SOME EFFECTS OF TUMOUR GROWTH UPON RAT MUSCLE PROTEIN METABOLISM

Michael John Raymonds

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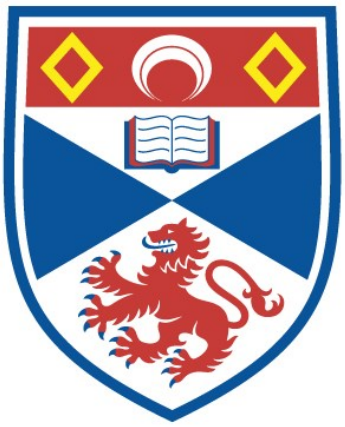

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SOME EFFECTS OF TUMOUR GROWTH UPON RAT MUSCLE PROTEIN METABOLISM

being a thesis presented by

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to the University of St. Andrews in application for the degree of Doctor of Philosophy

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CERTIFICATE

I hereby certify that Michael John Raymond has spent nine terms engaged in research work under my direction, and that he has fulfilled the conditions of General Ordinance No. 12 (Resolution of the University Court No. 1, 1967), and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

.....

DECLARATION

I hereby declare that the research reported in this thesis was carried out by me, and that the thesis is my own composition. No part of this work has been previously submitted for a higher degree,

The research was conducted in the Department of Biochemistry, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Dr. G.A.J. Goodlad.

ACADEMIC RECORD

I first matriculated at the University of St. Andrews in October, 1965, and graduated with the degree of Bachelor of Science, Second Class Honours (Division 1) in Biochemistry in June, 1969. I matriculated as a research student in the Department of Biochemistry, University of St. Andrews, in October, 1969.

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GENERAL INTRODUCTION

Neoplastic lesions, or tumours, consist of many cells whose multiplication is no longer subject to normal homeostatic control, and which may vary both morphologically and biochemically from the cells of the tissue of origin. The biological characteristics of the cancer cell would seem to indicate that it is a cell which has specialised to growth (Busch, 1954), and to survival under adverse conditions. For example, cells of the brain or heart die within minutes when exposed to an environment lacking in oxygen or containing cyanide. On the other hand, tumour cells have remained viable for as long as three days in an environment containing cyanide, and totally lacking in oxygen (Warburg, 1926). Another important property of cancer cells appears to be a loss of mutual adhesiveness (Mercer and Easty, 1961), which allows them to become detached from the tumour mass. The tumour cells may then spread through the bloodstream or lymph vessels and grow as secondary tumours (metastases) at sites distal to the original cancer.

The genesis of such a malignant growth has been attributed to many agents, but whatever the oncogenic agent, the pathogenicity of the growth is extreme, although the nature of the changes leading to the death

of the host is not clear. Some tumours will kill because of the anatomical site of their growth; as Mider (1955) put it: "unrelieved obstruction of the ureters, the aqueduct of Sylvius or the gastrointestinal tract is incompatible with life". Invasion of vital organs, either by primary tumour growth, or by metastases, again provides a readily understandable cause of death. However, the death of the host can equally be caused by a more general effect of the tumour upon the metabolism, and it was with some of these systemic effects of the growth of the tumour that this work has been concerned.

Tumour growth and metabolism

(i) The nature of the cancer cell

Neoplastic growth can vary over a wide spectrum from benign tumours to malignant growths. A benign tumour is localised, slow-growing, well-differentiated and generally harmless throughout the life-span of the organism. Benign 'cancer' is essentially an exaggeration of the process of hyperplasia, whereby an organ grows by cell division (as opposed to hypertrophy, or cell enlargement), and which is of normal occurrence even in healthy animals (Reid, 1965). Malignant tumours, on the other hand, may obliterate the organ in which they arise, and give rise to

metastases in other tissues. Malignant tumours can arise from the cells of all tissues; carcinomas originate in epithelial tissue such as skin and breast, while sarcomas arise from non-haematopoietic mesenchymal tissues, such as connective, vascular or muscular tissues. Malignant growths vary in the extent to which they retain the structure and functional capacity of the tissue in which they originated, but frequently there is anaplasia, or loss of characteristic tissue structure.

