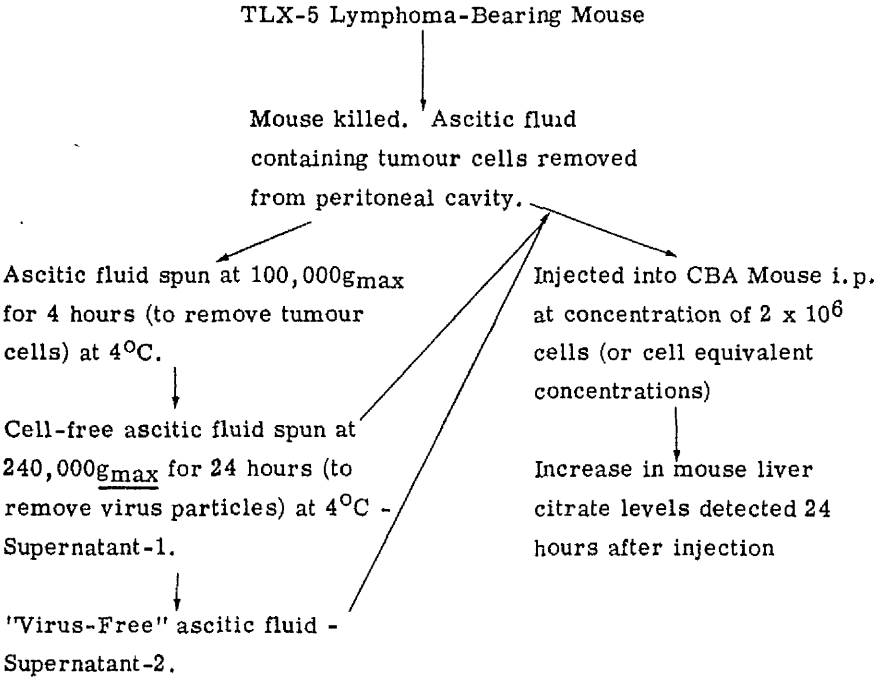
(e) Ammonium sulphate precipitation of SN1 and SN2

0-35 per cent saturation

4.85g $(NH_4)_2SO_4$ was added, slowly, with stirring, to 25 ml of SN1 and 25ml of SN2, at 4°C. The samples were then centrifuged at 4°C for 15 minutes at 20,000r.p.m., in

Figure 1

PREPARATION OF CELL-FREE ASCITIC FLUID
AND "VIRUS-FREE" ASCITIC FLUID



a Sorvall RC-5 centrifuge. The supernatants were removed and retained for further precipitation with $(NH_4)_2SO_4$. The pellets were redissolved in 25ml of sterile PBS, and represented the 0-35% $(NH_4)_2SO_4$ fraction.

35-65% $(NH_4)_2SO_4$ precipitation

4.6g $(NH_4)_2SO_4$ was added to SN1 supernatant retained from the previous precipitation step. This was added slowly, with stirring, at 4°C. The sample was centrifuged, as described in the previous step, and the pellet redissolved in 25ml of sterile PBS.

Traces of $(NH_4)_2SO_4$ were removed from the redissolved precipitates by dialysis at 4°C for 24 hours against 2 litres sterile PBS.

(f) Treatment of SN2 with Trypsin

25mg of trypsin (Sigma) was added to 1ml of SN2 and to 1ml of sterile PBS, samples were vortexed and incubated at 25°C for 30 minutes. 31.25mg of trypsin inhibitor (Sigma) was then added to each sample, vortexed and incubated at 25°C for a further 30 minutes. Control samples of SN2 and sterile PBS were incubated at 25°C for 60 minutes. Biological activity was determined for each sample.

(g) Resuspension of SN2 pellet (obtained from (b))

The pellet obtained during the preparation of SN2 was resuspended in an equivalent volume of sterile PBS by extensive homogenisation. In order to keep the sample cool, this process was carried out at 4°C. A control sample of SN2 was treated in the same way.

(vi) Treatment of Animals

Tumour cell suspension and cell-free preparations described in (v (a) - v (g)) were injected i.p. into experimental mice at a cell or cell-equivalent concentration of $2x10^6$ per 0.1ml, where mice were given 0.1ml. Mice were routinely killed 24 hours after injection, unless otherwise indicated, and their livers rapidly removed and hepatic citrate content determined, as a measure of the biological activity of the various preparations.

(vii) Sacrifice of Animals

All animals were killed rapidly by cervical dislocation. This was to prevent any undue stress to the animal which might adversely affect experimental results.

2. PREPARATION OF TISSUES AND ENZYMATIC DETERMINATION OF CITRATE

(i) Preparation of Tissue

Freeze-clamping

The clamp used was constructed from an ordinary set of pliers 26cm in length, to which had been welded 2 stainless steel plates whose dimensions were 6cmx5cmx1.5mm. These plates were of such a size that a mouse liver, when freeze-clamped, did not extrude beyond their edges.

Before freezing the liver, the tongs were pre-cooled in an aluminium tray containing liquid N_2. A stoneware mortar and pestle were also pre-cooled in this way. Preweighed homogenising vessels (Uniform) with a capacity of 15ml were precooled in Dry-Kold for at least 1 hour before use.

The mouse was killed and the liver rapidly exposed by a transverse upper abdominal incision, the liver removed, placed onto the precooled plate of the clamp, immediately clamped and the whole immersed into liquid N_2 (Calman and McAllister, 1975a, 1975b). The frozen liver was kept immersed in liquid N_2 for at least 3 minutes, then was reduced to a fine powder using the precooled mortar and pestle. To prevent the tissue thawing out during this procedure a small volume of liquid N_2 was added to the mortar. The powdered liver was then transferred to a preweighed, precooled homogenising vessel. After a rapid reweigh, the samples were stored in Dry-Kold or at -70°C.

(ii) Extraction and Assay of Liver Citrate

The reactions involved in the enzymatic determination of citrate are;

$$\text{Pyruvate} + \text{NADH} \xrightarrow{(1)} \text{Lactate} + \text{NAD}$$

$$\text{Citrate} \xrightarrow{(2)} \text{Oxaloacetate} + \text{Acetate}$$

$$\text{Oxaloacetate} + \text{NADH} + H^+ \xrightarrow{(3)} \text{Malate} + \text{NAD}$$

The enzymes catalysing the above reactions are:-

(1) Lactate dehydrogenase (E.C. 1.1.1.27), (LDH).

(2) Citrate Lyase (E.C. 4.1.3.6), (CL).

(3) Malate dehydrogenase (E.C. 1.1.1.37), (MDH).

Changes in the absorbance of NADH at 366nm. were measured The extinction coefficient of NADH being 3.30 at this wavelength. The procedure for determining citrate is that described by Bergmeyer (1965).

Reagents

(1) 0.6 M perchloric acid.

(2) 0.2 nM citrate standard.

(3) 0.03 M zinc chloride.

(4) 0.1 M triethanolamine buffer.

(5) 0.12 M sodium hydrogen carbonate.

(6) 10 mg β-NADH (reduced form) dissolved in 1.28ml of solution (5).

(7) 598U/ml LDH suspension.

(8) 1495U/ml MDH suspension.

(9) 100U/ml CL suspension.

All solutions and suspensions were kept under refrigeration. Enzymes and NADH were supplied by Boehringer, London. Solution (6) was used within a few hours of preparation. Solutions (1)-(4) could be kept up to 12 months if refrigerated, and according to the manufacturers, (Boehringer Mannheim) LDH and MDH suspensions were stable for several months if stored dessicated, under refrigeration. The CL suspension lost about 10% activity within 1 month of preparation.

Methods

4.0ml ice-cold perchloric acid was added to frozen, powdered liver in the homogenising vessel (the weight of liver present being first determined). This was mixed rapidly, then immediately homogenised using a precooled teflon pestle, with two series of passes at full speed. Samples were kept on ice for 30 seconds between each series of passes. A Potter Elvehjen (1936) type homogeniser was used, driven by a motor (Citenco) which

gave a maximum speed of approximately 7,200r.p.m..

The homogenate was centrifuged at 3,000gmax. for 10 minutes at 4°C, and the pellet discarded. Perchloric acid was removed from the tissue extract (supernatant) by precipitation with potassium carbonate. The pH of the supernatant was adjusted to 7.5 using 2M potassium carbonate, the final adjustment being made with 0.01M potassium carbonate. The sample was cooled on ice for 10 minutes and the precipitate removed by centrifugation at 3,000gmax. for 15 minutes at 4°C. The volume of the supernatant was then adjusted to 10ml with triethanolamine buffer. The citrate content of this tissue extract was then determined spectrophotometrically. A Gilford-250 Spectrophotometer was used, and measurements made at a wavelength of 366nm., pathlength 1cm and temperature of 25°C, in glass cuvettes, against air.

The following were pipetted into the cuvettes:-

triethanolamine buffer	2.0ml
β-NADH	0.06ml
zinc chloride	0.01ml
sample	0.09ml
LDH	0.01ml
MDH	0.01ml

After mixing, the absorbance was allowed to stabilise, and the final absorbance, (A1), noted. Then 0.01ml CL was added to cuvette, mixed, and the absorbance noted at 1-minute intervals until the reaction stopped (about 10 minutes). The final absorbance, (A2), was noted. A

* A recovery of between 90-95.5% was always obtained using the citrate standard solution.

standard citrate solution was included with each set of samples to be assayed. 0.09 ml of the standard solution diluted to a concentration of 0.18 uM was used.*

Citrate levels were calculated from:-

$\Delta A = (A1 - A2)$

$\Delta A \times 1.01 \times \frac{\text{supernatant volume (ml)}}{\text{weight of liver used (g)}}$ = umol. citrate per gram liver wet weight (Mc Allister, 1978)

3. STABILITY OF BIOLOGICAL MATERIALS AT -70°C.

(i) Stability of Citrate in Frozen, Powdered Liver

The livers of 10 untreated mice were freeze-clamped and powdered, as previously described. The powdered liver samples were "pooled", samples of approximately 700mg removed and their citrate content assayed. The remainder of the powdered livers was stored at -70°C, and samples removed at intervals up to 14 days and the analyses repeated.

(ii) Stability of SN1 and SN2

SN1 and SN2 were prepared as previously described. Aliquots were immediately injected i.p. into 2 seperate groups of 6 mice. The remainder of SN1 and SN2 was stored at -70°C. The treated mice were killed 24 hours after injection and their liver citrate content measured. This procedure was repeated at varicus time intervals up to 60 days, to determine the effect of storage at -70°C on the biological activity of SN1 and SN2.

4. DETERMINATION OF PROTEIN CONCENTRATIONS

(i) The Bradford Method

This method described by Bradford (1976) is a rapid

and sensitive method which can be used for the quantitation of microgram quantities of protein.

Reagents

(1) 1%(w/v) Coomassie Brilliant Blue G-250; 4.7%(w/v) ethanol and 8.5%(w/v) phosphoric acid.

(2) Standard Bovine serum albumin (BSA) solutions consisting of 5, 10, 15 and 20 ug BSA per 0.1ml PBS, respectively.

Method

0.1ml of standard protein solutions and samples were pipetted into separate 12 x 100mm test tubes. A blank consisting of 0.1ml PBS was also included. A volume of 2ml of the protein reagent (solution 1) was added to each tube, and the contents mixed by vortexing. The absorbance at 595nm. was measured after 2 minutes and before 60 minutes, in 3ml volume glass cuvettes against the reagent blank. The concentration of protein in the standard solutions was plotted against their corresponding absorbance, and linear regression used to find the line of "best fit" for the data points. This standard line was then used to determine the protein of the unknown samples. This method was sensitive when measuring protein concentrations in the range 10-100ug per ml. Samples with a protein concentration above this range were diluted with a known volume of PBS until the concentration fell within the measurable range.

(ii) Absorption of Protein at 230nm.

This method of measuring protein was also used, in most cases to supplement the data obtained using the Bradford method.

Reagents

Standard BSA solutions concisting of 5, 10, 15 and 20 ug per 2ml PBS respectively.

Method

The absorbance of samples and standards were measured at 230nm in 1ml volume quartz cuvettes against a PBS blank. The concentration of protein in the standard solutions was plotted against their corresponding absorbance, and linear regression used to find the line of "best fit" for the data points. The resultant standard graph was used to determine the concentration of protein in the unknowns.

5. ESTIMATION OF MOLECULAR WEIGHT BY GEL FILTRATION

Gel filtration is a valuable technique for the estimation of the molecular weights of unknown compounds, particularly proteins (Andrews, 1970). For the purpose of these studies Sephadex G-100 was used. This Sephadex-type has a molecular weight fractionation range between 4,000-150,000 daltons for peptides and globular proteins, and has a bed volume of 15-20ml per gram dry Sephadex (Pharmacia Fine Chemicals).

Preparation of the Gel

Since Sephadex G-100 has a minimum swelling time of 3 days at room temperature, the gel was allowed to swell for this period of time at room temperature, in excess PBS which was used as the solvent. The slurry was de-aerated before packing the column.

Packing the Column

The gel slurry was packed in a Pharmacia glass column with bed dimensions 1 x 30 cm. The column was mounted

vertically and the dead space under the supporting net and in the tubing filled with sterile PBS, which was used as the eluant. This was done by injecting the eluant into the outlet tubing and pumping it up through the bed support net. When the dead space was properly filled the outlet tubing was closed. The gel slurry was then poured carefully down the wall of the column, and the column completely filled with eluant. An eluant reservoir was connected to the top of the column and the last traces of air removed through the air vent in the column top piece. To obtain an even sedimentation, the flow was started soon after filling the column. Approximately 200ml of sterile PBS was allowed to pass through the column at a flow rate of 3.2ml per hour, in order to stabilise the column bed. A Gilson Minipuls-2 pump was used to maintain a controlled, steady flow rate. Before starting any experiments, the homogeneity of the bed was checked by running through a solution of Blue-Dextran 2000 (Pharmacia Fine Chemicals) at a concentration of 2mg per ml. The progress of the zone of this substance through the bed was monitored when 0.5ml was loaded onto the column, and the flow rate was 3.2ml per hour.

Procedure

A volume of 1.5ml of SN2 was layered onto the bed surface and 1.6ml fractions collected using a Gilson Microcol TDC-80 fraction collector at a flow rate of 3.2ml per hour. The protein content of the collected fractions was then determined by measuring their absorbance at 230nm. Since the gel in a newly packed column usually packs more

tightly the first time the column is used, this process was repeated 3 times, and the fractions obtained from the second and third runs retained, and assayed for biological activity.

Since more than 20 fractions were retained from each experiment, only those fractions containing measurable amounts of protein were assayed for biological activity, with a fraction containing no protein assayed as a control. Other controls included the initial starting material, SN2, and PBS which had passed through the column.

Biological activity was measured by injecting mice (10 per group) i.p. with 0.1ml of the eluted fractions, and measuring their hepatic citrate content 24 hours later.

Calibrating the Column

The usual method of column calibration (Andrews,1970) involves plotting a graph relating the molecular weights and gel filtration behaviour of standard compounds. The plot of elution volume, Ve, against the logarithm of molecular weight is now widely used, and gives a linear graph over the middle part of the working range of the column.

Reagents

Standard solutions of the following proteins were made up at a concentration of 2mg per ml of sterile PBS:-

Protein	Molecular weight x 10^{-3} (daltons)
cytochrome-C	12.4
chymotrypsinogen A	24.0
ovalbumin	45.0
BSA	67.0
aldolase	158.0

Procedure

Each protein solution was loaded onto the column and "run" separately. 0.5ml of the protein solution was loaded onto the column and fractions collected under conditions previously described. The protein content of each fraction was measured, and an elution profile was observed for each protein. The elution volume for each protein was measured at the position of half the maximum of the peak absorbance of the fractions collected.

The plot of the elution volume, Ve, against the logarithm of molecular weight was determined. Linear regression was used to determine the line of "best fit" for the data points. From the graph, the relative molecular weight of the biologically active component of SN2 was determined.

6 DETERMINATION OF MOLECULAR WEIGHT AND HOMOGENEITY BY POLYACRYLAMIDE GEL ELECTROPHORESIS

The molecular weights of proteins can be estimated by electrophoresis in polyacrylamide gels. The homogeneity of a protein sample during purification procedures can also be determined using this technique (Brewer, Pesce and Ashworth, 1974; Zahler, 1974).

Reagents for Gel Preparation

(1) 30% acrylamide - 1% methylenebisacrylamide.

(2) 1% N,N,N^1,N^1 - tetramethylenebisacrylamide.

(3) 1% freshly prepared ammonium persulphate

(4) 0.05% bromophenol blue.

(5) β-mercaptoethanol.

(6) 0.2M phosphate buffer.

(7) Standard protein solutions.

The following proteins were used at a concentration of 2mg per ml, dissolved in 0.01M phosphate-0.1%SDS-0.1% β- mercaptoethanol, pH 7.2

Protein	Molecular Weight x 10^{-3} (daltons)
Insulin	5.75
cytochrome-C	12.4
ovalbumin	45.0
BSA	67.0

Samples subjected to polyacrylamide gel electrophoresis were sample number 11 and 12 from gel filtration of SN2. These samples were found to be active in increasing hepatic citrate levels.

A method permitting the molecular weight determination of nanogram amounts of protein was used (Schultz and Wassarman, 1977). This method is based on the reaction of ^{3}H-labelled 1-methylaminonapth-alene-5-sulfonyl chloride ([^{3}H] dansyl chloride) with protein under denaturing conditions.

Reagents for the Reaction of [^{3}H] dansyl chloride with Protein

(1) 0.05M sodium bicarbonate - 1.4% SDS, pH 9.8.

(2) [^{3}H] dansyl chloride, 1uCi per ul in acetone.

(3) 5ug ovalbumin per ul solution.

(4) 25% trichloroacetic acid, 10% trichloracetic acid.

(5) 95% ethanol.

(6) 0.01M phosphate buffer-0.1% SDS-0.1% β-mercaptoethanol solution, pH7.2 (protein dissolving solution).

(7) Standard protein solutions, at a concentration of 2mg per ml, in sterile PBS. The proteins used were

insulin, cytochrome-C, ovalbumin and BSA. Samples were fraction number 11 and 12 from gel filtration of SN2 on Sephadex G-100.

Method

20ul volumes of standard and sample protein solutions were added to 30ul of 0.05M sodium bicarbonate-1.4% SDS solution in Eppindorf microfuge tubes, incubated at 60°C for 30 minutes, then 20uCi [^{3}H] dansyl chloride (20ul) added to the contents of each tube. The reaction mixture was then incubated at 37°C for 90 minutes. 10ul of ovalbumin was then added to each tube, followed by 90ul ice-cold 25% trichloroacetic acid. The pellet was precipitated by centrifugation in an Eppindorf-5412 centrifuge, at 4°C, and then washed seven times with 125ul portions of ice-cold 10% trichloroacetic acid. The pellet was then solubilised by incubation with 30ul protein dissolving solution (6) at 60°C for 1 hour.

Procedure for Electrophoresis of Samples

A Raven Scientific Electrophoresis Power Supply and Pharmacia Gel Electrophoresis Apparatus GE4 were used, and siliconised glass tubes of 100mm x 6mm (internal diameter). The gel mixture was made up as follows:-

5ml 0.2M phosphate buffer-SDS

3.3ml acrylamide solution

1ml Temed.

The mixture was degassed and then 0.7ml freshly-prepared ammonium persulphate solution added. The gel mixture was then pippeted into the glass tubes to a level of 80mm, and the gels overlayed with distilled H_2O and

allowed to polymerise (about 20 minutes).

To each of the standard protein and sample solutions was added:-

10ul 0.05% Bromophenol blue

1 drop glycerol

5ul β-mercaptoethanol.

These mixtures were incubated at 37°C for 30 minutes, and then carefully added to the top of each gel.

Phosphate buffer-SDS, diluted 1:1 with distilled H_2O, was used to fill the anode and the cathode compartments of the electrophoresis apparatus, and the gels run at 8 milliamperes per tube for 4 hours. The gels were then removed from the tubes, frozen using powdered Dry-Kold, and sliced into 1mm sections.

These sections were then placed into plastic scintillation vials and 10ml scintillation fluid added. The composition of the scintillation fluid was:-

5g PPO (2,5-diphenyloxazole)

0.5g BIS MSB (1,4-di-(2-methylstyryl)-benzene)

10ml NCS (tissue solubilizer, Amersham)

10ml hyamine hydrochloride

per litre toluene.

The gel slices were incubated at room temperature for 48 hours in this mixture (Aloya, 1979), then counted in a Liquid Scintillation Counter. The distance travelled by each protein relative to the boundary of the Bromophenol blue marker was measured at the position of maximum counts per minute in the gel slices. The Rf value for each protein was then calculated relative to cytochrome-C,

whose Rf was assumed to be 1. The calculated Rf values for each of the standard proteins was plotted against the logarithm of their respective molecular weights, and linear regression used to determine the line of "best fit" for the data points. This standard plot was then used to determine the molecular weight of the unknown components in fractions 11 and 12 and to determine the homogeneity of these samples.

(7) MEASUREMENT OF MOUSE SERUM LACTATE DEHYDROGENASE LEVELS

The 5 to 10 fold increase in the activity of LDH levels in serum of mice infected with LDV serves as a basis for detecting the virus (Notkins, 1965). The increase reaches a maximum 96 hours after infection.

Preparation of Serum

Whole blood was collected from mice under ether anaesthesia. The mice were swabbed down with absolute alcohol and a transverse lower abdominal incision made. The inferior vena cava was rapidly exposed and blood extracted from the vein using a wide-bore needle and 1ml syringe. By this stage of the experiment the mouse was dead.

The collected blood was then carefully transferred from the syringe into a glass centrifuge tube and incubated for 1 hour at 37°C to allow the blood to clot. The clotted blood was then centrifuged at 2000gmax. for 10 minutes and serum removed.

Great care had to be taken throughout this procedure to ensure that haemolysis did not take place, since red blood cells contain LDH.

Measurement of LDH Activity

This procedure was carried out by Dr. D. Thompson, the Department of Biochemistry, Gartnavel General Hospital, Glasgow. The method used was that described by McQueen, (1972). The reaction being measured was:-

Pyruvate + NADH $\rightleftharpoons$ Lactate + NAD

This reaction is catalysed by LDH.