(ii) Amino-acid and protein metabolism of tumours

An important property reported as being characteristi of tumour cells is the capacity to take up amino-acids and concentrate them. Ehrlich carcinoma ascites cells show highly concentrative uptake of such amino-acids as glycine and alanine both *in vivo* and *in vitro* (Christensen *et al.*, 1954; Christensen and Riggs, 1952; Christensen *et al.*, 1948). The ratio of the intracellular concentration of amino-acids to the extracellular concentration of amino-acids in tumour cells found by these workers ranged from 1.3 to 20.6. Mider *et al.* (1948) suggested that tumours act as 'nitrogen traps' in that they appear to concentrate amino-acids, but not to contribute to the nitrogen exchange of the host. Wiseman and

Ghadially (1958) advanced a hypothesis on this basis to explain the anorexia and cachexia of the host, stating that the tumour possesses enhanced power for the concentration of amino-acids, due to the maintenance of a low intracellular free amino-acid level by the rapid processes of protein synthesis in neoplastic cells. This view was consistent with the early work of LePage et al., (1952), who injected radioactively-labelled glycine into rats bearing transplanted tumours. The tumours were subsequently found to increase continuously in total weight and in total protein radioactivity, even under conditions of complete fast, while normal tissues lost radioactivity. Thus the proteins of the tumour were evidently not available to the host under conditions where normal tissues were grossly catabolised. El Mehairy (1950) reported an increase of alpha-amino-nitrogen in the blood of tumour-bearing mice. Wu and Bauer (1960) found reductions in the levels of some free amino-acids in muscle of rats bearing a Walker 256 carcinoma, and increases in the levels of most of these amino-acids in the plasma of the tumour-bearing animal. These results they took to be consistent with the liberation of amino-acids from muscle into plasma, followed by translocation to the tumour. It should be noted, however, that their method for the

estimation of amino-acids depended upon paper chromatography, so that the data obtained should be treated with some caution. In fact, their values for the amino-acid levels in normal rat muscle and plasma do not agree closely with more recent data obtained by column chromatographic analysis (Scharff and Wool, 1964; Schimassek and Gerok, 1965; Christophe *et al.*, 1971). Auerbach and Weisman (1958) found an increased concentration of plasma aromatic amino-acids in cancerous animals, and higher levels of serum tryptophan have been reported by Brackenridge (1960).

On the other hand, although Christensen *et al.*, (1948, 1954) found that ascites cells could concentrate amino-acids *in vivo* and *in vitro*, the *in vivo* experiments indicated that the concentrative capacity of the tumour cells was not equal to that of liver, although uptake into the tumour cells exceeded that into kidney and skeletal muscle. Christensen and Streicher (1948) have shown that concentration of amino-acids by tumour cells is a property shared by foetal tissue, and Riggs and Walker (1958) have demonstrated the same propensity in the tissues of young, growing rats. More recent studies have indeed indicated that most amino-acids enter cells by an active transport process (Christensen and Clifford, 1963; Johnstone and Scholefield, 1965; Pardee, 1967).

Greenlees and LePage (1955) found that labelled ascites cells transferred to unlabelled hosts lost 9% of their radioactivity per day. Forssberg and Revesz (1957) studied the loss of radioactivity of two Ehrlich ascites tumours labelled with ^{14}C-glycine and ^{35}S-methionine, and discovered that loss of radioactivity occurred when the tumour protein was labelled with glycine, but not when it was labelled with methionine. Finlayson et al.,(1959) used a number of Landschutz ascites cells labelled with several different radioactive amino-acids, and concurred with Forssberg and Revesz (1957), concluding that the loss of radioactivity observed was due entirely to intracellular turnover of the tumour protein, with consequent reutilisation of the labelled amino-acid. The work of Jordan et al. (1959) did not agree with this mechanism for the loss of radioactivity from labelled tumour protein. Employing a tissue culture of Walker 256 carcinoma cells, the proteins of which were labelled with ^{14}C-lysine, they discovered that transfer of labelled cells to unlabelled medium resulted in a net loss of 12.9% of the radioactivity per day from the cells. Part of this loss appeared to be due to turnover of the tumour protein, while the remainder could be accounted for as net transfer of radioactivity to the medium, possibly in the form of intact protein.

Transplanted tumours appear to vary considerably in their response to alterations in the protein content of the diet. Mice on a low-protein diet, and in negative nitrogen balance, exhibited tumour growth at 75% of the rate in control mice on an adequate protein intake (White, 1945; White and Belkin, 1945). This has been confirmed in hepatoma-bearing rats (Voegtlin and Thompson, 1949). The latent period, but not the growth rate, of the Walker 256 carcinoma is affected by nitrogen deprivation (Green et al., 1950). The growth rate of Sarcoma R-1 and the Flexner-Jobling hepatoma were both reduced with the host on a protein-free diet (Babson, 1954), the growth of the hepatoma being more dependent upon dietary protein than the sarcoma. Supplementation of a 12% casein diet with methionin increased the carcass weight and decreased the tumour weight of Sarcoma R-1 (Hilf, 1956). A negative nitrogen balance induced by cortisone administration decreases the rate of growth of the Walker tumour (Ingle et al., 1950), indicating that a tumour can obtain nitrogen from a host that is in negative nitrogen balance, but not with the ease that pertains when the host is in positive nitrogen balance. Force-feeding rats bearing the Walker tumour delayed the loss of carcass weight, but only temporarily (Begg and Dickinson, 1951; Stewart and Begg, 1953a,b; Terepka and

Waterhouse, 1956).

It now seems clear that the tumour does participate in some degree of dynamic exchange with host tissue nitrogen. It would appear, furthermore, that tumours have an advantage, shared to some extent by normal growing cells, of the ability to concentrate and conserve amino-acids for protein synthesis. They are not 'nitrogen traps' as Mider et al. (1948) visualized them, but they do seem to have a tendency to conserve their proteins and amino-acids to a greater extent than do most normal tissues.

(iii) Protein utilisation by the tumour

There is considerable evidence that plasma proteins may be taken up and utilised by cancer cells, without the initial step of extratumoural hydrolysis. The possibility was first suggested by Babson and Winnick (1954), who injected a radioactive amino-acid into tumour-bearing animals. Upon subsequent flooding of the rat with the non-radioactive isotope of the amino-acid, a dilution of protein radioactivity in normal and tumour tissues was noted. However, the injection of labelled plasma proteins followed by cold amino-acid did not produce a comparable dilution, and the uptake of label into the tumour cells was

very much greater than that into liver and kidney cells. Busch and Greene (1955) and Busch et al. (1956) showed that the uptake of free ^{14}C-glycine by tumour protein was very much less than the uptake into several other normal tissues such as pancreas and intestine. At the same time, these workers confirmed that the uptake of labelled amino-acid from labelled plasma protein was very much higher in tumour protein than in proteins from any other tissue. Furthermore, albumin labelled with any one of several amino-acids led to the same specific activity in tumour protein upon injection into a tumour-bearing animal. Injection of the separate amino-acids led to widely differing specific activities in the tumour protein (Busch et al., 1961). This finding appears to rule out the possibility that albumin is hydrolysed before entering the tumour cell.