The rate of decrease in absorbance of the sample at 340nm is a measure of the enzyme activity. An LKB/ Boehringer Kit was used according to the instructions supplied, and decreases in absorbance measured in an LKB spectrophotometer. Results were read from the template of the spectrophotometer, and are expressed in Units of activity per litre.

(8) MEASUREMENT OF BIOLOGICAL ACTIVITY IN THE SERUM OF TUMOUR-BEARING MICE

This experiment was carried out to determine whether the component present in SN1 and SN2, responsible for increasing mouse hepatic citrate levels, could be detected in the serum of tumour-bearing mice. Serum was collected, as previously described, 7 days after the mice had been injected with 2×10^6 viable tumour cells. Control mouse serum was collected from mice who had been injected with sterile PBS.

The collected serum from these 2 groups of mice (12 mice per group) was then centrifuged at 100,000gmax. for 4 hours at $4^{\circ}C$ and the supernatant retained. This procedure was carried out to ensure there were no tumour cells present in the serum. The supernatant was then

assayed for biological activity by injecting 0.1ml into normal mice. These mice were killed 24 hours later and their hepatic citrate content measured.

(9) MEASUREMENT OF MOUSE BODY TEMPERATURE

McAllister (1978) observed marked decreases in the body temperature of mice bearing TLX-5 lymphoma, over the 7 days of tumour growth. In this study, the body temperatures of mice injected with tumour cell-free preparations were measured over a period of 9 days and compared to the change observed in tumour-bearing mice.

Body temperatures of these mice were measured by placing a thermocouple in the inguinal region of each mouse, and allowing the reading to stabilise for at least 2 minutes.

(10) IN VITRO CULTURE OF TLX-5 LYMPHOMA CELLS

The tumour cells were cultured to determine whether if, after at least 5 passages through tissue culture, the "conditioned" cell-free growth medium from the cultured cells was biologically active in increasing mouse hepatic citrate levels.

Moreover, according to Notkins (1965), LDV can be removed from contaminated tumour cells by passaging them in culture for at least 4 passages. Consequently, these cells were examined by electron microscopy to determine whether or not LDV was present. The cells were also injected into normal mice i.p. at a concentration of 2×10^6 viable cells per 0.1ml to determine whether they were still active in increasing hepatic citrate levels.

Method

TLX-5 lymphoma cells were harvested from mice, as previously described. It was necessary to separate these cells from extraneous tissue debris, and this was done using a technique (Paul, 1970) which involved layering the cells onto a Ficoll-Hypaque solution at room temperature. This was then spun at 1200r.p.m. for 30 minutes at 25°C in an MSE Major centrifuge.

The cells were then washed 3 times with sterile PBS to remove traces of Ficoll-Hypaque, at 25°C, and then spun at 1800r.p.m. for 10 minutes. The pellet of cells was then resuspended in 10ml of RPMI-1640 (Gibco Biocult) medium and the number of viable cells determined.

A cell count of 0.5-1.0 x 10^6 viable cells per ml was found to be adequate for culturing in a 50ml flask (Sterilin). The amount of cells seeded was usually $5x10^6$, and these were maintained in medium, the constituents of which were:-

2ml (10,000 units) pennicillin-streptomycin (Gibco)

5ml of 200 mN L-glutamine (Flow)

5ml of 7.5% NaH_2CO_3 (Flow).

This mixture was made up to 500ml with RPMI-1640 and stored at 4°C. The cells were maintained at 37°C in medium supplemented with 20% Foetal Calf Serum (FCS) and in an humidified LEEC CO_2 incubator, with 95% air and 5% CO_2 passed into the incubator continuously.

Routine passage of cells involved vigorously agitating the culture vessel to detach the cells from it and to disaggregate clumps of cells. The cells were then spun at

room temperature for 10 minutes at 1800r.p.m., counted as before, and passaged in the manner previously described.

Cell-free "conditioned" growth medium was obtained by centrifuging the contents of the culture flask, after the cells had been passaged 5 times, at 100,000gmax. for 4 hours at 4°C. The supernatant was retained and its biological activity determined.

(11) ELECTRON MICROSCOPE EXAMINATION OF TUMOUR CELLS

Electron microscope examination of *in vivo* grown TLX-5 lymphoma cells was carried out by Mr. J. Parry of the University Department of Virology. Examination of *in vitro* grown tumour cells was carried out by Dr. J. Sommerville of the Department of Virology, Belvedere Hospital, Glasgow.

Method

Fixation and Dehydration

The cells were fixed for 30-60 minutes at room temperature in gluteraldehyde (2.5% solution in phosphate buffer + Mg^{++} + Ca^{++} + sucrose), rinsed twice with PBS, then post-fixed for 60 minutes in 1% osmium in phosphate buffer.

The cellular material was then dehydrated for 5 minutes in each of 30%, 50%, 90% and 95%alcohol. The material was finally dehydrated in 100% alcohol in a dessicator two times for 5 minutes and then for 20 minutes.

Embedding

The following procedure for embedding the material was carried out:-

(1) Material was placed in an alcohol-Epon mixture (50:50) for 12 hours.

(2) The alcohol-Epon mixture was changed to a 25:75 mixture and material retained in this for 6 hours.

(3) The material was finally put in a 100% Epon for 2 hours with 2 changes.

Embedded cells were then put in a fresh change of Epon in LKB Easy Molds, and placed in an oven for 72 hours at 65°C. The material was then sectioned using a Du Pont Diamond knife and an LKB Ultramicrotone 1.

The sections were then stained with uranyl acetate (50% in alcohol) and lead citrate, and examined using a Siemens Elniskopf 101 electron microscope. Kodak Electron Image film was used.

Statistical Treatment of Results

Results were calculated from the mean (X) and standard deviation (S.D.) of control and test populations using the students' unpaired t-test. Tables 1-21 also express students' t-values and significant levels for comparisons between test population and control group or zero time group.

CHAPTER 3

RESULTS

1. THE EFFECT OF TLX-5 LYMPHOMA CELLS AND CELL-FREE TUMOUR PREPARATIONS ON MOUSE HEPATIC CITRATE LEVELS

(i) Storage of Biological Material at -70°C

These studies involved working with biological material which is often very labile and susceptible to freezing and thawing procedures. Cell-free ascitic fluid (SN1, prepared by the ultracentrifugation of ascitic fluid from the peritoneal cavity of TLX-5 lymphoma bearing mice at 100,000gmax. for 4 hours at 4°C) was required in large volumes in order to characterise and isolate the biologically active component responsible for increasing mouse hepatic citrate levels. Large volumes of virus-free ascitic fluid (SN2, prepared by the ultracentrifugation of SN1 at 240,000gmax. for 24 hours at 4°C) were also required for these same reasons.

It was therefore thought not only convenient but necessary to be able to store these preparations at -70°C, but before this was done it was first necessary to determine whether SN1 and SN2 could be stored successfully at -70°C without losing their biological activity.

Measurement of the biological activity of SN1 and SN2 involved injecting mice i.p. with these preparations and measuring the hepatic citrate content of these mice 24 hours after treatment. For these results to be

relevant and to allow for stastical evaluation, it was necessary to include at least 6 mice or more in each experimental group. Stastical evaluation of the results was determined using the Student's unpaired t-test.

In the experiments described in this study it can be seen that large numbers of mice were involved, and it was therefore thought that if powdered, frozen livers from experimental mice could be stored for relatively short periods of time without affecting their citrate content, this would allow larger numbers of mice to be included in each group. Consequently, the effect of storage at -70°C on the hepatic citrate content of normal mice was investigated.

(a) SN1 and SN2

Storage of SN1 (Table 1) and SN2 (Table 2) at -70°C for periods of up to 60 days does not affect their biological activity, since the preparations will still increase hepatic citrate levels in treated mice when stored at -70°C for this length of time.

(b) Liver Samples

Storage of frozen, powdered liver at -70°C does not affect liver citrate content to any significant extent, as indicated in Table 3. It was therefore possible to store the frozen, powdered liver samples at -70°C for periods of up to 14 days, when measuring hepatic citrate content, without adversely affecting experimental results.

(ii) The Effect on Mouse Hepatic Citrate Levels of TLX-5 Lymphoma Cells and Tumour Cell-Free Preparations

Calman and McAllister (1975a, 1975b) observed increases in hepatic citrate levels in mice bearing a

variety of experimental tumours, including the TLX-5 lymphoma. In mice bearing this tumour, increases in hepatic citrate content were observed 24 hours after the inoculation of $2x10^6$ tumour cells, and attempts were therefore made in this study to reproduce these results, and to determine the levels of this metabolite in the livers of tumour-bearing animals over the course of tumour growth.

Later experiments by McAllister, Soukop and Calman (1977) showed that a cell-free preparation (SN1) of this tumour, when injected into normal mice, produced increases in hepatic citrate levels similar to the tumour cells. This observation led to the proposal that a factor or factors present in SN1 was responsible for bringing about the metabolite changes measured. These changes in hepatic citrate levels described by McAllister, Soukop and Calman (1977) were also observed in this study (Table 4).

Various methods were employed in an attempt to characterise the biologically active factor present in SN1. These included dialysis, heat-treatment and treatment with trypsin. Dialysis indicated whether the factor(s) involved was of a low molecular weight (less than 10,000 daltons), and heat-treatment determined whether it was heat-labile. Treatment with trypsin was used to determine whether the factor(s) was protein in nature.

During the course of these experiments, mouse spleen, thymus and epididymal fat pads were routinely removed and weighed in order to determine the effects of the various tumour cell-free preparations on the weights of these organs.

Moreover, the effect of virus-free ascitic fluid (SN2) on hepatic citrate levels was measured, since it was possible that ultracentrifugation under the conditions previously described might affect the biological activity of the preparation by removing the factor(s) involved. During the preparation of SN2 proteins with a molecular greater than 450,000 daltons would have pelleted out, and if biological activity could still be detected in the preparation, this would indicate that the factor(s) involved had a molecular weight of less than this. SN2 was then subjected to dialysis, heat-treatment and trypsin treatment.

(a) Hepatic Citrate

Increases in hepatic citrate content of mice bearing TLX-5 lymphoma are shown in Table 4. These increases were observed as early as 24 hours after the mice were inoculated with $2x10^6$ viable tumour cells, and could be detected throughout the period of tumour growth. Treatment of the mice with SN1 also brought about increases in hepatic citrate levels, which were observed up to 5 days after the initial injection of SN1 (Table 4).

The data presented in Table 4 also shows that the biological activity of SN1 is not affected by dialysis, but is totally destroyed by heat-treatment. SN2, as can be seen from Table 8, is as active as SN1 in producing significant increases in hepatic citrate levels, the most profound effect being observed 24 hours after treatment. Heat-treatment of SN2 (Table 9) and treatment with trypsin (Table 10) totally abolishes its biological

effect, but dialysis does not (Table 9). No biological activity could be detected in the resuspended pellet obtained during the high speed centrifugation of SN1 to produce SN2 (Table 9).

(b) Spleen Weight

Tumour-bearing mice showed an increase in spleen weight which was detected 24 hours after tumour implant (Table 5) and continued throughout the period of tumour growth, although at day 7 of tumour growth the observed increase did not differ significantly from the control mice, who were injected with sterile PBS. Increases in spleen weight were also detected in mice injected with SN1, but these increases did not become significant until 3 days after treatment and were not observed 7 days after injection of the preparation (Table 5). Dialysis of SN1 did not abolish its effect on spleen weight and appears to have enhanced this effect, since increases could be observed as early as 24 hours after injection. Heat-treatment of SN1 (Table 5) completely abolishes its effect on spleen weight.

Data presented in Table 8 shows that treatment of mice with SN2 will also increase spleen weight to the same extent as SN1, although increases were not significant 5 days after treatment but were significant 7 days after injection of the preparation.

(c) Thymus Weight

Thymus weight (Table 6) has been shown to decrease significantly in mice bearing TLX-5 lymphoma, these decreases being observed 24 hours after tumour implant

and continuing throughout the period of tumour growth. However, these decreases were not observed in mice treated with SN1, dialysed SN1 or heat-treated SN1, and on day 3 after treatment with SN1 thymus weights increased to a significant level. Day 5 after treatment with dialysed SN1, thymus weights also increased to a significant level. These increases then appear to return to normal in mice injected with SN1. Mice treated with SN2 do not show any significant changes in thymus weight, although small decreases in the weight of this organ were observed 3 days after treatment with this preparation (Table 8).

(d) Epididymal Fat Pads Weight

Data presented in Table 7 shows that increases in the weight of epididymal fat pads can be seen in mice bearing the TLX-5 lymphoma and in mice treated with SN1. These changes were observed 24 hours after the mice were treated with these preparations, although in tumour-bearing mice the increase did not reach a significant level 7 days after the inoculation of tumour cells.

Dialysis of SN1 abolished its ability to increase epididymal fat pad weights, as did heat-treatment. SN2 (Table 8) also had no significant effect on epididymal fat weights, although it did increase them slightly as did dialysed and heat-treated SN1, but not significantly.

(iii) Dose Response of Mouse Hepatic Citrate Levels to SN2

It was thought necessary to determine whether the biological activity of SN2 was "dose dependant", that is, to determine whether hepatic citrate levels were increased

to a greater extent with the injection of increasing cell equivalent concentrations of SN2. Therefore, a sample of SN2 was prepared at a cell equivalent concentration of $7x10^7$ per ml, and serial dilutions made by a factor of 10 to an eventual concentration of $7x10^3$ per ml. Mice were then injected with 0.1ml of these preparations and their hepatic citrate levels measured. Control mice were injected with sterile PBS, and liver citrate content was measured 24 hours after all the mice were treated. As can be seen from the data presented in Table 11 and in Figure 2, a "dose response" to serial dilutions of SN2 was observed, but the increases in hepatic citrate levels were not significant at a cell equivalent concentration of $7x10^4$ per ml or less. 10 mice were included in each group of experimental mice.

Another experiment was then carried out to determine the effects of multiple injections of SN2 on hepatic citrate content. A group of 10 mice were injected i.p. with 0.1ml of SN2 at a cell equivalent concentration of $2x10^7$ per ml. These mice received a total of 8 injections of this preparation; a single injection being given at 1-hour intervals for a period of 8 hours. Control mice were given 8-hourly injections of 0.1ml sterile PBS i.p., and hepatic citrate levels measured 24 hours after the final injection. A second control group were given 1 injection of the SN2 preparation, 0.1ml being injected i.p.

As can be seen from the data presented in Table 12, mice given 8x1 hourly injections of SN2 do develop an increase in hepatic citrate levels which is significantly

different from citrate levels observed in the PBS treated animals. However, this increase in hepatic citrate observed in mice given multiple injections of SN2 does not differ significantly from the levels measured in mice given a single injection, over the period of 24 hours, after which the livers were removed.

(iv) Precipitation of SN1 and SN2 with Ammonium Sulphate

Preliminary attempts to fractionate and isolate the biologically active factor(s) present in the ascitic fluid of tumour-bearing mice, involved using ammonium sulphate precipitation of the proteins present in SN1 and SN2. To determine the degree of purification of these and other cell-free tumour preparations, the protein content of each was measured, as well as their biological activity.

Results presented in Table 13 show that biological activity is present in the 35-65% ammonium sulphate precipitate of SN1, but not in the 0-35% ammonium sulphate precipitate of SN2.

The concentration of protein present in SN1, SN2 and the various preparations of SN1 and SN2 which have previously been described, are shown in Table 14. Heat-treatment of SN1 decreases its protein content by a relatively small amount, whereas dialysis does not appear to alter it to any relevant degree. Precipitation of SN1 with 35-65% ammonium sulphate results in considerable purification of the preparation in terms of protein content, without adversely affecting its biological activity, although there is some discrepancy in the concentration of protein measured in this preparation

depending on the method of measurement used.

Moreover, ultracentrifugation of SN1 to produce SN2 also achieves considerable purification in terms of protein content, as well as removing any viruses present in the preparation. The resuspended pellet, obtained during the preparation of SN2, contains a large amount of protein compared with that present in SN2, but it is inactive in increasing hepatic citrate levels. Precipitation of SN2 with 0-35% ammonium sulphate removes almost all of the protein present in SN2, and the redissolved precipitate is active in increasing hepatic citrate levels in treated mice.

(v) The Effect of Serum from Tumour-Bearing Mice on Mouse Hepatic Citrate Levels

To determine whether the biologically active factor present in SN1 and SN2 was present in the serum of tumour-bearing animals, that is, was a humoral factor, serum was collected from tumour-bearing mice and spun at 100,000gmax. for 4 hours at 4°C, to remove any cells which may have been present. The serum was then injected into normal mice i.p., and the hepatic citrate content of these mice measured 24 hours later. From the data presented in Table 15, hepatic citrate levels were increased to a significant extent in these animals, although not to the same degree as mice treated with SN2. The biologically active factor was therefore present in the serum of TLX-5 lymphoma bearing mice.

(vi) The Effect of TLX-5 Lymphoma Cells and Cell-Free Preparations on Mouse Body Temperature

McAllister (1978) observed significant decreases in mouse body temperature in mice bearing the TLX-5 lymphoma, over the period of tumour growth. These experiments set out to determine whether such an effect could be observed in mice treated with a variety of tumour cell-free preparations.

Data presented in Table 16 shows that after an initial increase in body temperature 24 hours after the inoculation of tumour cells, and 3 days later, there is a steady decline in body temperature until death of the animals occurs.

An initial increase in body temperature was observed in mice treated with SN1 on day 2 after treatment, but no other significant changes were observed until day 7, when body temperatures decreased significantly, returning to normal on day 8. Heat-treatment of SN1 did not abolish its effect on initially increasing mouse body temperature, but no further changes were observed until day 6 after treatment, when another increase was observed, returning to normal thereafter. Treatment of mice with dialysed SN1 also brought about an increase in body temperature, which was observed on days 1, 2 and 5 after the initial treatment. On day 7 after treatment a decrease in mouse body temperature was observed, returning to normal on day 8 (Table 16).

Table 1

STORAGE OF SN1 AT -70°C

Freshly prepared SN1 was injected into a group of at least 6 mice at a cell free equivalent concentration of 2×10^6 per 0.1ml, as indicated at Day 0. Mice were killed 24 hours later and their liver citrate content assayed. This process was repeated at the time intervals indicated, SN1 being stored at -70°C between times. Citrate is expressed as umol per gram liver wet weight, mean ($\bar{x}$) ± standard deviation, and statistical evaluation made against results obtained at day 0.

LIVER CITRATE CONTENT					
Day	0	1	10	28	60
$\bar{X}$	0.234 ± 0.019	0.295 ±0.045	0.246 ±0.030	0.267 ± 0.082	0.259 ± 0.032
t		1.476	0.841	1.022	1.710
DF		12	12	12	12
P		N.S.	N.S.	N.S.	N.S.

Abbreviations: t = test statistic using student's unpaired t-test.

DF = degree of freedom. = *$(n_1 + n_2) - 2$

P = statistical evaluation by student's unpaired t-test.

NS = non-significant.

Results were considered NS at $p > 0.05$

* Where:- n_1 = number of observations in group 1 (control group)

n_2 = number of observations in group 2.

Table 2

STORAGE OF SN2 AT -70°C

Freshly prepared SN2 was injected into a group of 6 mice at a cell-free equivalent concentration of $2x10^6$ per 0.1ml, as indicated at Day 0. Mice were killed 24 hours later and their liver citrate content assayed. This process was repeated at the time intervals indicated, SN2 being stored at -70°C between times. Citrate is expressed as umol per gram liver wet weight, $\bar{X} \pm$ S.D.

LIVER CITRATE CONTENT					
Day	0	1	10	28	60
$\bar{X}$	± .308 .062	± .288 .051	± .314 .049	± .316 .082	± .312 .073
T		.581	.165	.163	.085
DF		10	10	10	10
P		N.S.	N.S.	N.S.	N.S.

Table 3

STORAGE OF LIVER SAMPLES AT -70°C

Aliquots of frozen, powdered liver, pooled from 10 normal mice, were taken and assayed for liver citrate content at the times indicated. The liver was kept at -70°C. Citrate levels are expressed as umol per gram liver wet weight, $\bar{X} \pm$ S.D.

	LIVER CITRATE CONTENT			
Day	0	2	7	14
$\bar{X}$	0.133 ± 0.011	0.139 ± 0.010	0.192 ± 0.009	0.154 ± 0.027
t		0.598	0.664	0.267
DF		6	6	6
P		N.S.	N.S.	N.S.

Table 4

THE EFFECT OF TLX-5 LYMPHOMA CELLS AND CELL-FREE PREPARATIONS ON MOUSE HEPATIC CITRATE LEVELS

At least 6 mice were included in each group. TLX-5 bearing mice were injected i.p. with 0.1ml of a tumour cell preparation at $2x10^6$ viable cells per 0.1ml. Other experimental animals were treated, as indicated, with a cell free equivalent concentration of $2x10^6$ per 0.1ml. Control mice were injected i.p. with 0.1ml sterile PBS. Citrate levels are expressed as umol per gram liver wet weight, $\bar{x} \pm$ S.D. Mice were killed, after treatment, at intervals shown.

Table 5

THE EFFECT OF TLX-5 LYMPHOMA CELLS AND CELL-FREE PREPARATIONS ON MOUSE SPLEEN WEIGHT

Mice were treated as indicated in Table 4. Spleen weight is expressed in grammes, $\bar{x} \pm$ S.D.

Table 6

THE EFFECT OF TLX-5 LYMPHOMA CELLS AND CELL-FREE PREPARATIONS ON MOUSE THYMUS WEIGHT

Mice were treated as indicated in Table 4. Thymus weight is expressed in grammes, $\bar{x} \pm$ S.D.

* Dialysis of SN1 did not affect its ability to increase hepatic citrate content of treated mice at day 1. 3 and 7 after treatment. But 5 days after treatment, hepatic citrate content of treated mice fell, and did not differ significantly from that of the control mice. It did, however, differ significantly from the hepatic citrate content of mice treated with SN1 at day 5 ($p < 0.001$).