Metabolism of the tumour-bearing host

(i) The tumour-host relationship

The study of the metabolism of the tumour-bearing animal is inseparable from the study of the systemic effects of the tumour, for there is scarcely a tissue or metabolic process in the host animal which is not affected either directly or indirectly by the growth of a tumour. Begg (1955) has defined tumour-host relationships as

'those changes produced in the tissues of the host remote from the tumour, and in which no evidence of metastatic malignant cells is found'. It is true that many of the effects found in the cancerous animal, particularly cachexia, are also characteristic of haemorrhage, malnutrition and infection (Willis, 1948). However, it is equally true that the effects of a tumour may be noted when infection and haemorrhage are absent, invasion is minimal and nutrition adequate (Fenninger and Mider, 1954; Mider, 1955).

Clinically and experimentally, the most striking of the systemic effects of the tumour is the cachectic state of the cancerous patient or the tumour-bearing animal. Cachexia is characterised by weakness, anorexia, depletion of host components, electrolyte and water abnormalities, and a progressive fading of vital functions (Costa, 1963).

The effect of the tumour can be exerted long before the tumour has reached its greatest size or maximal growth rate. Thus, oedema in rats bearing the Walker 256 carcinoma is greatest during the early stages of tumour growth (McEwan and Haven, 1941). The caloric balance of rats is less positive during the period of tumour induction than either before transplantation or after the tumour becomes

palpable (Pratt and Putney, 1958).

Study of the water content of tissues of the tumour-bearing rat has led to inconsistent results. Rats bearing a Flexner-Jobling carcinoma, Jensen sarcoma (Schlotteman and Rubenov, 1932), hepatoma (Aoki, 1938) or Walker 256 carcinoma (McEwan, 1955) showed a slight increase in the water content of various organs such as liver, kidney and skeletal muscle. These increases were in general less than 10%, and the calculations did not take into account differing tumour weight, or the loss of weight due to lipid loss (Haven et al., 1949).

More recently, studies by Toal et al. (1961), in which the carcass was analysed separately from the tumour, showed a slight but consistent decrease in carcass water concentration, 25 to 30 days after Walker 256 tumour transplantation.

(ii) Protein metabolism

A prominent tumour-host effect is the ability of the tumour to cause mobilisation of host protein for its own utilisation. Sherman et al.,(1950) studied the nitrogen content of various tissues of rats bearing the Walker 256 carcinoma, and found that skeletal muscle contributed most

nitrogen to the tumour. In cancerous patients too, there is evidence of a fall in certain muscle protein components. Ritter (1958) separated muscle protein into three fractions by electrophoresis, and found that cancerous patients had decreased amounts of two of these fractions. Sobel (1966) fractionated abdominal muscle from rats bearing a Walker 256 carcinoma into 11 sub-fractions, and quantitatively compared the results with normal, starved and cortisone treated rats. Although he found approximately equal losses of carcass protein in severely starved animals, in animals which responded most acutely to cortisone treatment, and in the animals bearing the largest tumours, the patterns of loss of individual protein fractions from abdominal muscle were distinctly different with each treatment. In the case of the tumour-bearing animals, the loss appeared to be principally from the contractile proteins of muscle.

Not all tissues of the tumour-bearing animal suffer a depletion of protein. Renal nitrogen did not alter during the growth of the Walker 256 carcinoma in rats (Sherman et al., 1950), while the spleen and liver have actually been shown to increase in nitrogen content in the presence of a growing tumour (Sherman et al., 1950; Yeakel and Tobias, 1951; Clark and Goodlad, 1960). The observation of

Norberg and Greenberg (1951) that glycine was incorporated more readily into the plasma, liver and spleen of tumour-bearing mice than control mice, but less readily into muscle, is in keeping with the findings of Sherman et al. (1950). ^{14}C-labelled histidine behaved like glycine in respect to rate of incorporation into liver protein, and also exhibited increased incorporation into the nucleic acid of host liver (Reid et al., 1956).

The ability to demand nitrogen for protein synthesis is not confined to cancer. The pregnant rat maintained on a protein-free diet from the 11th day of gestation until parturition can produce viable, although small, young (Seegers, 1937). The growth of a tumour during pregnancy does not appear to materially affect the mean foetal weight (Paschkis et al., 1956). Liver regeneration after partial hepatectomy in mice bearing an ascites tumour produced limitation of tumour growth, and the rate of liver regeneration was less in the presence of the tumour (Straube and Hill, 1956), suggesting competition for a common amino-acid supply. Once again the pattern emerges of a rapidly growing normal tissue able to compete with a tumour for protein precursors.

(iii) Liver catalase

Cancer interferes with the iron-containing enzyme catalase, and with the production of cytochrome c (Kampschmidt et al., 1959).

The activity of liver catalase in the animal bearing a tumour has been studied more intensively than any other tissue enzyme in cancer. The depression of kidney catalase is much less than that of liver catalase, and the level of blood catalase in the tumour-bearing animal is normal (Greenstein and Andervont, 1942b). The demonstration of different rates of synthesis of catalase in different tissues (Theorell et al., 1951) may explain the variation in tissue response, but nevertheless, the effect of the tumour appears to be selective. Not all liver enzymes are depressed by the presence of a tumour, and not all tissues are affected to the same degree (Greenstein, 1954).

The catalase effect is not specific for the animal bearing a tumour. Depression of catalase has been observed after radiation (Feinstein et al.,1950), and during the growth of a chronic granuloma (Pounce and Shandwise, 1950). Nutrition (Rechcigl and Price, 1968) and hormonal status (Adams, 1952) both influence the level of catalase activity in the liver. Nevertheless, there is an effect on liver

catalase directly related to the presence of a tumour. The degree of depression of catalase activity bore a relation to the size of the tumour (Greenstein and Jenrette, 1941; Begg, 1951), and a malignant tumour produced a greater depression of catalase activity than a benign tumour of similar size and growth rate (Begg et al., 1953b). Further, liver catalase activity has been shown to return to normal upon surgical removal of the tumour (Greenstein and Andervont 1942a). Nor is the effect confined to mammals, for renal carcinoma in the frog (Lucke and Berwick, 1954) and tumour growth on the chorioallantoic membrane (Stavinski and Stein, 1951) has been reported to produce a depression of liver catalase activity.

Rechcigl et al. (1962) and Rechcigl and Sidransky (1962) carried out studies on the synthesis of catalase in rat hepatomas possessing unusually high levels of catalase activity. Contrary to the situation in normal tissues, the catalase level of these hepatomas was neither affected by cachexia in the tumour-bearing host, nor altered under the stress of a protein-free diet. It was shown that in rats bearing the Morris 5123 hepatoma the rate of catalase synthesis was about four times greater in the liver than in the tumour, whilst the first order rate constant for catalase destruction was practically the same in both types

of tissue. This may explain the lack of response of the 5123 hepatoma catalase to factors which cause a marked reduction of the activity of the enzyme in the tissues of the host.

Mechanism of tumour action on host metabolism

(i) The 'nitrogen trap' theory

It seems unlikely that any unitary hypothesis can be formulated to explain the systemic effects of tumour growth upon the metabolism of the host. Costa (1963) stated that "the devastations produced in the host by cancer are the compound result of a manifold impingement on organs and functions between destruction, attempted repair and homeostasis".

Certainly, the nitrogen trap theory as proposed by Mider et al, (1948), and expanded by Wiseman and Ghadially (1958), which depends upon the enhanced ability of the neoplastic cell to concentrate amino-acids to the eventual detriment of the host, has been unable to explain many of the effects observed in the protein metabolism of the host and tumour discussed above. It now seems that the proven ability of the tumour to retain a large proportion of its nitrogen under conditions of general protein

catabolism in the host (LePage et al., 1952; Greenlees and LePage, 1955), while contributing towards the ability of the tumour to grow under unfavourable conditions, cannot be directly responsible for tissue protein breakdown.

Because the depleting effect of the tumour on host tissues extends to many of the endocrine organs (Sherman et al., 1950; Kampschmidt et al., 1959), the possibility exists that disruption of the normal system of hormonal balance may occur.