Table 4

The Effect of TLX-5 Lymphoma Cells and Cell-Free Preparations on Mouse Hepatic Citrate Levels

		Liver Citrate Content			
Days After Injection		1	3	5	7
Controls	$\bar{X}$	0.141	0.165	0.149	0.155
	±	0.024	0.035	0.028	0.035
	n	10	10	10	10
TLX-5 Bearing Mice	$\bar{X}$	0.391	0.285	0.252	0.310
	±	0.043	0.041	0.031	0.031
	P	< 0.001	< 0.05	< 0.001	< 0.001
	n	8	8	8	8
SN1 Treated Mice	$\bar{X}$	0.312	0.330	0.281	0.169
	±	0.043	0.060	0.034	0.040
	P	< 0.001	< 0.001	< 0.001	N.S.
	n	8	8	8	8
* Dialysed SN1 Treated Mice	$\bar{X}$	0.249	0.237	0.200	0.250
	±	0.035	0.038	0.038	0.033
	P	< 0.001	< 0.02	N.S.	< 0.001
	n	10	10	10	10
Heat-Treated SN1 Treated Mice	$\bar{X}$	0.158	0.153	0.154	0.150
	±	0.030	0.029	0.017	0.025
	P	N.S.	N.S.	N.S.	N.S.
	n	10	10	10	10

Table 5

The Effect of TLX-5 Lymphoma Cells and Cell-Free Preparation on Mouse Spleen

		Spleen Weights			
Days After Injection		1	3	5	7
Controls	$\bar{X}$	.079	.077	0.080	0.114
n=10	±	0.070	0.004	0.011	0.011
TLX-5 Bearing Mice	$\bar{X}$	.143	.132	.137	.136
n = 8	±	.016	.025	.025	.038
	P	<0.001	<0.001	<0.001	N.S.
SN1 Treated Mice	$\bar{X}$	0.090	.142	.113	.114
n = 8	±	.015	.014	.016	.030
	P	N.S.	<0.001	<0.01	N.S.
Dialysed SN1 Treated Mice	$\bar{X}$	0.126	0.117	0.105	.135
n = 10	±	.006	.021	.002	.023
	P	<0.001	<0.01	<0.01	N.S.
Heat-Treated SN1 Treated Mice	$\bar{X}$	0.079	.076	.087	.078
n = 10	±	.004	.005	.029	.016
	P	N.S.	N.S.	N.S.	N.S.

Table 6

The Effect of TLX-5 Lymphoma Cells and Cell-Free Preparations on Mouse Thymus Weight

		Thymus Weight			
Days After Injection		1	3	5	7
Controls	$\bar{X}$	.021	.023	.023	.024
n = 10	$\pm$	.007	.010	.007	.004
TLX-5 Bearing Mice	$\bar{X}$	.009	.006	.007	.007
	$\pm$	.003	.003	.005	.002
n = 8	P	<0.01	<0.001	<0.001	<0.001
SN1 Treated Mice	$\bar{X}$	.031	.060	.031	.020
	$\pm$	.012	.005	.007	.006
n = 8	P	N.S.	<0.001	N.S.	N.S.
Dialysed SN1 Treated Mice	$\bar{X}$	.024	.025	.032	.030
	$\pm$	.005	.007	.005	.005
n = 10	P	N.S.	N.S.	<0.05	<0.05
Heat-Treated SN1 Treated Mice	$\bar{X}$	.019	0.20	.018	.021
	$\pm$	.006	.010	.002	.004
n = 10	P	N.S.	N.S.	N.S.	N.S.

Table 7

THE EFFECT OF TLX-5 LYMPHOMA CELLS AND CELL-FREE EXTRACTS ON MOUSE EPIDIDYMAL FAT PAD WEIGHT

Mice were treated as indicated in Table 4. Fat Pad weight is expressed in grammes, $\bar{X} \pm S.D.$

Table 8

THE EFFECT OF SN2 ON MOUSE HEPATIC CITRATE LEVEL, SPLEEN WEIGHT, THYMUS WEIGHT AND EPIDIDYMAL FAT PAD WEIGHT

Six mice were included in each group. Mice were treated with an i.p. injection of 0.1ml of a cell-free equivalent concentration of 2×10^6 per 0.1ml of SN2. Mice were killed after treatment at intervals shown. Controls were injected i.p. with 0.1ml sterile PBS.

Table 7

The Effect of TLX-5 Lymphoma Cells and Cell Free Extracts on Mouse Epididymal Fat Pad Weight

		Fat Pad Weight			
Days After Injection		1	3	5	7
Controls	$\bar{X}$	.494	.557	.662	.741
n = 10	±	.108	.198	.186	.210
TLX-5 Bearing Mice	$\bar{X}$	1.089	1.251	1.326	1.090
n = 8	±	.313	.236	.263	.362
	P	<0.001	<0.001	<0.001	N.S.
SN1 Treated Mice	$\bar{X}$	.881	1.301	1.486	1.515
n = 8	±	.176	.356	.172	.343
	P	<0.001	<0.01	<0.001	<0.01
Dialysed SN1 Treated Mice	$\bar{X}$	.706	.602	.546	.950
n = 10	±	.256	.341	.267	.253
	P	N.S.	N.S.	N.S.	N.S.
Heat-Treated SN1 Treated Mice	$\bar{X}$	.659	.806	0.444	.531
n = 10	±	.227	.299	.299	.090
	P	N.S.	N.S.	N.S.	N.S.

Table 8

The Effect of SN2 on Mouse Hepatic Citrate Level, Spleen Weight, Thymus Weight and Epididymal Fat Pad Weight

Days After Injection			1	3	5	7
Hepatic Citrate Content umol/g liver wet wt, $\bar{X} \pm$ S.D. n=6	Controls	$\bar{X}$	.141	.165	.149	.155
		$\pm$	.051	.036	.045	.024
	SN2Treated Mice	$\bar{X}$	.349	.320	.273	.149
		$\pm$	.051	.013	.025	.016
		P	<0.001	<0.001	<0.001	N.S.
Spleen WT, $\bar{X} \pm$ S.D. (g) n=6	Controls	$\bar{X}$	.079	.077	.080	.114
		$\pm$	.007	.076	.013	.011
	SN2Treated Mice	$\bar{X}$	.189	.154	.074	.077
		$\pm$	.005	.010	.012	.011
		P	<0.001	<0.001	N.S.	<.001
Thymus WT, $\bar{X} \pm$ S.D. (g) n=6	Controls	$\bar{X}$	.021	.023	.023	.024
		$\pm$	.005	.011	.005	.004
	SN2Treated Mice	$\bar{X}$	.033	.019	.024	.024
		$\pm$	.006	.010	.008	.005
		P	N.S.	N.S.	N.S.	N.S.
Fat-Pads WT, $\bar{X} \pm$ S.D. (g) n=6	Controls	$\bar{X}$	.494	.557	.662	.741
		$\pm$	.094	.198	.186	.210
	SN2Treated Mice	$\bar{X}$	.706	.862	.682	.776
		$\pm$	.071	.077	.101	.127
		P	N.S.	N.S.	N.S.	N.S.

Table 9

EFFECT OF HEAT-TREATED SN2 DIALYSED SN2 AND RESUSPENDED SN2 PELLET ON MOUSE HEPATIC CITRATE LEVEL

There were at least 6 mice in each group. Mice were injected i.p. with 0.1ml of a cell-free equivalent concentration of 2×10^6 per 0.1ml of heat-treated and dialysed SN2 and of resuspended SN2 pellet. Control mice were injected i.p. with $0.1 cm^3$ of sterile PBS. Hepatic citrate levels were assayed 24 hours after injection of mice, and are expressed as umol per gram liver wet weight, $\bar{X} \pm$ S.D.

	Controls n = 8	Heat-Treated SN2 n = 8	Dialysed SN2 n = 9	Resuspended SN2 Pellet n = 8
$\bar{X}$	0.168	0.127	* 0.298	0.176
$\pm$	.029	.032	.037	.026
t		1.585	2.175	0.289
DF		14	15	14
P		N.S.	<0.05	N.S.

* The hepatic citrate content of mice treated with dialysed SN2, although it is lower than that of mice treated with SN2 (Table 8), this difference is not significant.

Table 10

THE EFFECT OF TRYPSIN TREATED SN2 ON MOUSE HEPATIC CITRATE LEVELS

All preparations were injected i.p. at a cell equivalent concentration of $2x10^6$ per 0.1ml. Control group 1 were injected i.p. with 0.1ml sterile PBS. Control group 2 were injected i.p. with 0.1ml of sterile PBS with trypsin and trypsin inhibitor. Control group 3 were injected i.p. with 0.1ml of SN2. The experimental group were injected i.p. with 0.1ml of SN2 treated with trypsin plus trypsin inhibitor. Citrate values are expressed as umol per gram liver wet weight, $\bar{X} \pm$ S.D.

Group	Control 1 n = 5	Control 2 n = 5	Control 3 n = 6	Experimental n = 6
$\bar{X}$	0.169	0.163	.269	0.162
$\pm$	.058	.029	.048	.013
t		0.116	2.802	0.250
DF		8	9	9
P		N.S.	<0.05	N.S.

The mean citrate value in the experimental group (0.162) also differed significantly from the mean citrate value of control group 3 (0.269) at $P < 0.01$.

Table 11

"DOSE RESPONSE" OF MOUSE HEPATIC CITRATE LEVELS TO SN2

Mice were injected i.p. with 0.1ml of SN2 at cell equivalent concentrations as shown, with 10 mice in each group. Control mice were injected i.p. with 0.1ml sterile PBS. Hepatic citrate content was measured 24 hours after mice had been injected. Citrate is expressed as umol per gram liver wet weight, $\bar{X} \pm$ S.D., n=6 in each group.

Concentration of SN2 (cell equivalent per ml)	Hepatic Citrate	
	$\bar{X}$	P
Controls	0.168 ± 0.052	
$7x10^3$	0.187 ± 0.040	N.S.
$7x10^4$	0.204 ± 0.077	N.S.
$7x10^5$	0.234 ± 0.031	<0.01
$7x10^6$	0.266 ± 0.034	<0.01
$7x10^7$	0.320 ± 0.066	<0.001

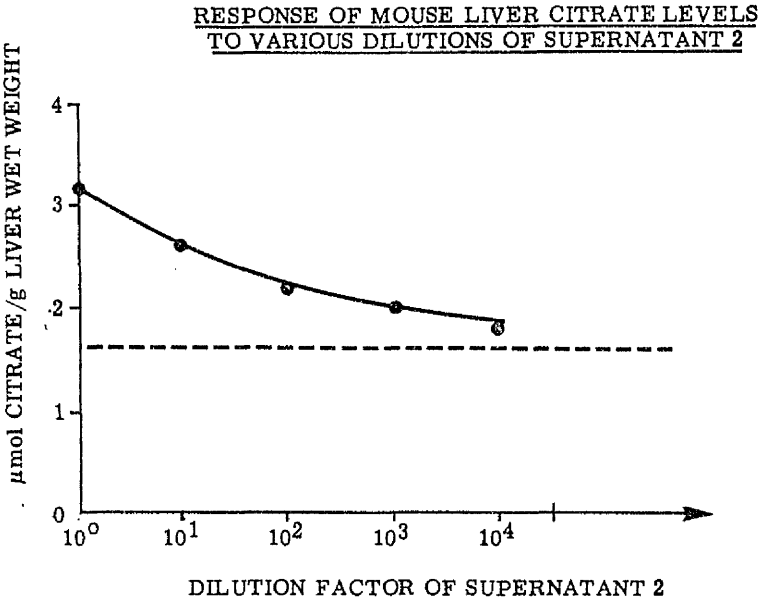
Table 12

THE EFFECT OF MULTIPLE INJECTIONS OF SN2 ON MOUSE HEPATIC CITRATE LEVELS

Experimental mice were injected i.p. with 0.1ml of SN2 at a cell equivalent concentration of $2x10^7$ per ml. These mice received 8 injections of SN2, a single injection being administered hourly. Control group 1 were given 8 injections of 0.1ml sterile PBS i.p., a single injection administered hourly. Control group 2 were given a single injection of SN2, 0.1ml being administered i.p. at a cell equivalent concentration of $2x10^7$ per ml. 10 mice were included in each group, and hepatic citrate levels measured 24 hours after the final injection. Citrate is expressed as umol per gram liver wet weight. Results obtained from Experimental group were compared with those obtained from Control groups 1 and 2.

		Hepatic Citrate Levels		
		Control 1	Control 2	Experimental
	$\bar{X}$	0.178	0.256	0.278
	$\pm$	0.043	0.018	0.030
Control 1	t			4.855
	p			<0.001
Control 2	t			0.848
	p			N.S.

Figure 2



Initial cell concentration of ascitic fluid was $7x10^7$/ml. Dilutions of Supernatant 2 obtained from this ascitic fluid were made with sterile PBS. Control mice injected i.p. with sterile PBS had a liver citrate content of 0.168 µmol/g liver wet weight. At concentrations lower than $7x10^5$ cell equivalents/ml, when 0.1ml was injected i.p. into mouse, liver citrate levels were not significantly altered from that of controls.

Table 13

THE EFFECT OF AMMONIUM SULPHATE FRACTIONATION OF SN1 AND SN2 ON THEIR ABILITY TO INCREASE MOUSE HEPATIC CITRATE LEVELS

At least 6 mice were included in each group. Mice were injected i.p. with 0.1ml of cell equivalent concentrations of $2x10^6$ per 0.1ml of all preparations. Control group 1 were injected i.p. with 0.1ml of sterile PBS. Control group 2 were injected i.p. with 0.1ml of untreated SN1. Control grop 3 were injected i.p. with 0.1ml untreated SN2. Experimental group 1 were injected with 0.1ml of the redissolved 0-35% ammonium sulphate precipitate of SN1 and experimental group 2 with 0.1ml of the redissolved 35-65% ammonium sulphate precipitate of SN1. The redissolved 0-35% ammonium sulphate precipitate of SN2 was injected into mice in experimental group 3. Citrate levels are expressed as umol per gram liver wet weight, $\bar{x} \pm$ S.D.

		CONTROL GROUPS			EXPERIMENTAL GROUPS		
		1	2	3	1	2	3
n =		6	6	6	6	6	8
Citrate	$\bar{X}$	.144	.245	.289	.176	.240	.267
	$\pm$	.039	.029	.030	.040	.034	.028
	P		<0.001	<0.001	N.S.	<0.001	<0.001
Spleen (g)	$\bar{X}$	.079	.094	.090	.084	.089	.094
	$\pm$	.007	.015	.006	.009	.008	.005
	P		<0.01	N.S.	N.S.	<0.05	<0.01
Thymus (g)	$\bar{X}$	.026	.029	.026	.028	.023	.029
	$\pm$	.005	.005	.006	.002	.010	.007
	P		N.S.	N.S.	N.S.	N.S.	N.S.
Fat Pads (g)	$\bar{X}$	.553	.745	.671	.538	.568	.698
	$\pm$	.082	.170	.068	.134	.148	.170
	P		<.05	N.S.	N.S.	N.S.	N.S.

Table 14

PROTEIN CONCENTRATION OF SN1 AND SN2 PREPARATIONS

Protein concentrations shown in column 1 were determined by the Bradford Method (1976). Preparations were at a cell equivalent concentration of $2x10^7$ per 1.0ml. Concentrations shown in column 2 were determined by absorption of protein at 230nm. Results are expressed as ug per 1ml of sample.

Preparation	Protein (ug/ml) Column 1	Column 2
SN1	1777	1838
Heat-Treated SN1	1010	59.4
Dialysed SN1	1850	---
0-35% $(NH_4)_2SO_4$ SN1 precipitate	71	61
35-65% $(NH_4)_2SO_4$ SN1 precipitate	41.5	1320
SN2	31	37
Resuspended SN2 Pellet	1660	1742
0-35% $(NH_4)_2SO_4$ SN2 precipitate	25	14

Table 15

EFFECT OF SERUM FROM TUMOUR-BEARING MICE ON HEPATIC CITRATE LEVELS

Serum was collected from TLX-5 bearing mice, centrifuged at 100,000gmax for 4 hours at 4°C and 0.1ml injected i.p. into 6 mice. Control mice were injected with 0.1ml sterile PBS, and a second control group injected i.p. with 0.1ml of SN2 at a cell-free equivalent concentration of 2×10^6 per 0.1ml. Hepatic citrate levels were measured 24 hours after treatment of mice and are expressed as umol per gram liver wet weight, $\bar{X} \pm$ S.D.

	Control 1 (PBS)	Control 2 (SN2)	TLX-5 Bearing Mouse Serum
n =	6	6	5
$\bar{X}$	0.209	0.369	0.310
$\pm$	0.052	0.063	0.079
t		4.666	2.447
DF		12	11
P		<0.001	<0.05

Table 16

THE EFFECT OF TLX-5 LYMPHOMA CELLS AND TUMOUR CELL-FREE PREPARATIONS ON MOUSE BODY TEMPERATURE.

The body temperatures of mice injected with TLX-5 lymphoma cells and cell-free preparations, as shown, were measured daily for a period of 8 days after the initial i.p. injections of 0.1ml of cell or cell free equivalent concentrations of 2×10^6 per 0.1ml. Control mice received 0.1ml sterile PBS i.p.. Body temperatures were measured in °C, $\bar{X} \pm$ S.D.

Day After Injection		1	2	3	4	5	6	7	8
PBS n=6	$\bar{X}$	36.7	36.65	37.06	36.83	36.96	37.01	37.16	36.96
	±	1.6	.48	.68	.61	.25	.64	.48	.56
TLX-5 n=6	$\bar{X}$	38.0	36.83	37.6	36.45	35.66	33.78	25.92	Animals
	±	3.75	.18	.98	.32	.78	.75	.77	
	P	<0.001	N.S.	N.S.	N.S.	<0.01	<0.001	<0.001	Dead
SN1 n=6	$\bar{X}$	37.27	37.76	37.36	37.36	37.21	36.10	36.18	36.93
	±	1.19	.45	.21	.37	.32	.42	.66	.76
	P	N.S.	<0.001	N.S.	N.S.	N.S.	N.S.	<0.05	N.S.
Heat-Treated SN1 n=6	$\bar{X}$	37.10	37.66	37.20	37.28	37.11	37.90	37.70	37.65
	±	1.51	.23	.37	.34	.59	.49	.14	.33
	P	N.S.	<0.01	N.S.	N.S.	N.S.	<0.02	N.S.	N.S.
Dialysed SN1 n=6	$\bar{X}$	37.3	37.65	36.71	37.23	37.81	36.5	36.55	36.46
	±	.99	.37	.38	.43	.53	.22	.93	.32
	P	<0.05	<0.01	N.S.	N.S.	<0.01	N.S.	<0.05	N.S.

2. THE PURIFICATION AND MOLECULAR WEIGHT DETERMINATION OF THE BIOLOGICALLY ACTIVE COMPONENT OF TLX-5 LYMPHOMA CELL-FREE ASCITIC FLUID

Gel filtration and gel electrophoresis are useful techniques for the purification and molecular weight determination of unknown compounds of biological origin. Gel filtration is particularly useful in that the biological activity of substances eluted from the gels can be determined, in this case by injecting mice with the eluted fractions and measuring their hepatic citrate content. Unfortunately, the biological activity of substances subjected to electrophoresis in polyacrylamide gels could not be determined in this case, since it was not possible to elute these substances from the gels. However, this method was useful in determining the homogeneity of biologically active fractions obtained from the gel filtration of SN2, and in determining the relative molecular weight of the biologically active component. These methods have been described in detail in Chapter 2 of this thesis.

(a) Gel Filtration of SN2 on Sephadex G-100

Since SN2 represented a relatively pure tumour cell-free preparation in terms of protein content, and was free of viral contamination, it was used in these experiments. The elution profile of SN2 on Sephadex G-100 is shown in Figure 3. As fractions were eluted from the gel their absorbance at 230nm was measured and plotted against

* Table 18 shows that the protein content of the eluted fractions is less than that of the starting material, SN2 Fractions 11 and 12 had a relatively high protein content and were active in increasing the hepatic citrate content of treated mice (Table 17). Fraction 4, although having a high protein content relative to the other fractions, was inactive in increasing mouse hepatic citrate levels (Table 17).

elution volume. The biological activity of the eluted fractions containing protein was measured and is shown in Table 17. The protein content of these eluted fractions, measured by their absorbance at 230nm, is shown in Table 18.*

Determination of the relative molecular weights of the eluted fractions in which biological activity was detected (fraction numbers 11 and 12) involved comparing the elution profile of the unknowns with that of proteins of a known molecular weight. The elution profiles of cytochrome-C (Figure 4), ovalbumin (Figure 5), chymotrypsinogen (Figure 6), aldolase (Figure 7) and BSA (Figure 8) are shown. The log of the molecular weights of these proteins was plotted against their respective elution volumes (Figure 9) and linear regression used to determine the line of "best fit" for the data points. This standard graph was then used to determine the relative molecular weight of the biologically active component of SN2 present in fractions 11 and 12, which was found to be between 6948 -7585 daltons.

(b) Gel Electrophoresis

Electrophoresis of the biologically active fractions obtained from gel filtration of SN2 on Sephadex G-100 was carried out on polyacrylamide gels. The relative mobility of the active factor, as determined by its peak of counts per minute, that is, the extent of reaction of the components of these active fractions with [^{3}H] dansyl chloride, were compared with the mobility of ovalbumin and BSA (Figure 10) and cytochrome-C and insulin (Figure 11).

* The insulin used was bovine insulin (Sigma).

** It may be, however, that insulin target cells in the mouse do not respond when bovine insulin is used. Therefore further experiments, using insulin from different sources, would be necessary before insulin could be excluded as the active protein factor. Moreover, the concentration of active insulin injected into the mice may have been very low, and future experiments might involve using greater concentrations of insulin.

The Rf values of each of the standard proteins relative to the mobility of cytochrome-C in the gels was plotted against the log of their respective molecular weights (Figure 14) and linear regression used to find the line of "best fit" for the data points. Fractions 11 (Figure 12) and 12 (Figure 13) were shown to contain 2 peaks of activity in identical positions within the gel, and the relative mobility of these proteins within the gel was used to determine their molecular weights. The molecular weights of the proteins present, as determined from the standard graph, were 103,276 and 6886 daltons, respectively.