(ii) Hormones and the tumour-host relationship

The study of the role of the tumour in alteration of the hormonal balance of the host is complicated by several observations that a reciprocal relationship can exist. Quite apart from the involvement of hormones in tumourigenesi (Gardner, 1957), many tumours appear to be hormone dependent (Noble, 1957). Goranson et al.(1955) found that the presence of alloxan diabetes in the host animal lowered the percentage of takes and reduced the size of intraperitoneally transplanted Novikoff hepatomas. In the animals in which the tumour did take, blood-sugar was lowered in the diabetic tumour-bearing animals as opposed to the tumour-bearing controls, and the diabetic animals lived longer. It may be that the increased availability of glucose to the tumour

due to the increased blood level in the diabetic animal, may well have delayed the cachectic effect of the tumour, since there is evidence that neoplastic tissue has a considerable capacity for gluconeogenesis (Kit and Graham, 1956).

Clinically, the picture is further complicated by the apparent ability of many primary tumours to synthesize large amounts of substances with hormonal activity, simulating hyperfunction of a particular endocrine gland. Thus, a wide variety of cancers, particularly hepatic and adrenal carcinomas, have been reported in association with hypoglycaemia (Lipsett et al., 1964; Lowbeer, 1961). Pancreatectomy did not reduce hypoglycaemia in a mouse bearing a transplanted hepatoma (Silverstein et al., 1960).

Adrenocortical hyperplasia has been reported in patients with malignant tumours occurring in regions other than the adrenals or adenohypophysis. The most common types of tumour associated with this condition are bronchial carcinomas and thymomas (Riggs et al., 1961) although similar symptoms have been observed occasionally in subjects with tumours of the pancreas, CNS, breast, thyroid, prostate and oesophagus. These subjects showed increased excretion of 17-ketogenic steroids in the urine and increased cortisol secretion rates (Prunty et al., 1963).

Lucas et al. (1962) studied five patients with Cushing's syndrome and non-endocrine tumours, and found significant amounts of an ACTH-like substance in the plasma, and in extracts of the primary tumours and secondary deposits. Evidence of adrenal hyperfunction was abolished by adrenalectomy, suggesting that the condition was due to an excess production of corticotrophin-like material and not glucocorticoids by the tumour. The high glucocorticoid excretion was not abolished by dexamethasone indicating that corticotrophin production by the tumour is not under negative feedback control, unlike that of the pituitary. Whether or not this corticotrophin-like substance is ACTH remains to be shown.

Other syndromes which have been reported to result from interference with the hormonal control systems of the host by paraendocrine tumour activity have been hyperthyroidism (Tisne et al., 1955; Dowling et al., 1960) and excessive production of serotonin (Oates and Sjoerdheim, 1964), gonadotrophin (McArthur, 1963; Hung et al., 1963), anti-diuretic hormone (Schwartz et al., 1957; Armatruda et al., 1963) and insulin-like factors (Hobbs and Muller, 1966).

In animals bearing experimentally transplanted tumours, there is evidence that production or utilisation of some of the protein anabolic hormones is impaired

(Goodlad, 1964; Claus et al., 1962; Babson, 1954; Goranson, 1955; Begg, 1955), whilst the levels of protein catabolic hormones may be increased. For example, there is evidence to suggest that the adrenals of tumour-bearing animals may be hyperfunctional, with respect to both glucocorticoid and aldosterone production (Begg, 1958; Hilf et al., 1960; Kavetsky et al., 1962; Rechcigl et al., 1961).