(c) The Effect of Insulin on Mouse Hepatic Citrate Levels

Since the low molecular weight protein of the biologically active component of SN2 had a molecular weight similar to that of insulin, which has a molecular weight of 5750 daltons, it was decided to measure the effect of insulin, if any, on the hepatic citrate levels of normal mice. Moreover, McAllister (1978) found decreases in plasma glucose levels of TLX-5 lymphoma bearing mice, but since he did not measure plasma insulin, he could not attribute these low levels of glucose to increased levels of circulating insulin.*

However, data shown in Table 19 shows that when insulin is injected i.p. into normal mice at a concentration of protein similar to that injected when mice were treated with fraction 11 and 12 from the gel filtration of SN2, this has no effect on hepatic citrate levels. This biologically active factor does not appear to be insulin.**

Table 17

EFFECT OF FRACTIONS ELUTED FROM SEPHADEX G-100 COLUMN ON MOUSE HEPATIC CITRATE LEVELS

1.5ml of SN2 at a cell equivalent concentration of $8x10^7$ per 1ml was used as starting material. Fractions were collected every 30 minutes from the Sephadex G-100 column at a rate of 3.2ml per hour. 0.1ml of the fractions indicated below were injected i.p. into groups of 10 mice, and hepatic citrate levels assayed 24 hours after injection. Control mice received 0.1ml of PBS buffer which had passed through the column. Citrate is expressed as umol per gram liver wet weight, $\bar{X} \pm S.D.$ Mice injected with SN2 received $0.1cm^3$ of a cell-free equivalent concentration of $8x10^6$ per $0.1cm^3$.

Fraction No.	Elution Volume cm^3	Hepatic Citrate $\bar{X} \pm S.D.$	P
4	6.4	0.139 ± 0.054	N.S.
9	14.4	0.085 ± 0.023	N.S.
10	16.0	0.160 ± 0.052	N.S.
11	17.6	0.265 ± 0.049	<0.001
12	19.2	0.245 ± 0.065	<0.05
13	20.8	0.205 ± 0.053	N.S.
20	32.0	0.140 ± 0.019	N.S.
SN2	---	0.242 ± 0.023	<0.001
Controls	---	0.140 ± 0.062	---

Table 18

PROTEIN CONTENT OF SN2 FRACTIONS ELUTED FROM SEPHADEX G-100 COLUMN

Protein concentrations were determined by measuring the absorbance of the protein at 230nm. Figures shown are for fractions collected when the cell equivalent concentration of the starting material was $2x10^6$ per 0.1ml and are expressed as ug per 1ml. This experiment was carried out in duplicate and the results shown are the means of two experiments.

Fraction Number	Elution Volume (cm^3)	Protein Content ug/cm^3
4	6.4	9.35
9	14.4	0.99
10	16.0	2.61
11	17.6	8.52
12	19.2	10.32
13	20.8	2.74
20	32.0	1.09
SN2	--	37.0

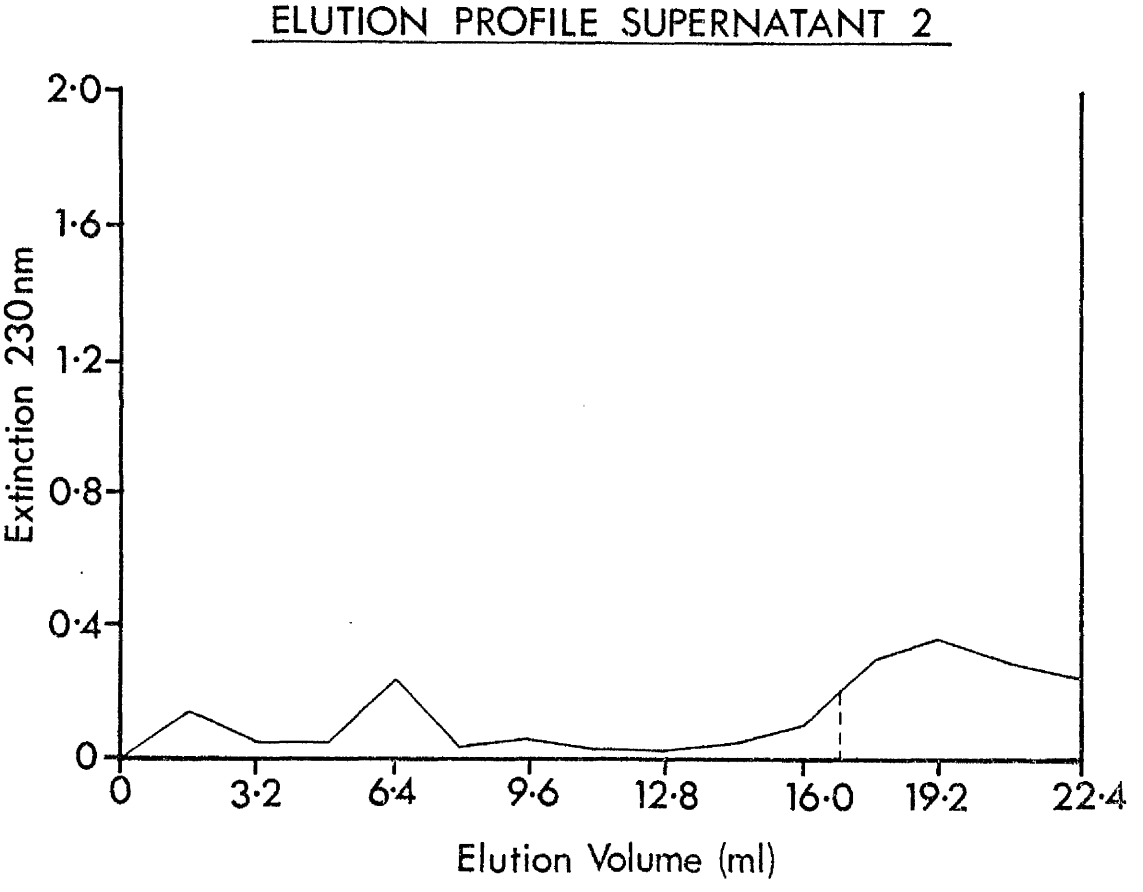
Table 19

EFFECT OF INSULIN ON MOUSE HEPATIC CITRATE LEVELS

A group of 6 mice were injected i.p. with 0.1ml of insulin at a concentration of 9 ug insulin per 0.1ml of sterile PBS. Control mice were injected i.p. with 0.1ml sterile PBS.

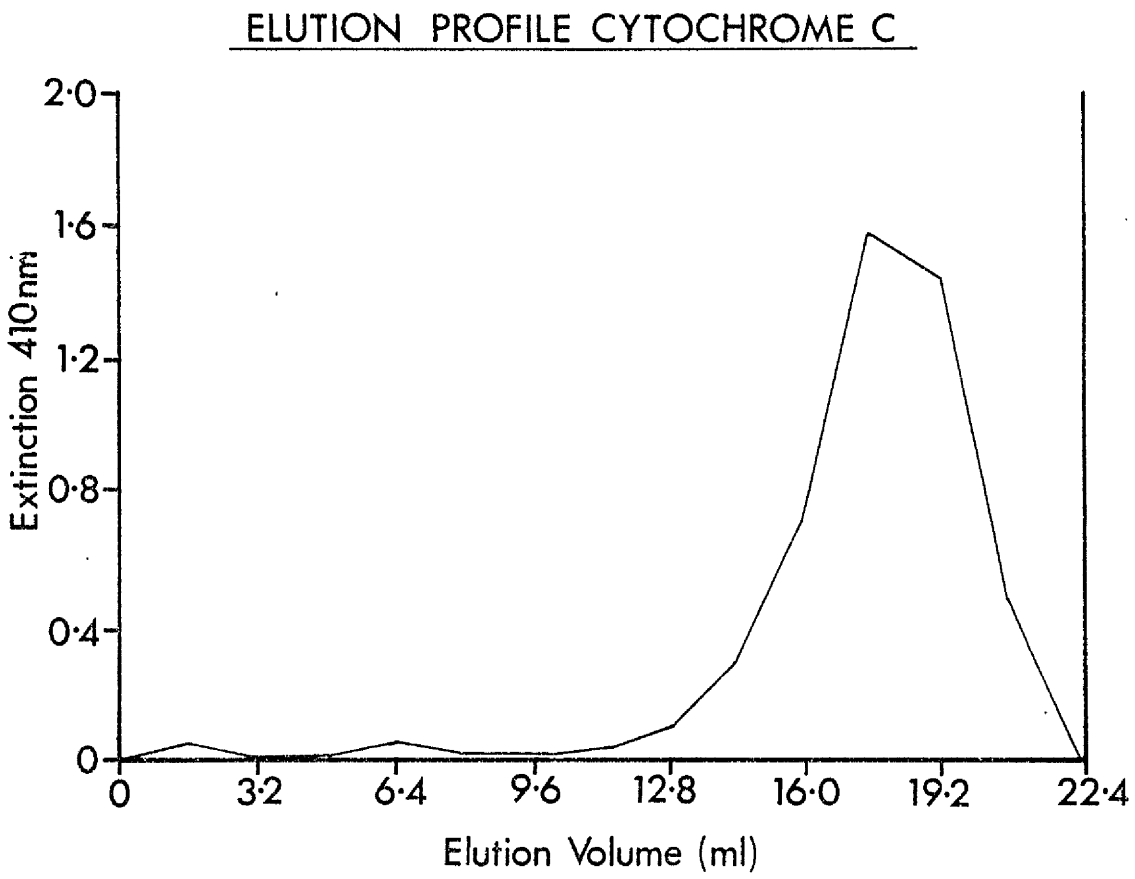
	Controls	Insulin Treated
$\bar{X}$	0.209	0.282
$\pm$	0.048	0.041
t		1.923
DF		10
P		N.S.

Figure 3



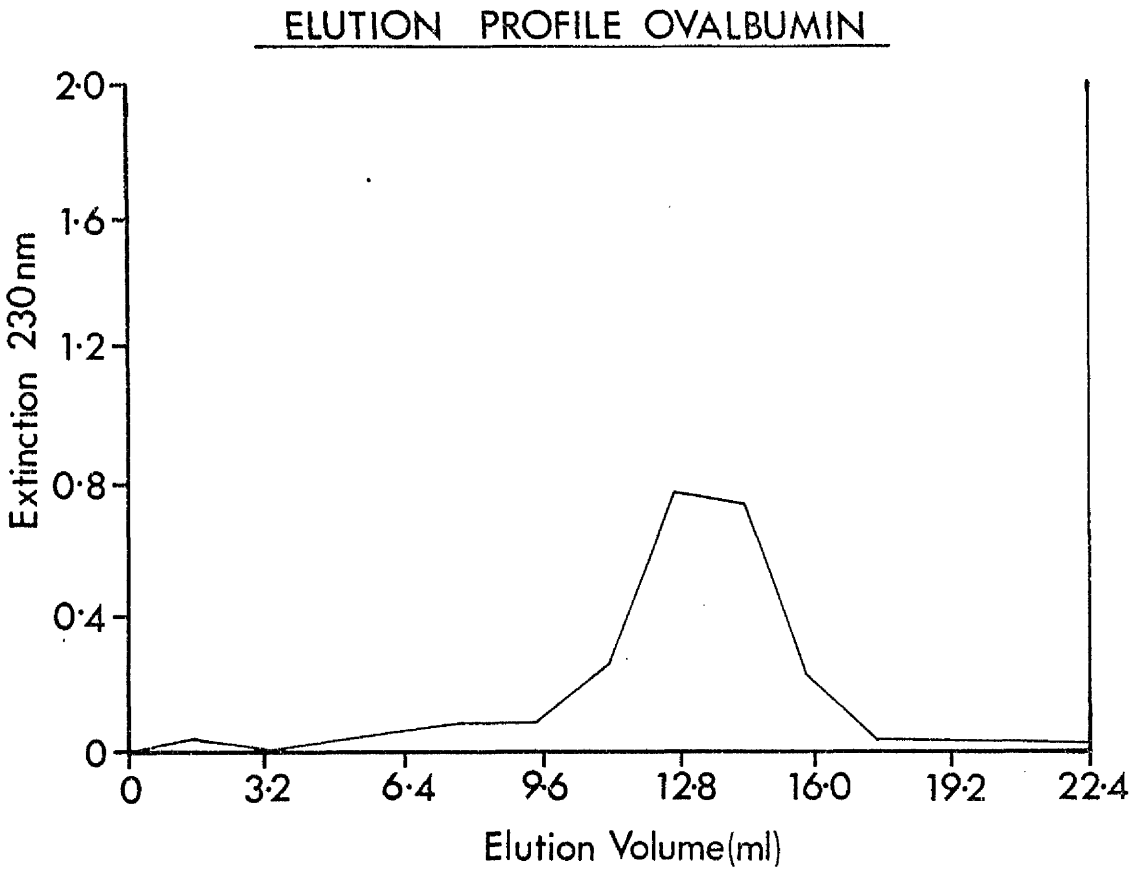
Sample volume was 1.5ml. SN2 was loaded onto the column of Sephadex G-100 at a cell equivalent concentration of $8x10^8$ per ml. Bed dimensions were 1x30 cm. Flow rate was 3.2ml per hour. Fractions were collected at 30 minute intervals, and their absorbances at 230nm recorded. The eluant was sterile PBS.

Figure 4



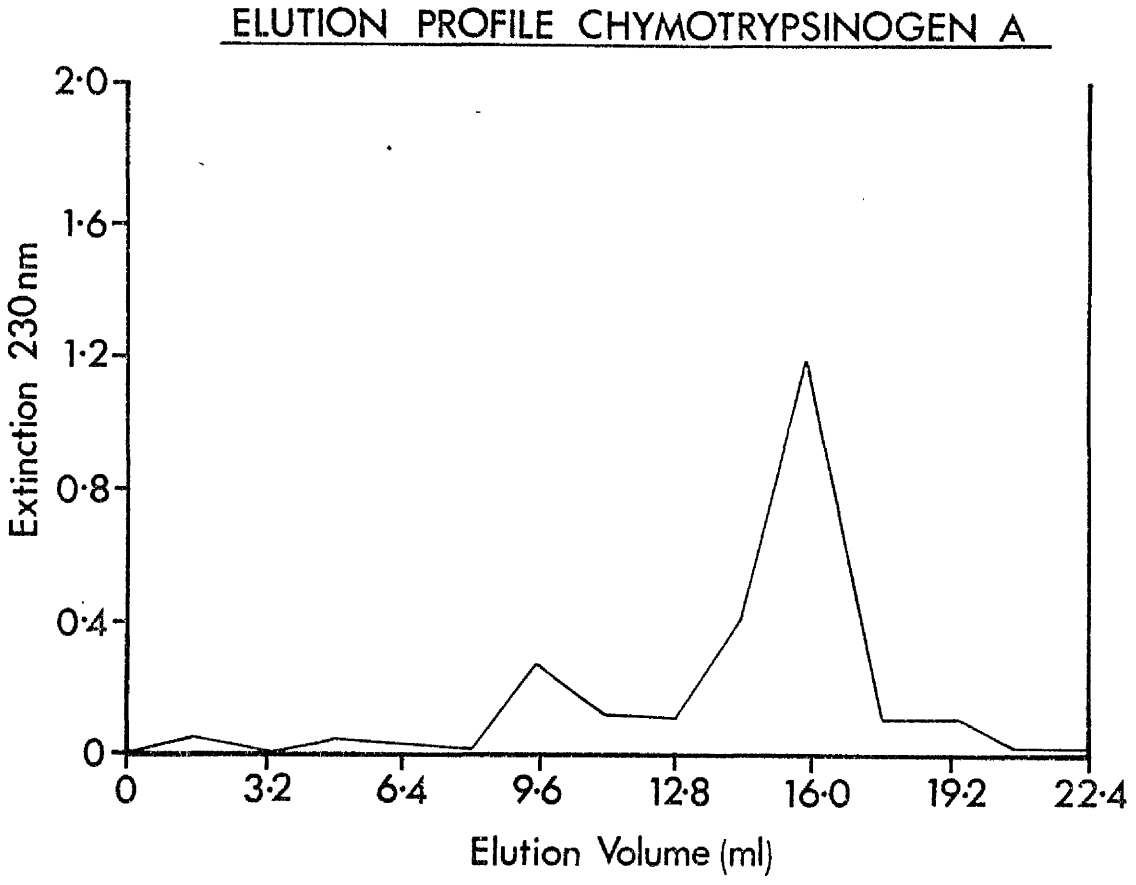
Sample volume of cytochrome-C was 0.5ml, loaded onto a Sephadex G-100 column at a concentration of 2mg per ml. Bed dimensions were 1x30 cm. Flow rate was 3.2ml per hour. Fractions were collected at 30 minute intervals, and their absorbance at 230 nm recorded. The eluant was sterile PBS.

Figure 5



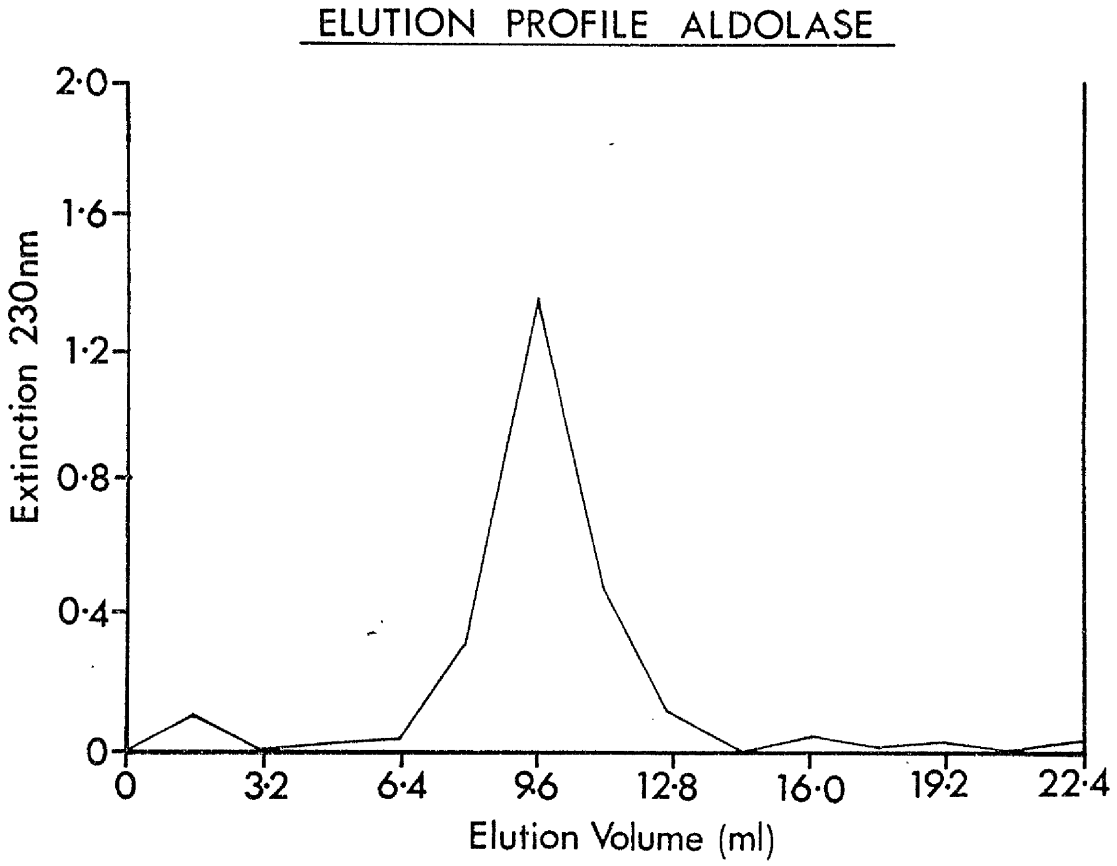
Sample volume of ovalbumin was 0.5ml loaded onto a Sephadex G-100 column at a concentration of 2mg per ml. Bed dimensions were 1x30 cm. Flow rate was 3.2ml per hour. Fractions were collected at 30 minute intervals, and their absorbance at 230nm recorded. The eluant was sterile PBS.

Figure 6



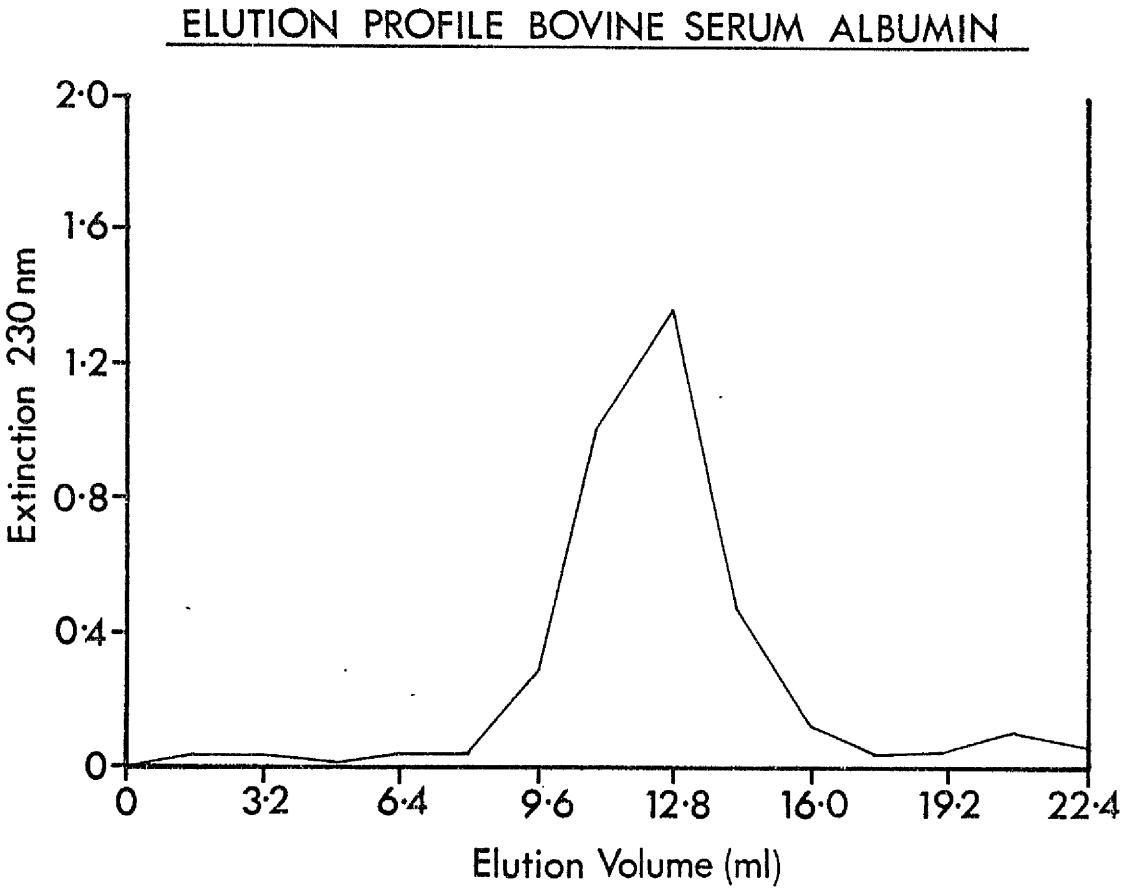
Sample volume of chymotrypsinogen was 0.5ml loaded onto a Sephadex G-100 column at a concentration of 2mg per ml. Bed dimensions were 1x30 cm. Flow rate was 3.2ml per hour. Fractions were collected at 30 minute intervals, and their absorbance at 230nm recorded. The eluant was sterile PBS.

Figure 7



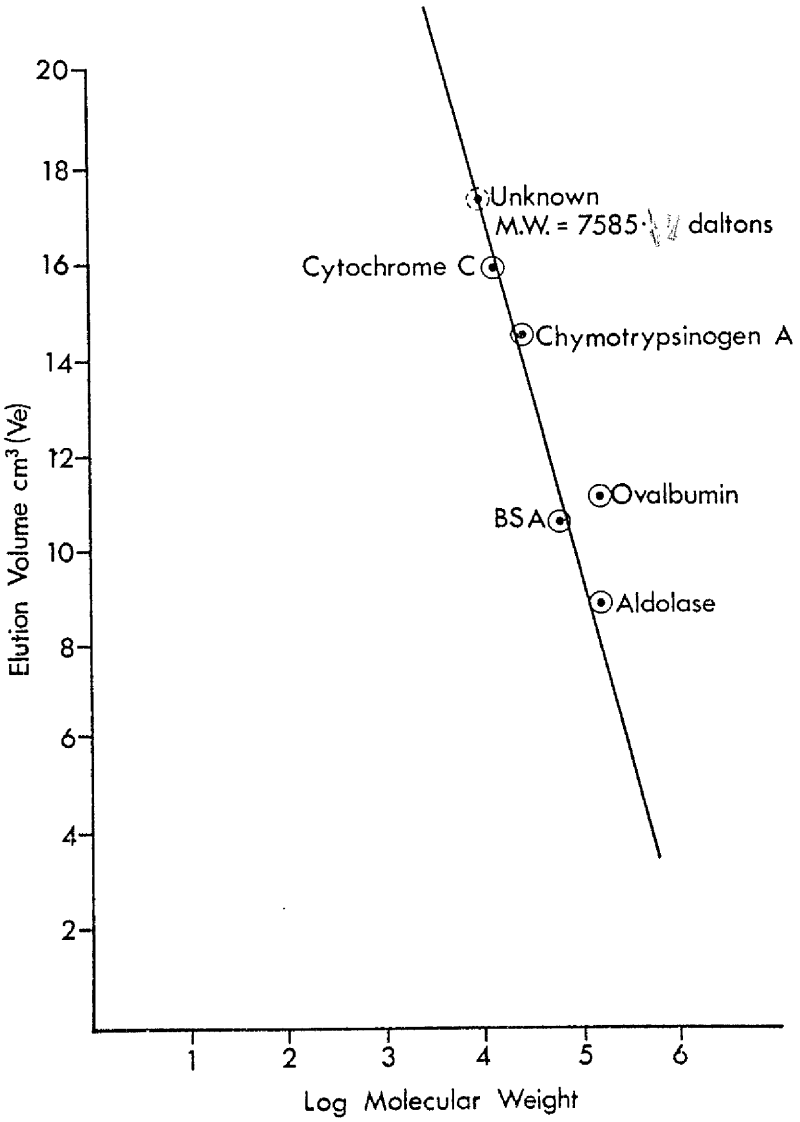
Sample volume of aldolase was 0.5ml loaded onto a Sephadex G-100 column at a concentration of 2mg per ml. Bed dimensions were 1x30 cm. Flow rate was 3.2ml per hour. Fractions were collected at 30 minute intervals and their absorbance at 230nm recorded. The eluant was sterile PBS.

Figure 8



Sample volume of BSA was 0.5ml loaded onto a Sephadex G-100 at a concentration of 2mg per ml. Bed dimensions were 1x30 cm. Flow rate was 3.2ml per hour. Fractions were collected at 30 minute intervals, and their absorbance at 230nm recorded. The eluant was sterile PBS.

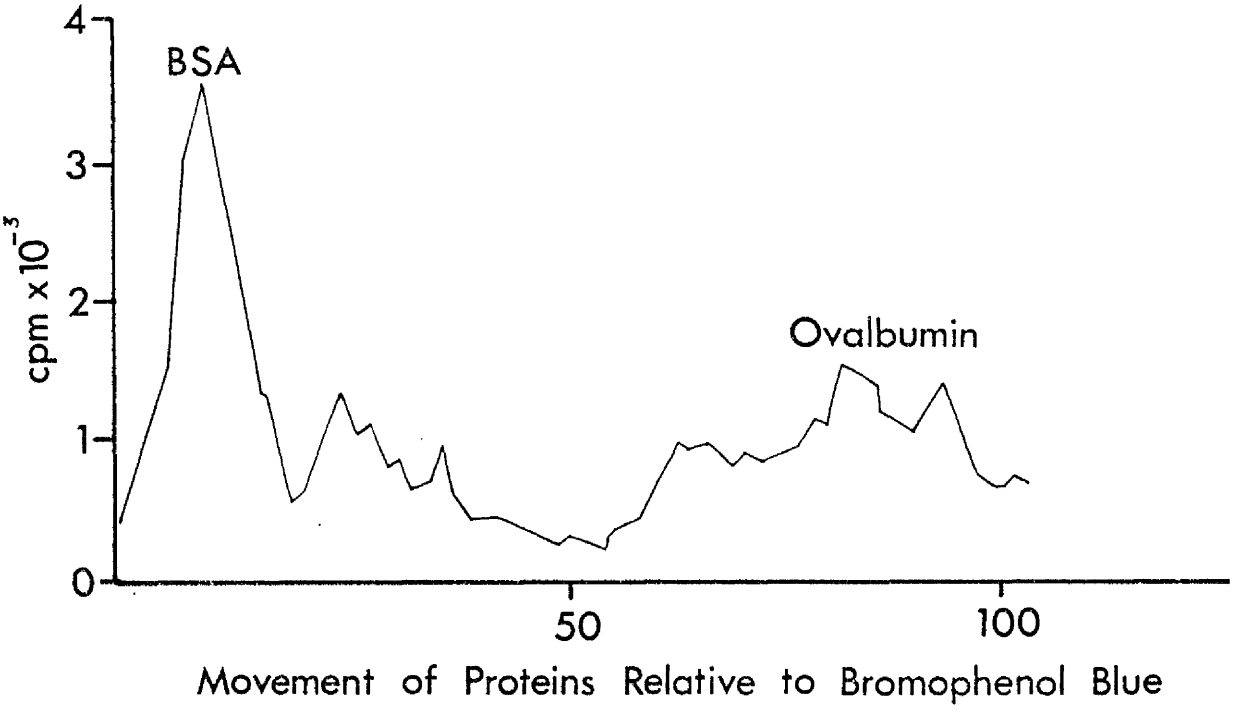
Figure 9



The elution volumes of the various proteins determined from their elution profiles on Sephadex G-100 was plotted against the log of their respective molecular weights. This standard graph was used to determine the molecular weight of the unknown protein in biologically active fractions.

Figure 10

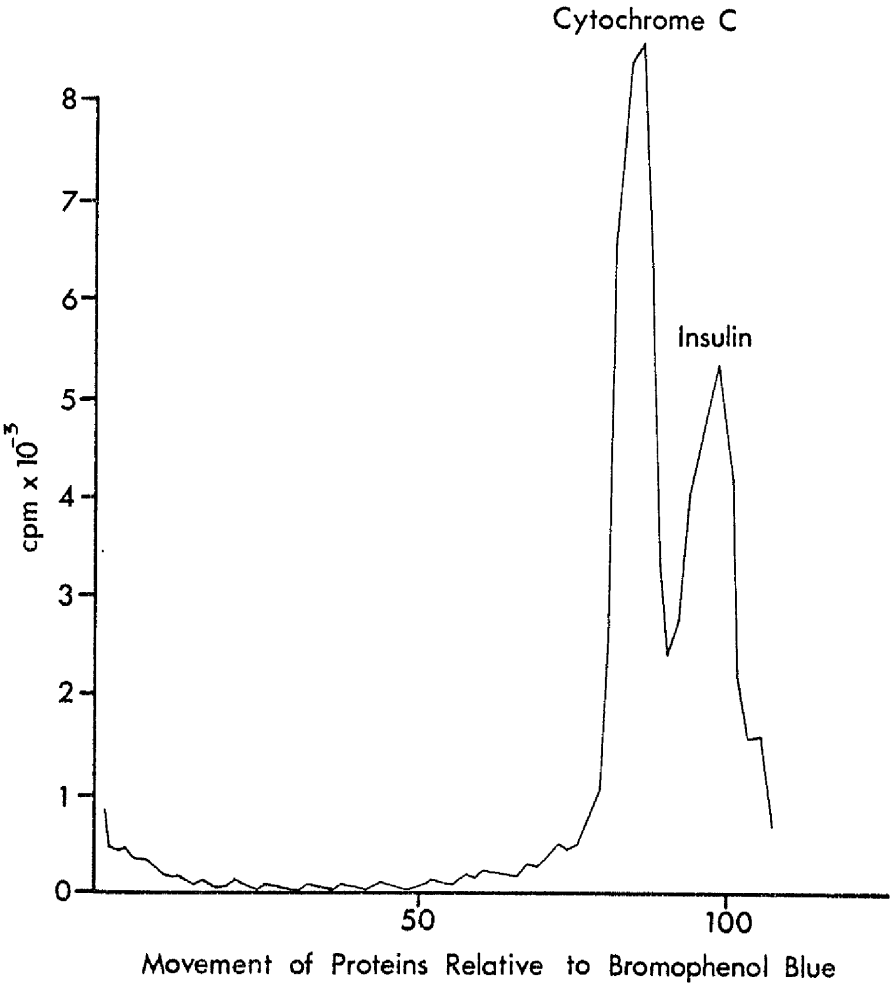
Mobility of Ovalbumin and BSA in Polyacrylamide Gels



Samples were electrophoresed in 10% polyacrylamide gels for 4 hours at 8 milliamperes per tube. Gel dimensions were 80mmx6mm (internal diameter).

Figure 11

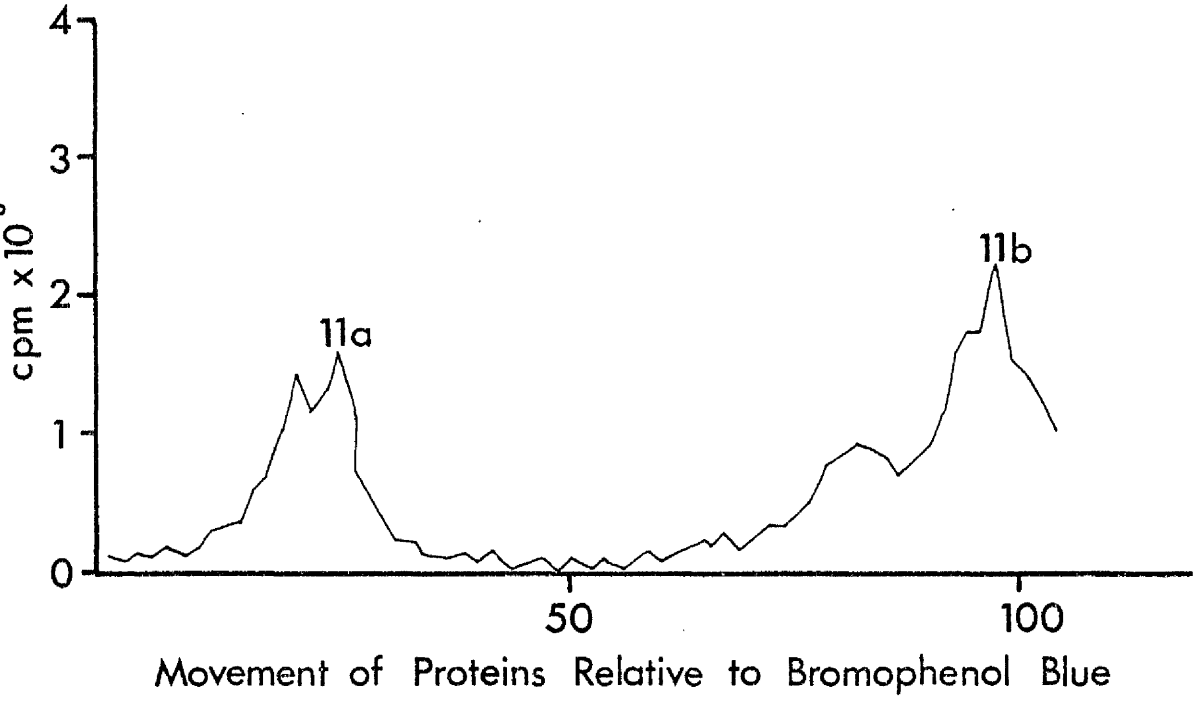
Mobility of Cytochrome-C and Insulin-Polyacrylamide Gels



Samples were electrophoresed in 10% polyacrylamide gels for 4 hours at 8 milliamperes per tube. Gel dimensions were 80mmx6mm (internal diameter).

Figure 12

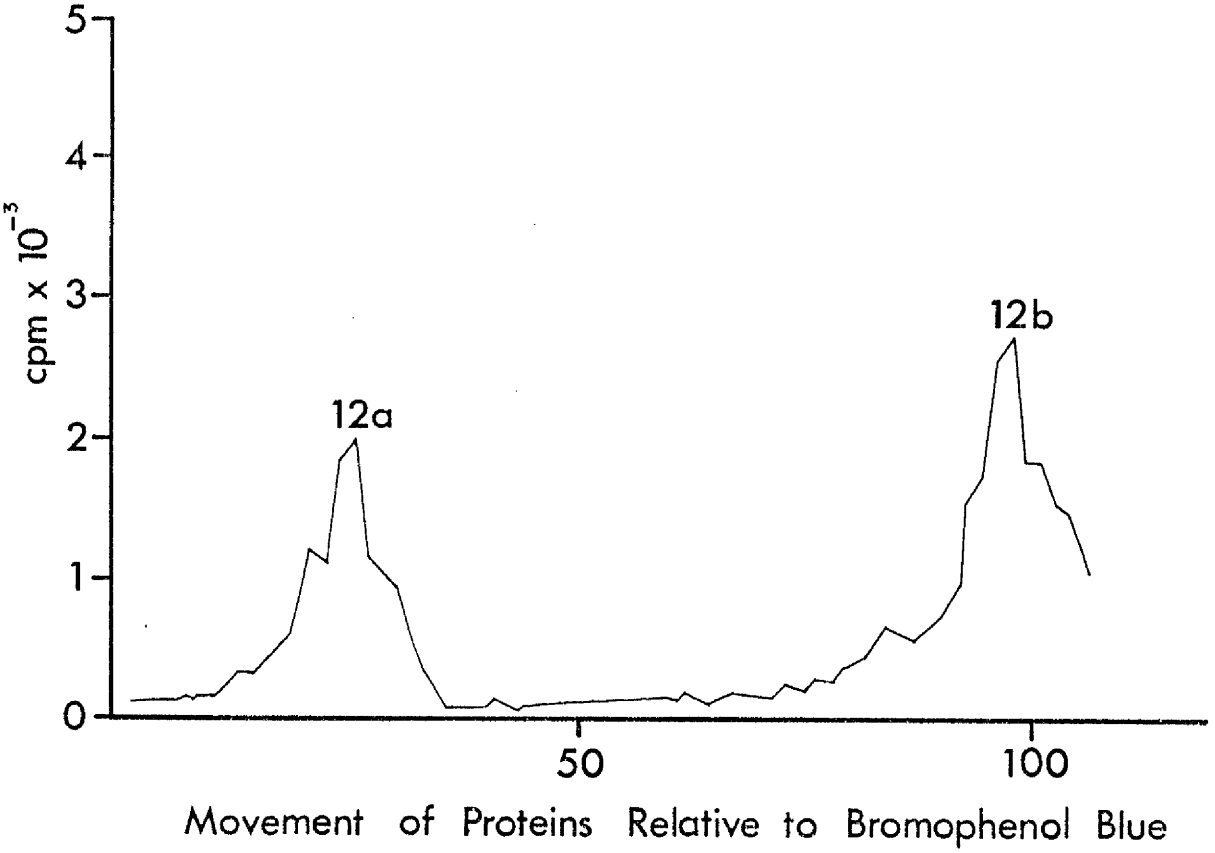
Mobility of Protein in Fraction 11 (from Gel Filtration of SN2) in Polyacrylamide Gels



Samples were electrophoresed in 10% polyacrylamide gels for 4 hours at 8 milliamperes per tube. Gel dimensions were 80mmx6mm (internal diameter).

Figure 13

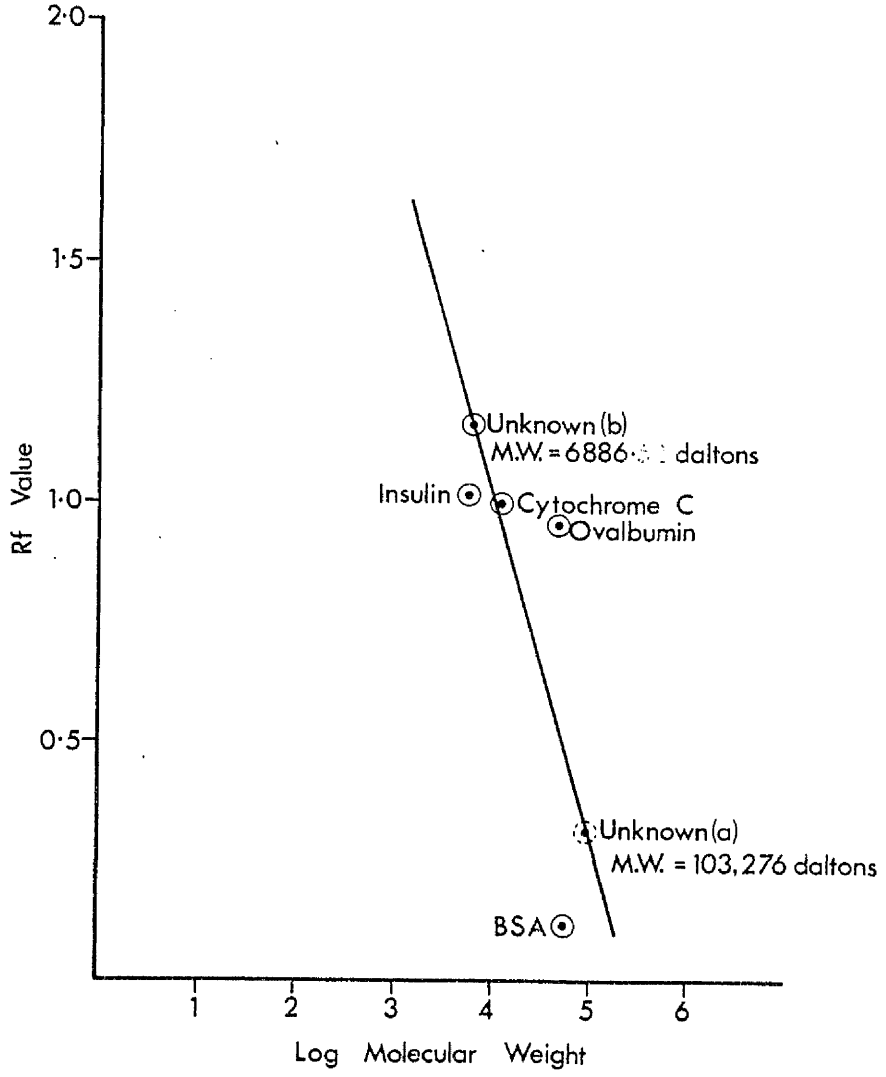
Mobility of Protein in Fraction 12 (From Gel Filtration of SN2) in Polyacrylamide Gels



Samples were electrophoresed in 10% polyacrylamide gels for 4 hours at 8 milliamperes per tube. Gel dimensions were 80mmx6mm (internal diameter).

Figure 14

Rf of Standard Proteins versus Log Molecular Weight



The Rf values of standard proteins determined by their mobility in 10% polyacrylamide gels was plotted against the log of their respective molecular weights. This standard graph was used to determine the molecular weights of the unknown proteins in fractions11 and 12 obtained from gel filtration of SN2.

3. STUDIES ON THE VIRAL CONTAMINATION OF TLX-5 LYMPHOMA

As has previously been stated, experimental tumour systems are frequently contaminated, not only with viruses but with bacteria (Notkins, 1965; Kampschmidt and Schultz, 1969). The most frequent contaminant of murine tumours is the lactate dehydrogenase virus (LDV), whose most profound effect is an elevation of plasma or serum lactate dehydrogenase (LDH) activity of infected mice. This elevation of LDH activity reaches a peak 96 hours after infection with the virus (Notkins, 1965) and serves as one of the methods of detection of the presence of the virus. Another method which can be used to detect the presence of this virus involves the examination of tumour cells using electron microscopy. These 2 methods of detection were used in this study to determine whether TLX-5 lymphoma cells were contaminated with this virus.

One method which can be used to eliminate this viral agent from contaminated tumours is by passaging the tumour cells through tissue culture several times. Thus the TLX-5 lymphoma cells were grown in tissue culture for at least 5 passages and injected into normal mice to determine whether they were still active in increasing hepatic citrate levels.

The possibility that the increase in hepatic citrate levels observed in tumour-bearing mice was due to the presence of viral contaminants of the tumour cells and

not to the presence of the tumour had to be investigated. Passage of the tumour cells through tissue culture also would be able to help distinguish whether the active factor purified from cell-free ascitic fluid was of host or tumour cell origin. If biological activity could be detected in the "conditioned" cell-free growth medium from the tumour cells this would indicate that the factor was initially of tumour cell origin.

Moreover, ultracentrifugation of SN1 at 240,000gmax. for 24 hours at 4°C would have the effect of removing viral particles from the preparation. Thus SN2 is a virus-free preparation.