The catabolic action of corticosteroids upon protein distribution in the tissues of the animal body is in many respects similar to that of a growing tumour, in that it causes a breakdown of carcass protein and a deposition of liver protein (Silber and Porter, 1953; Goodlad and Munro, 1959). On the other hand, livers of rats bearing a Walker 256 carcinoma exhibited a much greater increase in ribonucleic acid content relative to protein than did rats treated with cortisone (Clark and Goodlad, 1960). The levels of hepatic alanine alpha-oxoglutarate transaminase in hypercortical rats and tumour-bearing rats (Rosen et al., 1959; Harding et al., 1961; Beaton et al., 1957; Goodlad and Clark, 1962), do not support the hypothesis that increase concentrations of glucocorticoid are responsible for the effects of the tumour growth on liver metabolism. Sobel (1966) showed that although tumour growth and cortisone

administration produced a similar loss of protein in rat abdominal muscle, the depletions occurred in different protein fractions.

Goodlad (1964) has suggested that adrenal hypertrophy in rats bearing a Walker 256 carcinoma could be related to the level of hepatic Δ^4-steroid hydrogenase activity. This suggestion is based on the correlation between the level of this enzyme system and adrenal size shown by Urquhart et al. (1958), and the observation of an elevation in the activity of the enzyme in rats bearing a Walker 256 carcinoma (Goodlad and Clark, 1961).

There is little doubt that the levels and possibly the action of several hormones are altered in the tumour-bearing animal, but the evidence suggests that these alterations are due in the main to atrophy or hypertrophy of the endocrine glands (Begg, 1958; Goodlad, 1964; Claus et al., 1962). These effects on the endocrine system could result either from the general protein depletion occurring in the cancerous animal (Sherman et al., 1950) or to paraendocrine activity in the tumour (Lipsett, 1964), and in any event will inevitably contribute to the observed effects of tumour growth in the host animal. Nevertheless, these effects must be secondary to some primary action of the tumour upon the endocrine system, so that the alterations

in host metabolism cannot be attributed solely to hormonal imbalance.

(iii) The secretion of a cancer toxin

The idea of a cancer toxin from neoplastic tissue is not new (Ewing, 1934), and a diffusible substance produced by a tumour has been suggested as a possible explanation of the systemic effects of the tumour (Greenstein, 1943). Adams (1950) demonstrated that the injection of a tumour suspension into mice led to an early depression of liver catalase activity. The activity then returned to normal, with a second drop when the tumour became established. The effect of tumour growth upon liver catalase activity has been discussed above. From this and subsequent experimen Adams concluded that tumour tissue contains a factor, present in only low or negligible concentrations in normal tissue, which produces a fall in liver catalase activity when injected into normal animals (Adams,1952; Adams, 1953). In 1948, Nakahara and Fukuoka had succeeded in preparing a fraction from human tumours, which depressed the activity of liver catalase when injected into normal animals. Nakahara and Fukuoka named this material 'toxohormone', a name which was intended to convey the combination of humoral and toxic properties exhibited by the substance (Nakahara

and Fukuoka, 1948). Greenfield and Meister (1951) confirmed this work using transplanted tumours in experimental animals, namely a mammary tumour in mice, and a fibrosarcoma and the Walker 256 carcinoma in rats.

A detailed account of the development of the extraction and purification procedures which eventually led to the production of a toxohormone fraction active in doses of 10 ug per rat, is given in section 3 (page 107)

An active fraction has been prepared from the spleen of healthy mice (Day et al., 1954), suggesting that toxohormone production may not be confined to tumour tissues. However, the catalase depression produced in these experiments was relatively small, and did not approach the 50% depression required by Nakahara and Fukuoka (1948). Nakahara and Fukuoka (1954) reported the production of toxohormone by tumour tissue slices in vitro, when incubated with amino-acid mixtures in the presence of adenosine triphosphate. Under identical experimental conditions, normal tissue slices (liver and skeletal muscle failed to produce demonstrable amounts of toxohormone. It would seem, therefore, that while toxohormone may be present in small amounts in certain normal tissues, it is produced in quantity only by neoplastic tissues.

The attribution of the depression of liver catalase activity to the action of toxohormone requires acceptance of the hypothesis of humoral transfer of this agent.