(a) Serum LDH Activity

Serum was collected from mice injected with tumour cells and with SN2. Control mice were injected with sterile PBS. Serum was collected from these mice 96 hours after the mice had been treated. The LDH activity of the collected serum is shown in Table 20. It can be seen from these results that activity of this enzyme is increased not only in tumour-bearing mice but in mice treated with the virus-free preparations, SN2.

(b) The Effect of In Vitro Grown TLX-5 Lymphoma Cells and Cell-Free Growth Medium on Mouse Hepatic Citrate Levels

Data presented in Table 21 shows that in vitro grown TLX-5 cells passaged through tissue culture for 5 passages was effective in increasing mouse hepatic citrate levels in treated mice. The "conditioned" cell-free medium from these cultured cells was also effective in increasing

hepatic citrate content. Inspection of these in vitro grown cells using a light microscope did not reveal the presence of host cells in the preparation, but the possibility still exists that host cells, particularly lymphocytes, are present in the cultured cells in very small amounts. Nevertheless, since the cell-free growth medium from these cells was effective in increasing hepatic citrate content, this was thought to indicate that the biologically active factor was primarily of tumour cell origin since it was present at a concentration high enough to affect liver citrate levels.

(c) Electron Microscope Examination of In Vivo and In Vitro Grown TLX-5 Lymphoma Cells

Electron microscope examination of in vivo grown TLX-5 lymphoma cells revealed the presence of virus particles. These virus particles are shown in Plates 1, 3 and 4, and are indicated by the arrows. They are rounded or elliptical particles, the rounded particles having an average diameter of 70mu, and the elliptical particles an average length of 70.07mu. Plate 2 shows a calibration photograph for Plate 1.

Electron microscope examination of in vitro grown TLX-5 lymphoma cells, grown in tissue culture for 5 passages, did not reveal the presence of any viral particles.

Table 20

MOUSE SERUM LACTATE DEHYDROGENESE

The serum LDH activity of mice treated as indicated was measured. Serum was collected 96 hours after mice had been treated. All mice were injected i.p. with 0.1ml of a cell or cell equivalent concentration of $2x10^6$ per 0.1ml. Control mice received 0.1ml sterile PBS. Activity of LDH is expressed as U per litre, $\bar{X} \pm S.D.$

LDH ACTIVITY

	Controls	TLX-5 Bearing Mice	SN2 Treated Mice
$\bar{X}$	1637.5	5317.1	4128.0
$\pm$	139.7	431.1	156.7
t		11.021	8.297
DF		13	11
P		<0.001	<0.001

Table 21

THE EFFECT OF IN VITRO GROWN TLX-5 LYMPHOMA CELLS AND "CONDITIONED" CULTURE MEDIUM ON MOUSE HEPATIC CITRATE LEVELS

There were at least 6 mice in each group. Control group 1 were injected i.p. with 0.1ml of culture medium in which no cells had been grown. Experimental group 1 had been injected i.p. with 0.1ml of a cell concentration of $1x10^6$ viable cells per 1.0ml of TLX-5 cells grown in vitro. Experimental group 2 received 0.1ml of a cell concentration of $1x10^6$ viable cells per 1ml of in vivo grown TLX-5 cells, harvested after a fifth passage through tissue culture. Experimental group 3 received 0.1ml of the cell-free conditioned culture medium from the in vitro grown cells. Citrate levels are in umol per gram liver wet weight, $\bar{X} \pm$ S.D.

	Control Group	Experimental 1	Experimental 2	Experimental 3
$\bar{X}$	0.181	0.267	.307	0.267
$\pm$	0.020	0.052	.030	0.027
T		3.226	4.823	3.226
DF		14	12	14
P		<0.01	<0.001	<0.01

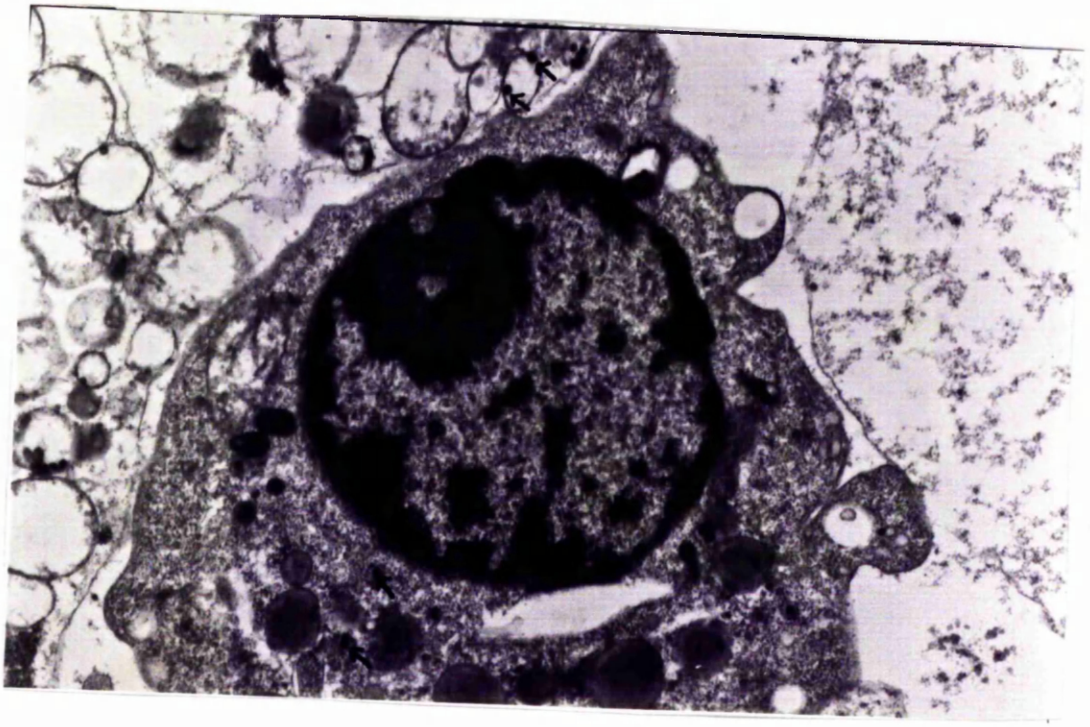


Plate 1 *In vivo* grown TLX-5 lymphoma cell preparation. Final magnification 13,745x. Virus particles are indicated by the arrows.

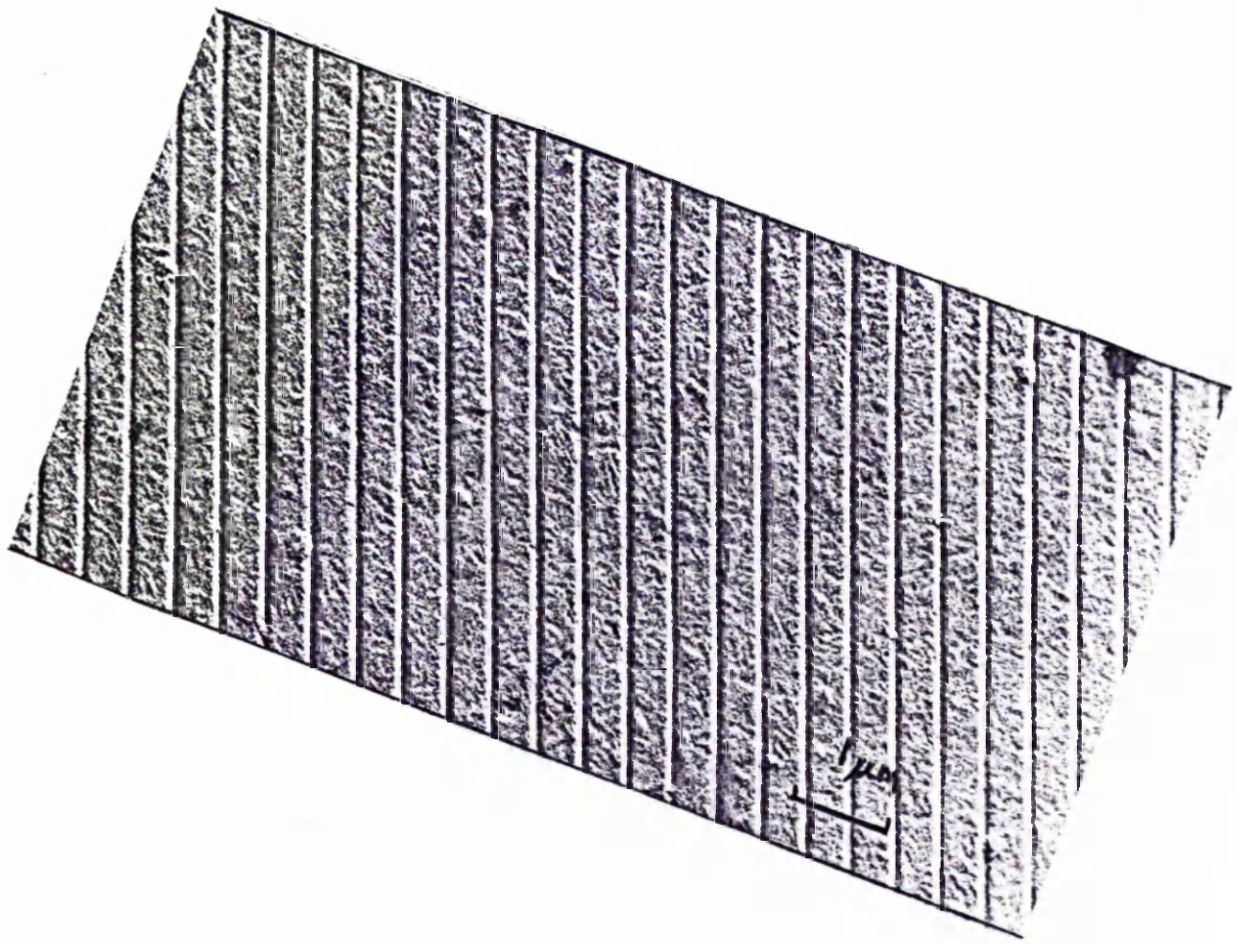


Plate 2 Calibration photograph for plate 1. 1 um length is indicated on photograph.

Plate 3 In vivo grown TLX-5 Lymphoma cell preparation. Final magnification 43,200x. Virus particles are indicated by the arrows.

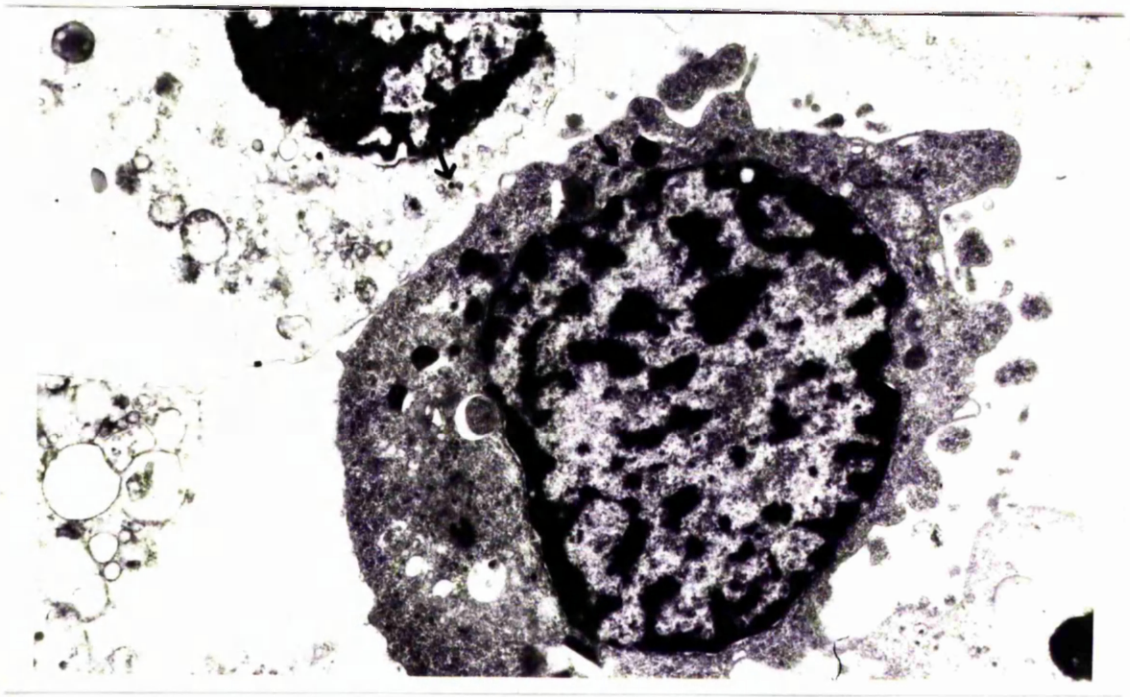


Plate 4 In vivo grown TLX-5 lymphoma cell preparation Final magnification 9750x. Virus particles are indicated by the arrows.

CHAPTER 4

D I S C U S S I O N

(i) Hepatic Citrate Levels

Haven, Randall and Bloor (1949) showed that in rats bearing the Walker 256 carcinosarcoma the citrate content of liver, kidney and spleen was higher than that of control animals. In later studies, Calman and McAllister (1975(a), 1975(b)) observed significant increases in the hepatic citrate content of mice bearing the TLX-5 lymphoma, Sarcoma-180 and C3H mammary tumours. In the case of TLX-5 lymphoma bearing mice, these changes were observed as early as 24 hours after the injection of 2×10^6 viable tumour cells. This was thought to indicate that products present in the ascitic fluid of tumour-bearing mice were responsible, as this was found not to be an allogeneic effect. Later experiments (McAllister, Soukop and Calman, 1976; McAllister, Soukop and Calman, 1977) justified this proposal, since these early changes in hepatic citrate content were observed in mice treated with a cell-free ascitic fluid preparation of TLX-5 lymphoma (SN1).

Data presented in this study showing changes in hepatic citrate content, at 2 day intervals over a 7 day period, in mice injected with TLX-5 lymphoma cells or SN1, shows that these changes follow a similar pattern to those described by Calman and McAllister (1975(a), 1975(b)) and McAllister, Soukop and Calman (1976), suggesting that a factor or factors present in SN1 are involved in the aetiology of these changes.

Experiments were then carried out to define the nature of the biologically active component of SN1, and to determine whether one factor or more was responsible for this biological activity. SN1 was subjected to dialysis, which was found to have no effect on its ability to increase hepatic citrate levels. Heat-treatment, however, completely destroyed its biological activity. These results indicate that the biologically active component(s) of SN1 is heat-labile, and may have a molecular weight greater than 10,000 daltons.

It was then necessary to determine whether this biologically active component(s) was primarily of tumour or host origin, since it was possible that it may have been produced by the host in response to the presence of the growing tumour. The TLX-5 cells were grown in vitro for several passages, and a cell-free preparation of "conditioned" growth medium prepared. Mice injected with this preparation showed changes in hepatic citrate content similar to those of mice injected with SN1. These results indicate that this biologically active factor(s) is present in the "conditioned" growth medium from the TLX-5 lymphoma cells and is probably primarily of tumour origin. Examination of the in vitro grown tumour cells using a light microscope did not reveal the presence of host cells in the preparation, but the possibility remains that there may have been host lymphocytes present in the preparation, in very low concentrations.

Considerable purification in terms of protein content was obtained upon high speed centrifugation of SN1 at 240,000gmax for 24 hours (to give SN2), and upon precipitation of SN1 with ammonium sulphate. Both of these purification procedures did not alter the biological activity of the preparation, and since ultracentrifugation results in a virus-free preparation, subsequent studies were carried out using SN2. Large volumes of SN1 and SN2 were required for these and other purification procedures, and it was possible to store SN1 and SN2 at -70°C for periods of up to 60 days without affecting their biological activity.

Dialysis of SN2 did not affect its biological activity, but heat-treatment did. When SN2 was treated with trypsin its biological activity was completely destroyed, indicating that the biologically active factor(s) involved is protein in nature. The effect of SN2 on hepatic citrate levels was also shown to be concentration dependant. Since the active factor(s) was not removed by dialysis or by ultracentrifugation under the conditions described, it was thought likely to have a molecular weight in the range 10,000-45,500 daltons.

Gel filtration is a valuable technique for the estimation of the molecular weights of unknown compounds (Andrews, 1970) particularly proteins, and can be applied to the isolation and purification of

proteins. Sephadex is particularly useful for the chromatography of substances of biological origin, which are often very labile. In gel filtration quantitative yields are obtained as there is no irreversible retention of small amounts of substances on the column, and it is therefore useful for analytical purposes. The choice of the appropriate Sephadex type depends on the molecular size of the substances to be separated, since each Sephadex type fractionates within a particular molecular weight range determined by the degree of swelling of the gel.

Molecules with a molecular weight in the range 10,000-45,500 daltons can be separated upon columns of Sephadex G-100. This Sephadex type has a molecular weight fractionation range between 4,000-150,000 daltons for peptides and globular proteins (Andrews, 1970). Fractions eluted from a Sephadex G-100 column were tested for biological activity, and activity was detected in fractions in which molecules of a relative molecular weight of 6948.6-7587.7 daltons would be eluted.

Moreover, the molecular weight of a protein can also be estimated by electrophoresis in polyacrylamide gels. The homogeneity of protein samples during purification procedures can also be determined using this technique. The advantages of using this method are that it can rapidly separate complex mixtures of protein with high resolution, and requires only a small

amount of protein. The main factors which effect separation are time of electrophoresis, the concentration of acrylamide in the gels, and the sample volume. Short electrophoresis times and high acrylamide concentrations (10% or greater) favour the separation of low molecular weight compounds.

Biologically active fractions, obtained from the gel filtration of SN2, were subjected to polyacrylamide gel electrophoresis under the conditions described in Chapter 2. The mobility of the components of these fractions within the gel was found to be identical. These fractions were found to contain 2 main components, one with a relative molecular weight of 6886.5 daltons, the other with a relative molecular weight of 103,276 daltons. The presence of this high molecular weight component in these fractions presents a dilemma, since molecules of this size would not have eluted from the Sephadex G-100 column at the same point as the 6886.5 daltons component, nor would it be present in SN2. It is thought that this molecule may be present due to the contamination of glassware during the purification procedures, or may be ovalbumin which was introduced as a "carrier-protein" during the [^{3}H] dansyl chloride reaction of the proteins. The low molecular weight component of 6886.5 daltons is thought to be the biologically active component of SN2.

Moreover, biological activity could also be detected in the serum of tumour-bearing mice, indicating that

this factor is a humoral factor. Since this factor has a molecular weight similar to that of insulin, normal mice were treated with insulin and their hepatic citrate content measured. Insulin was found to have no effect on hepatic citrate levels, but it is of interest that marked decreases in serum insulin have been reported in rats bearing Walker 256 carcinosarcoma, accompanied by decreases in serum glucose levels (Goodlad, Mitchell, McPhail and Clark, 1975). It was concluded by these workers that these decreases in serum insulin and glucose reflected the general disturbances of energy metabolism in the host associated with tumour growth.

Although these results show that there is a factor present in the ascitic fluid and sera of tumour-bearing mice, responsible for increasing hepatic citrate levels, and that this factor is heat-labile, probably primarily of tumour origin, protein in nature with a molecular weight between 6,800-7,600 daltons, these results give no insight into the mechanisms underlying the production of this metabolic abnormality.

These observed changes in hepatic citrate content are opposite to those found in animals under conditions of forced gluconeogenesis (Wieland, 1966; Start and Newsholme, 1968) although McAllister, Soukop and Calman (1976, 1977) argue that an accumulation of citrate in the livers of tumour-bearing mice would inhibit phosphofructokinase, since citrate is one of

several modifiers of the enzyme (Start and Newsholme, 1970). This would result in a reduction of the glycolytic flux in the liver and favour gluconeogenesis, since phosphofructokinase is a rate-limiting enzyme in glycolysis. This mechanism, however, does not appear to regulate gluconeogenesis in the liver of normal animals, since under gluconeogenic conditions the hepatic citrate content decreases (Start and Newsholme, 1968; Herrera and Frienkel, 1968).

(ii) Spleen

Hypertrophy of the spleen of tumour-bearing animals has been frequently observed (Stewart and Begg, 1953; Konda and Smith, 1973). Nakahara and Fukuoka (1958) and Adams and McCoy (1959) showed that rapid enlargement of the spleen occurred in mice injected with "toxohormone". Injection of cell-free ascitic fluid from TLX-5 lymphoma bearing mice (McAllister, Soukop and Calman, 1977) was also shown to induce spleen hypertrophy.

It has also been shown in this study that the spleen weight of TLX-5 lymphoma bearing mice and mice injected with SN1 increases significantly. Dialysis of SN1 does not affect its ability to increase the spleen weights of treated mice, but heat-treated SN1 will not produce this effect. SN2 treated mice also show an increase in spleen weight.

Konda and Smith (1973) were of the opinion that hypertrophy of the spleen was due to the immune response, and presented evidence showing that the

functional, immunologically competent cells of the spleen were increased during tumour-bearing. They also suggested that tumour cell products may be involved in producing these effects.

It would appear that a factor or factors present in SN1, which is non-dialysable, heat labile and present in the high speed supernatant of SN1 and in the 35-65% ammonium sulphate precipitate, is responsible for increasing spleen weight. From this data the molecular weight of this factor or factors is between 10,000-45,500 daltons, but no further investigations into the nature of the factor or factors involved was carried out.

(iii) Thymus

Thymic atrophy is a frequent finding in tumour-bearers, and Konda and Smith (1973) showed a decrease in thymus cell numbers over the course of tumour growth. The functional, immunologically competent cells of the thymus, as well as the spleen, were also shown to be increased during tumour-bearing. Ertl (1973) showed that in rats bearing the Walker 256 carcinosarcoma commencement of thymic atrophy was related to the onset of progressive cachexia, and these results were confirmed by McAllister, Soukop and Calman (1976, 1977).

Moreover, Nakahara (1952) demonstrated thymus involution in animals injected with "toxohormone", but in this study thymus involution was not seen in mice injected with SN1 although it could be seen in tumour-bearing mice. Decreases in thymus weight were detected

in mice injected with SN2, but these changes did not reach a significant level.

(iv) Epididymal Fat Pads

Haven, Bloor and Randall (1949) showed that in rats bearing the walker 256 carcinosarcoma carcass lipids varied inversely with the size of the tumour, but McAllister, Soukop and Calman (1976) did not observe a loss of carcass lipid in mice bearing the TLX-5 lymphoma. They did observe, however, increases in the epididymal fat pad weight of these mice during the course of tumour growth.

Data presented herein confirms the results of McAllister, Soukop and Calman (1976) and shows that in TLX-5 lymphoma-bearing mice and mice injected with SN1, significant increases in epididymal fat pad weight does occur. Dialysis and heat-treatment of SN1 destroys its activity in producing these changes. Also, mice injected with SN2 do not show any significant changes in epididymal fat pad weight. These results indicate that the factor or factor(s) present in SN1 responsible for bringing about these changes is heat-labile, and removed by ultracentrifugation.

From these results it would appear that there are several different factors present in the cell-free ascitic fluid from TLX-5 lymphoma-bearing mice responsible for bringing about the observed systemic effects of the tumour, that is, increases in hepatic citrate, spleen hypertrophy, thymic atrophy and increases in epididymal fat pad weight.

(v) Virology Studies

Ohashi (1961) was able to isolate "toxohormone" from the spleens of mice infected with Friend virus, suggesting that the possibility of viral infection showing a "toxohormone"-like activity must not be overlooked. Kampschmidt and Schultz (1963) showed that the effects of tumour extracts on plasma iron (which is more sensitive to the effects of "toxohormone" than liver catalase) can be attributed to lipopolysaccharide from bacterial contaminants in the tumour. Kampschmidt and Schultz (1963) investigated several different transplantable tumours and found that in none of these could a "toxohormone" factor be isolated in the absence of bacteria. They did not discount, however, the possibility of viral contamination of these tumours being involved in producing a "toxohormone"-like effect.

Moreover, Davis, Cross and Lapis (1962) presented evidence that anaemia associated with the NK/LY ascites tumour in mice is caused by a replicating agent viroid in nature, which can be isolated from the tumour cells. One virus particularly associated with murine tumours is LDV.

Since the initial report on the association of LDV with mouse tumours (Riley, Lilly, Huerto and Bardell, 1960) a large number of experimental mouse tumours were found to be contaminated with this virus (Notkins, 1965). LDV was first recognized by its ability to increase plasma LDH. It then became apparent that a number of

other enzymes were elevated in the plasma of infected mice. These enzymes included isocitrate dehydrogenase and malate dehydrogenase (Notkins, Greenfields, Marshall and Bane, 1963; Plagemann, Gregory, Swim and Chan, 1963). The effect of LDV on the activity of glycolytic enzymes of infected mice, and the widespread contamination of muring tumours with this agent led to the consideration of the possibility that the TLX-5 lymphoma used in this study might be contaminated with this virus. If this was the case, then the interpretation of experimental results would be difficult since LDV, as well as causing splenomegaly (Notkins, 1965) may also be causing the observed increase in hepatic citrate levels of tumour-bearing mice, and mice treated with cell-free preparations of the tumour.

However, tumours can be freed from LDV contamination by passage through rats or by several passages through tissue culture (Notkins, 1965). In the absence of a specific serological test for LDV, the characteristic elevation of plasma or serum LDH activity, reaching a peak 96 hours after infection and the high virus titre 24 hours after infection, are sufficiently unique to determine whether LDV is present (Pope and Rowe, 1964).

Although there have been reports of a close correlation between the growth of certain murine tumours and increases in plasma LDH (Hsieh, Suntzeff and Cowdry, 1955; Manso, Sugiura and Wroblewski, 1958) these

experiments had been performed before it was recognized that many tumours were contaminated with LDV. Various workers then reinvestigated the relationship between tumour growth and plasma enzyme elevation in the presence and absence of LDV (Adams, Rowson and Salaman, 1961; Notkins and Greenfield, 1962; Notkins, Greenfield, Marshall and Bane, 1963; Plagemann, Watanabe and Swim, 1962; Riley, 1963; Yaffe, 1962). They showed that the early rise in plasma enzyme activity was due, not to the presence of a tumour, but to the presence of LDV. These reports indicated, however, that in the absence of LDV the activity of LDH, MDH and isocitrate dehydrogenase in tumour-bearing animals remained within the normal range until the tumours became palpable and then enzyme elevation roughly paralleled the growth of the tumour.

The problem of the possible contamination of TLX-5 lymphoma with LDV was approached in the following ways:-

(1) SN1 was extensively centrifuged to give a virus free preparation, SN2 (Notkins, 1965).

(2) TLX-5 lymphoma cells were passaged in tissue culture to remove endogenous virus contamination (Notkins, 1965).

(3) Serum LDH activity was measured in tumour-bearing mice and mice injected with SN2.

(4) Tumour cells grown <u>in vitro</u> and <u>in vivo</u> were examined by electron microscopy for the presence of virus particles.

Extensive centrifugation of SN1 did not adversely affect its biological activity, and increases in hepatic

citrate content were observed in mice treated with the virus free tumour preparation, SN2. TLX-5 lymphoma cells grown in vivo for several passages were also shown to significantly increase hepatic citrate levels.

However, the serum LDH activity of mice injected with in vivo grown TLX-5 cells was significantly increased, as was LDH activity in the serum of mice injected with SN2. Since the TLX-5 lymphoma is a particularly fast growing tumour, and since serum LDH activity was measured 96 hours after the injection of tumour cells or SN2, it is possible that a factor present in the tumour cell preparation and in SN2, which is not a virus, is involved in producing this effect. Also, since red blood cells contain high levels of LDH, the slightest degree of haemolysis in the collected serum would increase LDH activity measured. Much more work is required before this phenomenon can be clearly understood.

Electron microscope examination by Notkins (1965) of a thin section of an osmium fixed pellet prepared from viremic plasma revealed rounded particles averaging 40 mu in diameter, and elliptical or oblong particles 36-40 mu wide and 45-75 mu long. These particles had a prominent, ring-shaped nucleoid 26-29 mu in diameter, consisting of a dense ring with an electron luscent core. Around the nucleoid was a 5-7 mu thick layer, limited by what appeared to be a thin outer membrane. Notkins (1965) also observed particles similar in size

and shape within macrophages obtained from the peritoneal cavity of infected mice.

Electron microscope examination of *in vivo* grown TLX-5 lymphoma cells revealed the presence of particles in the preparation. These particles had an average length of 74 mu, and were not present in *in vitro* grown tumour cells. It is therefore possible that these virus particles are LDV particles, but further characterisation is required before they can be positively identified.

C O N C L U S I O N S

This work has demonstrated the presence of a factor in the ascitic fluid of TLX-5 lymphoma bearing mice capable of increasing hepatic citrate levels. This factor is present in the serum of tumour bearing mice and is probably of tumour origin. It is non-dialysable, heat-labile, and protein in nature since treatment with trypsin destroys its biological activity. Gel filtration and gel electrophoresis experiments indicate that the factor has a molecular weight between 6,800-7,600 daltons.

Although *in vivo* grown TLX-5 lymphoma cells are contaminated with a virus, this factor does not appear to be a virus since it is present in SN2, and *in vitro* grown tumour cells which are free from viral contamination also bring about increases in hepatic citrate levels.

Moreover, a factor present in SN2 and therefore not a virus is active in increasing serum LDH activity in mice injected with SN2. The injection of SN2 into normal mice also brings about increases in the spleen weight of these mice. This factor involved in increasing spleen weight is non-dialysable, and heat-labile, with a molecular weight which is probably in the region 10,000-45,500 daltons.

Although SN1 treatment increases the weight of mouse epididymal fat pads, dialysis, heat-treatment and ultracentrifugation of SN1 destroy this effect. Results indicate that there are several different factors present in the ascitic fluid from tumour-bearing mice responsible for bringing about these observed systemic effects of the tumour.

Gel electrophoresis of this purified factor obtained from gel-filtration experiments was not conclusive in determining the homogeneity of the purified preparation. Further work using gels with a higher acrylamide concentration might be conclusive in determining this. Injection of mice with insulin was ineffective in increasing hepatic citrate levels, therefore this factor was not insulin.

No attempt has been made in this work to study the mechanisms underlying the metabolic abnormalities produced by this biologically active component from TLX-5 lymphoma cells, and further work would be required to investigate the mechanisms involved.

REFERENCES.

Adams, D.H. (1959) The effect of sarcoma 37 on the intracellular distribution of mouse liver catalase. British Journal of Cancer, 13, 704-710.

Adams, D.H., Rowson, E.K. and Salaman, M.H. (1961) The effect of tumours, of leukemia, and of some viruses associated with them, on the plasma lactate dehydrogenase activity of mice. British Journal of Cancer, 15, 860-867.

Aloya, V.J. (1979) Scintillation counting of ^{3}H and ^{14}C-containing gel slides: a one-step method. Analytical Biochemistry, 99, 1, 161.

Andrews, P. (1970) Estimation of molecular size and molecular weights of compounds by gel filtration. In Methods of Biochemical Analysis, ed. Glick, D. Vol. 18. John Wiley and Sons.

Annau, E., Manginelli, A. and Roth, A. (1951) Increased weight and mitotic activity in liver of tumour-bearing rats and mice. Cancer Research, 11, 304-306.

Aoki, C, (1938) Uber den cholesterin, phosphatiden und fettsaurengehalt der organe von hepatomgeimpften ratten. Gann, 32, 101-105.

Argyris, T.S. and Argyris, B.F. (1962) Differential response of skin epithelium to growth-promoting effects of a subcutaneously transplanted tumour. Cancer Research, 22, 74-77.

Begg, R.W. (1958) Tumour-host relations. In Advances in Cancer Research, ed. Greenstein, J.P. and Haddow, A. Vol. 5, pp. 1-54. London: Academic Press.

Bodansky,O. and Scholler, J. (1956) Increases in plasma lactate dehydrogenase activity in tumour-bearing rats. Cancer Research, 16, 894-899.

Bergmeyer, H.U. (1965) Methods of Enzymatic Analysis. London: Academic Press.

Bladen, H.A. Jr. and Notkins, A.L. (1963) Electron microscope demonstration of the lactate dehydrogenase agent. Virology, 21, 269-271.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye-binding. Analytical Biochemistry, 72, 248-254.

Brewer, J.M., Pesce, A.J. and Ashworth, R.B. (1974) Practical aspects of column chromotography. In Experimental Techniques in Biochemistry, pp. 84-92. Prentice Hall.

Brewer, J.M., Pesce, A.J. and Ashworth, R.B. (1974) Electrophoresis. In Experimental Techniques in Biochemistry, pp. 84-92. Prentice Hall.

Busch, H. (1962) An Introduction to the Biochemistry of the Cancer Cell, pp. 128-159. Prentice Hall.

Cahill, G.F. Jr. (1970) Insulin and proinsulin. New York Journal of Medicine, 283, 762.

Calman, K.C. and Mc Allister, R.A. (1975a) Metabolic abnormalities in tumour-bearing animals. British Journal of Surgery, 32, 161.

Calman, K.C. and Mc Allister, R.A. (1975b) Metabolic abnormalities in tumour-bearing animals. British Journal of Cancer, 32, 247.

Carey, R.W., Pretlow, T.G., Ezdlini, E.Z. and Holland, J.F.

(1966) Studies on the mechanism of hypoglycaemia in a patient with massive intraperitoneal leimyosarcoma. American Journal of Medicine, 40, 458-469.

Cavallini, D., Mondovi, B., De Marco, C. and Ferro-Luzzi, G. (1959) The biosynthesis of coenzyme A in pigeon liver extract. Enzymologia, 20, 359-365.

Ceriotti, G. and Spandrio, L. (1955) On the in vitro anticatalase activity of tumour extracts. Biochimica et Biophysica Acta, 18, 303.

Ceriotti, G., Spandrio, L. and Berti, E. (1975) On the in vitro inhibition of catalase by amino acids. Biochimica et Biophysica Acta, 23, 262.

Chan, P.L. and Sinclair, N.R. (1972) Immunologic and virologic properties of chemically and gamma-irradiation induced thymic lymphomas in mice. Journal of the National Cancer Institute, 48, 6.

Cheung, H.T., Cantarrow, W.D. and Sundharadas, G. (1979) Characteristics of a low molecular factor extracted from mouse tumours that affects in vitro properties of macrophages. International Journal of Cancer, 23, 344-352.

Cohen, G., Dembiec, D. and Marcus, J. (1970) Measurement of catalase activity in tissue extracts. Analytical Biochemistry, 34, 30-38.

Cohen, S.M., Ogawa, S. and Shulman, R.G. (1979) ^{13}C-NMR studies of gluconeogenesis in rat liver cells; utulisation of labelled glycerol by cells from euthyroid and hyperthyroid rats. Proceedings of the National Academy

for Science, U.S.A., 79, 4, 1603-1607.

Costa, G. (1977) Cachexia, the metabolic component of neoplastic disease. Cancer Research, 37, 2327-2335.

Costa, G. (1977) Summary of the informal discussion of general effects of cancer on nutrition. Cancer Research, 37, 2365.

Costa, G. and Holland, J.F. (1962) Effects of Krebs 2 carcinoma on the lipide metabolism of male Swiss mice. Cancer Research, 22, 1081.

Curatola, L., Colucci, M., Cambini, A.L., Poggi, A., Marasca, L., Donati, M.B. and Semeraro, N. (1979) Evidence that cells from experimental tumours activate coagulation factor X. British Journal of Cancer, 40, 228.

Dalton, A.J. (1954) Histologic changes in adrenal glands of tumour-bearing mice. Journal of the National Cancer Institute, 5, 99-109.

Davies, A.J.S., Cross, A.M. and Lapis, K. (1962) Anaemia associated with the NK/lymphoma in mice. The British Journal of Cancer, Vol. XVI, 770.

Day, E.D., Gabrielson, F.C. and Lipkind, J.B. (1954) Depressions in the liver catalase activity of mice injected with homogenates of normal mouse spleen. Journal of the National Cancer Institute, 15, 239-252.

De Duve, C. (1963) The lysosome concept. Ciba Symposium on Lysosomes, ed. De Reuck, A.V.S. and Cameron, M.P., pp. 1-35. J. and A. Churchill, Ltd., London.

Devlin, T.M. and Pruss, M. (1958) Oxidative phosphorylation

in transplanted Novikoff hepatoma of the rat. Federation Proceedings, 17, 211.

Devlin, T.M. and Costa, G. (1964) Phosphorylation and ATPase activity of liver mitochondria from Swiss mice bearing Krebs 2 carcinoma. Proceedings of the National Academy of Science, U.S.A., 116, 1095-1098.

De Wys, W.D. (1970) Working conference on anorexia and cachexia of neoplastic disease. Cancer Research, 30, 2816-2818.

De Wys, W.D. (1972) Studies correlating the growth rate of a tumour with its metastasis and providing evidence for tumour-related systemic growth retarding factors. Cancer Research, 32, 374-379.

De Wys, W.D. (1977) Anorexia in cancer patients. Cancer Research, 37, 2354-2358.

De Wys, W.D. (1979) Anorexia as a general effect of cancer. Cancer, 43, 2013-2019.

Dickens, F. and Weil-Malherbe, H. (1941) Metabolism of normal and tumour tissue; metabolism of intestinal mucous membrane. Biochemical Journal, 35, 7-15.

Eisenstein, A. (1973) Effects of adrenal corticol hormones on carbohydrate, protein, and fat metabolism. American Journal of Clinical Nutrition, 26, 113-120.

Elleman, C.J. and Eldinger, D. (1977) Suppressive factors in ascitic fluid and sera of mice bearing ascites tumours. Journal of the National Cancer Institute, 59, 3.

Engel, R. (1941) Epidemien von seroser hirnhautentzundung

in kassa und umgebung. Kin Wchnschr. 20, 1004-1008.

Engel, R. (1942) Indikationen zur bluttransfusion ouf dem gebiete der inneren medizin. Zentralbl. f. Chir., 69, 980.

Ertl, N. (1973) Systemic efects during growth of malignant experimental tumours. Oncology, 415-425.

Exton, J.H., Corbin, J.G. and Park, C.R. (1969) Control of gluconeogenesis in liver. The Journal of Biological Chemistry, 244, 15, 4095-4102.

Exton, J.H., Friedman, N., Wong, E.H.A., Brineaux, J.P., Corbin, J.D. and Park, C.R. (1972) Interaction of glucocorticoids with glucagon and epinephrine in the control of gluconeogenesis and glycogenolysis in liver and of lipolysis in adipose tissue. The Journal of Biological Chemistry, 247, 3579-3588.

Evgenieva, T.P. (1970) Vopr. Oncol., 16, 54. (In Russian).

Fenninger, L.D. and Mider, G.B. (1954) energy and nitrogen metabolism in cancer, In Advances in Cancer Research, ed. Greenstein, J.P. and Haddows, A. Vol. 2, pp. 229-253. London: Academic Press.

Field, E.J. and Caspary, E.A. (1972) Lymphocyte sensitisation in advanced malignant disease: a study of lymphocyte depressive factor. British Journal of Cancer, 26, 164.

Folkman, J (1974) Tumour angiogenesis. In Advances in Cancer Research, ed. Klein, G. and Weinhouse, S. Vol. 19, pp. 331-357. New York: Academic Press.

Folkman, J., Merler, E., Abernathy, C. and Williams, G. (1971)

Isolation of a tumour factor responsible for angiogenesis. Journal of Experimental Medicine, 133, 275.

Fujii, S., Kawachi, T., Okuda, H., Haga, B. and Yamamura, Y. (1960) The isolation of a basic protein having toxohormone activity from tumour tissue. Gann, 51, 223-229.

Fukuoka, S. and Nakahara, W. (1952) Toxohormone and thymus involution in tumour-bearing animals. Gann, 43, 55-62.

Ghadially, F.N. and Parry, E.W. (1965) Ultrastructure of the liver of tumour-bearing animals. Cancer, 18, 4.

Goodlad, G.A.J., Mitchell, A.J.H., Mc Phail, L. and Clark, C.M. (1975) Serum insulin and somatedin levels in the tumour-bearing rat. European Journal of Cancer, 11, 733.

Greenbaum, A.L., Gumaa, K.A. and Mc Lean, P. (1971) The distribution of hepatic metabolites and the control of the pathways of carbohydrate metabolismin animals of different dietary and hormonal status. Archives of Biochemistry and Biophysics, 143, 617-633.

Greene, A.A. (1960) Oxidative phosphorylation in liver mitochondria of the rat bearing Walker carcinoma 256. Cancer Research, 20, 233-236.

Greenfield, R.E. and Price, V.E. (1954) Liver catalase: Manometric determinations. Journal of Biological Chemistry, 209, 355-361.

Greenstein, J.P. (1942a) Distribution of acid and alkaline phosphatase in tumours, normal tissue and tissues of tumour bearing rats. Journal of the National Cancer Institute, 2.

Greenstein, J.P. (1942b) Titration of liver catalase activity activity of normal and tumour-bearing rats and mice. Journal of the National Cancer Institute, 2, 525-530.

Greenstein, J.P. (1943) Further studies of liver catalase activity of tumour-bearing animals. Journal of the National Cancer Institute, 3, 397-404.

Greenstein, J.P., Jenrette, W.V. and White, J. (1941) The liver catalase of tumour-bearing rats and the effect of extirpation of the tumours. Journal of the National Cancer Institute, 2, 283-291.

Grusdeva, K.V. (1966) Doctor of Science Thesis, Omsk, U.S.S.R. (in Russian).

Hanschumaker, R.F., Mueller, G.C. and Strong, F.M. (1951) An improved enzymatic assay for coenzyme A. The Journal of Biological Chemistry, 189, 335-342.

Harrison, M.F. (1953) Effect of starvation on the composition of the liver cell. The Biochemical Journal, 55, 204-211.

Haven, F.L., Bloor, W.R. and Randall, C. (1949) Lipids of the carcass, blood plasma and adrenals of the rat in cancer. Cancer Research, 9, 9.

Haven, F.L. and Bloor, W.R. (1956) Lipids in cancer. In Advances in Cancer Research, ed. Greenstein, J.P. and Haddow, A. Vol. 4, pp. 288-314. London: Academic Press.

Henderson, D.C., Chang, R.W.S. and Turk, J.L. (1979) Infection with lactate dehydrogenase virus alters host response to tumours. British Journal of Cancer, 39, 453.

Henriksen, O. and Law, L.W.(1977) Biological characterisation of a tumour growth-promoting factor from ascitic fluid of tumour-bearing mice. International Journal of Cancer, 19, 866-869.

Herrman, J. and Staib, W. (1969) Beziehung zurischen lipolyse gluconeogenese und insulinsekretion nach cortisolgabe an ratten. European Journal of Biochemistry, 7, 427-433.

Herrera, E. and Frienkel, N. (1968) Interrelationship between liver composition, plasma glucose, ketones and hepatic acetyl coenzyme A and citric acid during prolonged starvation in the male rat. Biochimica et Biophysica Acta, 170, 244-253.

Hill, B.R. and Levi, C. (1954) Elevation of a serum component in neoplastic disease. Cancer Research, 14, 513.

Higgins, H.M., Miller, J.A., Price, J.M. and Strong, F.M. (1951) Levels and intracellular distribution of coenzyme A and pantothenic acid in rat livers and tumours. Proceedings of the Society of Experimental Biology and Medicine, 75, 462.

Higgins, P.J., Tong, C., Borenfreund, E., Okund, R.S. and Bendich, A. (1979) Tissue distribution and biochemical properties of an interspecific tumour-associated gamma foetal antigen. British Journal of Cancer, 40, 253.

Holmberg, B. (1965) Isolation of a polypeptide from tumour tissue with a toxic effect on cells in culture. European Journal of Cancer, 1, 199.

Holmberg, B. (1968) Further characterisation of a polypeptide from tumour tissue which exerts a toxic effect on cells in culture. European Journal of Cancer, 4, 263-271.

Hornbrook, K.R., Burch, H.B. and Larry, O.H. (1965) Changes in substrate levels in liver during glycogen synthesis induced by lactate and hydrocortisone. Biochemical and Biophysical Research Communications, 18, 206-211.

Holroyde, C.P., Gabuzda, T.G., Putnam, R.C., P. and Richard, M.A. (1975) Altered glucose metabolism in metastatic carcinoma. Cancer Research, 35, 3710-3714.

Hsieh, K.M., Suntzeff, V. and Cowdry, E.V. (1955) Serum lactate dehydrogenase activity as indication of neoplastic disease and regression. Proceedings of the Society of Biological Medicine, 89, 627-629.

Jakubovic, A., Keilova, H. and Sorm, F. (1961) On inhibition of liver catalase activity (ii) The influence of cell-free ascitic fluid on liver catalase activity in mice. Neoplasia, 8, 45-51.

Johnson, M.E.M., Das, N.M., Butcher, F.R. and Fain, J.F. (1972) The regulation of gluconeogenesis in isolated rat liver cells by glucagon, insulin, dibutyryl cyclic adenosine monophosphate and fatty acids. The Journal of Biological Chemistry, 247, 10, 3229-3235.

Kawai, A., Tamura, M., Tanimoto, S., Honna, H. and Kuzyya, N. (1969) Studies on adrenocortical function in patients with lung cancer. Metabolism, 18, 609-619.

Kay, D. (1961) Techniques for Electron Microscopy. Oxford, England, Blackwell Scientific Publications Ltd., pp. 179-181.

Kampschmidt, R.F. (1965) Mechanism of liver catalase depression in tumour-bearing animals: a review. Cancer Research, 25.

Kampschmidt, R.F., Adams, M.E. and Mc Coy, T.A. (1959) Some systemic effects of toxohormone. Cancer Research, 19.

Kampschmidt, R.F. and Schultz, G.A. (1963) Absence of

toxohormone in rat tumours free of bacterial contamination. Cancer Research, 23.

Kampschmidt, R.F., Mayne, M.A., Goodwin, W.L. and Clabaugh, W.A. (1960) Duplication of some of the systemic effects of tumours by extracts from these tumours. Cancer Research, 20, 3, 368-372.

Kampschmidt, R.F. and Upchurch, H.F. (1963) Effect of bacterial contamination of the tumour on tumour-host relationships. Cancer Research, 23.

Kido, H., Tomihara, Y., Watanabe, I., Ohsawa, N. and Katanuma, N. (1979) Amplification of growth inhibition by glucocorticoid on L5178Y and L1210 lymphoblasts in vivo. Cancer Research, 39, 3258-3261.

Knox, E.W. and Sharma,C. (1968) Enzymologia Biologica et Clinica, 9, 21-30.

Konda, S., Nakao, Y. and Smith, R.T. (1973) The stimulatory effect of tumour-bearing upon the T and B cell subpopulations of the mouse spleen. Cancer Research, 33, 2247-2256.

Konda, S. and Smith, R.T. (1973) The effect of tumour-bearing upon changes in cell distribution in membrane antigen characteristics in murine spleen and thymus cell subpopulations. Cancer Research, 33, 1878.

Kuzin, A.M., Sharoukhova, K.S. and Chudinova, I.A. (1955) Effect of tumour extracts on the catalase and coenzyme A of the liver of normal mice. Biokhimiya, 20, 126-128.

Landau, B.R., Wells, N., Craig, J.W. and Leonard, J.R. (1962) The mechanism of hepatoma-induced hypoglycaemia. Cancer, 15, 1188-1196.

Le Page, G.A., Greenlees, J.L., Edmonds, M.P. and Groth, D.P. (1955) Investigation of diverse systems for cancer chemotherapy screening; incorporation of glycine-2-14C into ascites tumour cell purines as a biological test system. Cancer Research, (Supplement 3), 102-105.

Le Page, G.A., Potter, R., Bush, H., Heidelburger, C. and Hurlberg, R.B. (1952) Growth of carcinoma implants in fed and fasted rats. Cancer Research, 12, 153-157.

Le Vine, R. (1964) Analysis of the actions of the hormonal antagonists of insulin. Diabetes, 13, 4.

Lundholm, K. (1975) Skeletal muscle metabolism in cancer disease. A study with special reference to the development of cancer cachexia. Thesis, Goteburg, Elanders boktrveken.

Lundholm, K., Bylund, A.C., Holm, J. and Schersten, F. (1976) Skeletal muscle metabolism in patients with malignant tumour. European Journal of Cancer, 12, 465-473.

Lundholm, K., Edstrom, S., Ekman, L., Karlberg, I., Bylund, A. and Schersten, T. (1978) A comparative study of the influence of malignant tumour on host metabolism in mice and man. Cancer, 42, 453.

Lundholm, K. and Schersten, T. (1975) Incorporation of leucine into human skeletal muscle protein. A study of tissue amino acid pools and their role in protein biosynthesis. Acta Physiologica Scandinavia, 93, 433-441.

Mahler, H.R. and Cordes, E.H. (1966) In Biological Chemistry. Harper and Row, New York.

Manso, C., Suguira, K. and Wroblewski, F. (1958) Glutathionine reductase and lactate dehydrogenase activities

of tissues of rodents with transplanted tumours. Cancer Research, 18, 682-689.

Marshal, M.J., Neal, F.E. and Goldberg, D.M. (1979) Isoenzymes of hexokinase, 6-phosphogluconate dehydrogenase, phosphoglucomutase and lactate dehydrogenase in tumour-bearing animals. British Journal of Cancer, 40, 380.

Mc Allister, R.A., Soukop, M. and Calman, K.C. (1976) Metabolic abnormalities in tumour-bearing animals. British Journal of Cancer, 34, 312.

Mc Allister, R.A., Soukop, M. and Calman, K.C. (1977) Correction of changes in liver metabolites of mice following curative tumour resection. British Journal of Cancer, 46, 405.

Mc Allister, R.A. (1978) Studies on metabolic changes in liver of tumour-bearing mice. A Thesis submitted to the University of Glasgow for the Degree of Doctor of Philosophy.

Mc Ewen, H.D. and Haven, F.L. (1941) Effect of carcinoma 256 on the water content of liver. Cancer Research, 1, 148-150.

Mc Fadzean, A.J.S. and Yeung, T.T. (1956) Hypoglycaemia in primary carcinoma of the liver. American Medical Association Archives of Internal Medicine, 98, 720-731.

Mc Queen, M.J. (1972) Optimal assay of LDH and α-HBD at 37°C. Annals of Clinical Biochemistry, 9, 21.

Medigrecenau, F. (1970) On the relative size of the organs of rats and mice bearing malignant new growths. Proceedings of the Royal Society of London (Biology), 82, 286.

Mergenhagen, S.A. and Notkins, A.L. (1967) Adjuvanticity of lactic dehydrogenase virus: Influence of virus infection on the establishment of immunologic tolerance to a protein antigen in adult mice. The Journal of Immunology, 99, 3, 576-581.

Mertvetzov, N.P. (1969) Antogonizm v deistvii insulina i gidrokortizona v protesse fermentativnoi induktsii. Biokhimiya, 34, 381-384.

Meyer, J.P. and Meyer, J.A. (1977) Prevention by splenectomy of thymus weight depletion in the presence of progressively growing tumour in rats. Journal of the National Cancer Institute, 59, 3.

Mider, G.B. (1951) Some aspects of nitrogen and energy metabolism in cancer subjects: A review. Cancer Research, 11.

Mider, G.B. (1954) Proceedings of the Cancer Research Conference, First Conference, Honey Harbour, Ontario, pp. 120-137.

Mider, G.B., Tesluk, J. and Morton, J.J. (1948) Effects of Walker carcinoma 256 on food intake, body weight and N_2 metabolism in growing rats. Acta of the Unio International Contra Cancrum, 6, 409-420.

Mischenko, I.P. (1940) Processes of the Synthesis and analysis in cancer's host as revealed by the data on nitrogen metabolism. Kharkov, 3, 109-115. (In Russian).

Mizukami, T., Kobayashi, A. and Nishio, T. (1969) Cancer and endocrine metabolism with special reference to pituitary adrenal system function. Clinical Endocrinology (Tokyo), 16, 679-684.

Mollering, H. and Bergmeyer, H.U. (1975) Kinetic determinations of CoA. In Methods of Enzymatic Analysis, ed. Bergmeyer, H.U. Vol. 4. London: Academic Press.

Mullen, J.L., Buzby, G.P., Gertner, M.H., Stein, T.P., Hargrove, W.C., Oram-Smith, J. and Rosato, E.F. (1980) Protein synthesis dynamics in human gastrointestinal malignancies. Surgery, 87, 33.

Nair, B.K., De Ome, K.B. (1973) A growth stimulating factor from solid mouse mammary tumours. Cancer Research, 33.

Nanni, G. and Casu, A. (1961) In vitro uncoupling of oxidative phosphorylation in normal mitochondria by serum of sarcoma-bearing rats. Experientia, 17, 402-404.

Nanni, G. (1961) Influenza di estratti di un sarcoma da innesto del ratto sulla moffologia dei mitocondri di fegato e di rene. Sperimentale, 111, 55-65.

Nakagaura, S. (1952) Proceedings of the Japanese Academy, 28, 305-310. (In Japanese).

Nakahara, W. and Fukuoka, F. (1948) A toxic cancer tissue constituent as evidenced by its effect on liver catalase activity. Japanese Medical Journal, 1, 271-277.

Nakahara, W. and Fukuoka, F. (1949) Toxohormone: A characteristic toxic substance produced by cancer tissue. Gann, 40, 45-69.

Nakahara, W. and Fukuoka, F. (1958) The newer concept of a cancer toxin. In Advances in Cancer Research, Vol.5, pp.157-177, ed. Greenstein, J.P. and Haddow, A. London: Academic Press.

Nakahara, W. (1960) A chemical basis for tumour-host relations. Journal of the National Cancer Institute, 24,77.

Nakata, Y., Suematsu, T., Nakata, K., Matsumoto, K. and Sakamoto, Y. (1964) Activities of various aminotransferases

in tumour-bearing rats. Cancer Research, 24, 1689.

Nepom, J.T., Hellstrom, I. and Hellstrom, K.E. (1976) Purification and partial characterisation of a tumour-specific blocking factor from sera of mice with growing chemically induced sarcomas. The Journal of Immunology, 117, 5.

Nepom, J.T., Hellstrom, I. and Hellstrom, K.E. (1977) Antigen specific purification of blocking factors from sera of mice with chemically induced tumours. Proceedings of the National Academy of Science, U.S.A., 74, 10, 4605-4609.

Newsholme, E.A. and Start, C. (1974) Regulation of carbohydrate metabolism in liver. In Regulation in Metabolism, Ch.6, 247. John Wiley and Sons, Ltd. London.

Newsholme, E.A. and Start, C. (1972) General aspects of the regulation of enzyme activity and the effects of hormones. In Handbook of Physiology and Endocrinology, ed. Steiner,D.F. and Freinkel, N. Vol. 1, Ch. 23. WashingtonD.C.: American Physiological Society.

Notkins, A.L. (1965) Lactate dehydrogenase virus. Bacteriological Reviews, 29, 2.

Notkins, A.L., Berry, R.J., Moloney, J.B. and Greenfield, R.E. (1962) Relationship of the lactate dehydrogenase factor to certain murine tumours. Nature, 193, 79-80.

Notkins, A.L. and Greenfield, R.E. (1962) Infection of tumour-bearing mice with the lactic dehydrogenase agent. Proceedings of the Society of Experimental Biology and Medicine, 109, 988-991.

Notkins, A.L., Greenfield, R.E., Marshal, D. and Bane, L. (1963) Multiple enzyme changes in the plasma of normal and tumour-bearing mice following infection with the lactate dehydrogenase agent. Journal of Experimental Medicine, 117, 185-195.

Notkins, A.L., Mergenhagen, S.E., Rizzo, A.A., Scheele, C. and Waldman, T.A. (1966) Elevated gamma-globulin and increased antibody production in mice infected with LDH virus. Journal of Experimental Medicine, 123, 347.

Notkins, A.L. and Schochat, S.J. (1963) Studies on the multiplication and the properties of the lactate dehydrogenase agent. Journal of Experimental Medicine, 117, 735-747.

Okuda, H., Ikegami, H. and Fujii, S. (1972) Purification of toxohormone. Gann, 63, 605-613.

Olivares, J. (1970) The toxohormone problem. In Oncology, ed. Clark, R.L., Cumley, J.E., Mc Cay, J.E. and Copeland, M.M. Vol. 3, Ch. 6, pp. 158-163. Chicago: Medical Year Book Publishers.

Paul, J. (1970) Cell and Tissue Culture. Livingstone, E.S. Edinburgh and London.

Plagemann, P.G.W., Gregory, K.F., Swim, H.E. and Chan, K.W. (1963) Plasma lactate dehydrogenase elevating agent of mice: Distribution in tissues and effect on lactate dehydrogenase isozyme patterns. Canadian Journal of Microbiology, 9, 75-86.

Plagemann, P.G.W., Watanabe, M. and Swim, H.E. (1972) Plasmic lactate dehydrogenase elevating agent of mice: Effects on levels of additional enzymes. Proceedings of the Society of Experimental Biology and Medicine, 111, 749.

Pope, J.H. and Rowe, W.P. (1964) Identification of WM1 as LDH virus and its recovery from wild mice in Maryland. Proceedings of the Society of Experimental Biology and Medicine, 116, 1015-1019.

Rapp, G.W. (1973) Some systemic effects of malignant tumours: Coenzyme A levels. Cancer Research, 31, 357.

Rechigi. M., Grantham, F. and Greenfield, R.E. (1961) Studies on the cachexia of tumour-bearing animals. 1. Body weight changes, carcass composition and metabolic studies. Cancer Research, 21, 238-251.

Riley, E.E. Jr. (1959) Depression of liver catalase by various agents. Cancer Research, 19, 285-289.

Riley, V. (1963) Transmissible agents and anaemia of mouse cancer. New York State Journal of Medicine, 63, 1523-1531.

Riley, V. (1963) Synergistic glycolytic activity associated with transmissible agents and neaplastic growth. In Control Mechanisms in Respiration and Fermentation, ed. Wright, B. pp. 211-241. The Ronald Press Co., New York.

Riley, V. (1963) Enzymatic determination of transmissible replicating factors associated with mouse tumours. Annals of the New York Academy of Science, 100, 762-790.

Riley, V. (1963) Evidence for a minute infectious entity. Proceedings of the American Association for Cancer Research, 4, 57.

Riley, V., Lilly, F., Huerto, E. and Bordell, D. (1960) Transmissible agent associated with 26 types of experimental mouse neoplasms. Science, 132, 545-547.

Riley, V. and Spackman, D. (1976) Melanoma enhancement by

viral induced stress. Pigment Cell, 2, 163-173.
Riley, V. and Wroblewski, F. (1960) Serial LDH activity in plasma of mice with growing or regressing tumours. Science, 132, 151-152.
Rinard, G.A., Okuno, O. and Haynes, R.L. (1969) Stimulation of gluconeogenesis in rat liver slices by epinephrine and glucocorticoids. Endocrinology, 84, 622-631.
Rounds, D.E. (1970) A growth modifying factor from cell lines of human malignant origin. Cancer Research, 30, 2847.
Rubin, H. (1970) Overgrowth stimulation factor released from Rous sarcoma cells. Science, 167, 1271-1272.
Saez, S. (1974) In Mammary Cancer and Neuroendocrine Therapy, pp. 101-122, ed. Stoll, B.A. Butterworth, London.
Saez, S. (1971) Adrenal function in cancer: Relation to the evolution. European Journal of Cancer, 7, 381-387.
Salter, J.M., Ezrim, J.C., Laidlow, J.C. and Cornell, A.G. (1960) Metabolic effects of glucagon in human subjects. Metabolism, 9, 753-768.
Samundjan, E.M. (1966) A Thesis presented for the degree of Doctor of Science, Kiev.
Santisteban, G.A., Riley, V. and Fitzmaurice, M.A. (1972) Thymolytic and adrenal corticol responses to LDH elevating virus. Proceedings of the Society of Experimental Biology and Medicine, 139, 202-206.
Schersten, T., Wahlquist, L. and Jilderos, B. (1971) Lysosomal enzyme activity in liver tissue, kidney tissue and tumour tissue from patients with renal carcinoma. Cancer, 27, 2.

Schultz, R.M. and Wassarman, P.M. (1977) ^{3}H Dansyl chloride: A useful reagent for the quantition and molecular weight determination of nanogram amounts of protein. Analytical Biochemistry, 77, 25-32.

Seabra, A. and Deutsch, H.F. (1955) Studies on catalase inhibition as related to tumours. Journal of Biological Chemistry, 214, 447.

Shapot, V.S. (1972) Some biochemical aspects of the relationship between the tumour and the host. In Advances in Cancer Research, Vol. 15, pp. 253-286, ed. Klein, G., Weinhouse, S. and Haddow, A. London: Academic Press.

Shapot, V.S. (1979) On the multiform relationships between the tumour and the host. In Advances in Cancer Research, Vol. 30, ed. Klein, G., Weinhouse, S. and Haddow, A. London: Academic Press.

Shapot, V.S. and Blinov, B.A. (1974) Blood glucose levels and gluconeogenesis in animals bearing transplantable tumours. Cancer Research, 34, 1827-1832.

Sherman, C.D., Morton, J.J. and Mider, G.B. (1950) Potential sources of tumour nitrogen. Cancer Research, 10, 374-378.

Shils, M.E. (1979) Principles of nutritional therapy. Cancer, 43, 2093-2102.

Shils, M.E., Friedland, I.M., Fine, A.S. and Shapiro, D.M. (1956) Quantitative biochemical differences between tumour and host as a basis for cancer chemotherapy: 111. Thiamine and coenzyme A. Cancer Research, 16, 581-584.

Shonfield, A., Baggot, D. and Gundersen, K. (1961)

Hypoglycaemia and polycythaemia associated with primary hepatoma. New England Journal of Medicine, 265, 231.

Sibley, J.A. and Lehninger, A.L. (1949) Aldolase in the serum and tissues of tumour-bearing animals. Journal of the National Cancer Institute, 9, 303-309.

Sidransky, H. and Verney, E. (1979) Effect of nutritional alterations on protein synthesis in transplantable hepatomas and host livers of rats. Cancer Research, 39, 1995-2000.

Sidransky, H. and Verney, E. (1979) Effect of inhibitory and stimulatory agents on protein synthesis in hepatomas and host livers of rats. Journal of the National Cancer Institute, 63, 1.

Spackman, D. and Riley, D. (1976) The modification of cancer by stress: Effects of plasma corticosterone elevation on immunological system components in mice. Federation Proceedings of the Federation of American Societies of Experimental Biology, 35, 1693.

Start, C. and Newsholme, E.A. (1968) The effects of starvation and alloxan diabetes on the contents of citrate and other metabolic intermediates in rat liver. The Biochemical Journal, 107, 411-415.

Start, C. and Newsholme, E.A. (1970) A switch in the regulation of glycolysis and gluconeogenesis in rat liver. FEBS Letters, 6, 171-173.

Start, C. and Newsholme, E.A. (1973) Regulation of carbohydrate metabolism in the liver. In Regulation in Metabolism, Ch. 6, pp.247-291. John Wiley and Sons, London.

Stewart, A.G. and Begg, R.W. (1953) Systemic effects of tumours in force-fed rats. 11. Effect on the weight of carcass, adrenals, thymus, liver and spleen. Cancer Research, 13, 556-559.

Suda, M., Tanaka, T., Sue, F., Harano, Y. and Morimura, H. (1966) Dedifferentiation of sugar metabolism of the liver of tumour-bearing rat. Gann Monograph, 1, 127-141.

Tashiro, T. and Sakurai, Y. (1970) Effect of alkylating agents on glycogen phosphorylase activity of ascites tumour cells. Gann, 61, 35-40.

Theologides, A. (1972) Pathogenesis of cancer cachexia: A review and a hypothesis. Cancer, 29, 484-488.

Theologides, A. (1979) Cancer cachexia. Cancer, 43, 2004.

Ting, C.C., Tsai, S.C. and Rogers, M.J. (1977) Host control of tumour growth. Science, 197.

Toporek, M. (1973) Effects of whole blood or albumin fraction from tumour-bearing rats on liver protein synthesis. Cancer Research, 33, 2579-2583.

Treves, J.A., Cohen, I.R. and Feldman,M. (1976) Suppressor factor secreted by T-lymphocytes from tumour-bearing mice. Journal of the National Cancer Institute, 57, 2.

Tuan, D., Smith, S., Folkman, J. and Merler, E. (1973) The isolation of the non-histone proteins of rat Walker 256. Their association with tumour angiogenesis. Biochemistry, 12, 3159.

Vaage, J. and Agarwal, S. (1979) Endogenous growth factor indicated by increased ornithine decarboxylase activity in malignant cells treated with host serum or ascites

fluid. Cancer Research, 39, 1511-1517.

Waterhouse, C. and Kemperman, J.H. (1971) Carbohydrate metabolism in subjects with cancer. Cancer Research, 31, 1273-1278.

Weber, G., Convery, H.J., Lea, M.A. and Stamm, N.B. (1966) Feedback inhibition of key glycolytic enzymes in liver: Action of free fatty acids. Science, 154, 1357-1370.

Weber, G. (1968) In The Biological Basis of Medicine, Vol. 2, p.263, ed. Bittar, E.G. Academic Press: New York.

Weinhouse, S. (1979) A report of the U.S.-Japan conference on gene derepression and host-tumour relationships in carcinogenesis. Cancer Research, 39, 3279-3280.

Weiss, J.B., Brown, R.A., Kumar, S. and Phillips, P. (1979) An angiogenic factor isolated from tumours: A potent low molecular weight compound. British Journal of Cancer, 40, 493-496 (Short communication).

Wieland, O. (1966) Ketogenesis and its regulation. In Advances in Metabolic Disorders, Vol. 3, pp. 1-47, ed. Levine, R. and Luft, R. New York: Academic Press.

Willmott, N., Baxter, A., Calman, K.C. and Malcolm, A. (1980) Failure of RA 233 to affect the growth of intravenously injected murine lymphoma cells. In Press.

Wiseman, G. and Ghadially, F.N. (1958) A biochemical concept of tumour growth, infiltration and cachexia. British Medical Journal, 2, 18-21.

Wu, C. and Bauer, J.M. (1960) A study of the free amino acids and glutamine synthesis in tumour-bearing rats.

Cancer Research, 20.

Yaffe, D. (1962) The distribution and in vitro propogation of an agent causing high plasma lactate dehydrogenase activity. Cancer Research, 22, 573-580.

Yeakel, E.H. and Tobias, G.L. (1951) Liver nitrogen in tumour-bearing rats. Cancer Research, 11, 830-833.

Young, V.R. (1977) Energy metabolism and requirements in the cancer patient. Cancer Research, 37, 2336-2347.

Zahler, W.L. (1974) Analytical polyacrylamide gel electrophoresis and molecular weight determination. In Methods in Enzymology, Vol. XXXll, Biomembranes Part B, p.70, ed. Fleischer, S. and Packer, L. New York: Academic Press.