Masamune et al. (1958, 1959) isolated glycoprotein from the fluids obtained from different tumours, and this glycoprotein showed a clear toxohormone effect on liver catalase activity. Hirsch and Pfutzer (1953) reported that not only the cells, but also the cell-free fluid of Ehrlich ascites carcinoma and Yoshida sarcoma were active in depressing liver catalase activity when injected into normal mice. Gastric juice from patients suffering from gastric cancer has also been shown to possess toxohormone activity (Kawamorita et al., 1951; Iwatsuru et al., 1954).

Nagagawa (1952) reported the isolation of a liver catalase depressing factor from the urine of cancer patients, by means of benzoic acid adsorption, and the presence of toxohormone in urine from cancerous patients has since been confirmed by Sato et al. (1953) and Fuchigami et al.,(1956).

Lucke et al. (1952) utilised the method of parabiosis for the study of the problem. The principle involved is that capillary anastomoses develop at the parabiotic junction, through which a small quantity of blood crosses continuously between the partners. Lucke included in his experiments

normal rats, normal parabiotic pairs, rats bearing a Walker 256 carcinoma, and parabiotic pairs of which one partner was tumour-bearing. Tumours were implanted from six to forty-nine days after parabiosis was established, and assays made from twelve to eighteen days after implantation showed that when one partner was tumour-bearing, the liver catalase level was depressed in both animals, the degree of depression being approximately the same as in single tumour-bearing rats. Parabiosis *per se* did not affect liver catalase activity. It was thus established that the tumour substance responsible for the depression of liver catalase activity can be transferred across a parabiotic junction.

Plaza de los Reyes *et al.*(1953) reported that fresh plasma of cancer patients reduced liver catalase activity when injected into normal rats, and Kuzin *et al.* (1955) also found that the alcohol precipitable fraction separated from the blood of tumour-bearing animals exerted a similar effect.

On the basis of the above evidence, it can be concluded that toxohormone is secreted from the tumour into the body fluids of cancer patients and tumour-bearing animals.

Several other effects of toxohormone, apart from

the depression of liver catalase, have now been described (Trojanowski, 1970). Most of these appear to be concerned with iron metabolism, but all of the activities reported for toxohormone have also been associated with tumour growth. A list of these activities is shown in Table 23, (page 120), and their implications will be assessed in the general discussion (page 154).

The transplantable tumour in cancer research

(i) Reservations

Results of various kinds obtained with the use of transplanted tumours have been extrapolated more or less freely to spontaneous tumours. In recent years, it has become increasingly obvious that such inferences are unjustifi in certain cases, and that serial transplantation by itself may lead to artificial situations, complicated by factors which do not enter into autologous tumour-host systems.

The environment of growing neoplastic tissue is well-suited to viral growth (Koprowska and Koprowski, 1953), which, unlike most instances of bacterial contamination, is not obvious to the eye of the researcher, and does not lend itself to simple detection. Such viral contaminants may or may not damage the tumour cell itself, but in any event are likely to complicate the results of experiments

designed to investigate the growth, ultrastructure or biochemistry of tumours.

Klein (1959) pointed out that unless the most stringent steps are taken to ensure the genetic identity of the donor and recipient in a transplantation experiment, isoantigenic differences are certain to exist between the host and the tumour. Because transplantation is usually between animals belonging to a highly inbred strain, these differences are unlikely to be sufficient to cause rejection of the transplant, but may nevertheless produce reactions in the host not attributable to the effects of the tumour *per se*.

There is evidence too that serial transplantation itself causes differences in the action of the tumour. Greenlees and LePage (1955), studying the uptake of ^{14}C-glycine into tumour proteins during early transplant generations of a mammary carcinoma of the mouse, found an accumulation of counts in the tumours during starvation only after the fifth transplant generation. Kampschmidt and Upchurch (1972) followed the alterations produced in the host by a sarcoma induced by the intramuscular injection of 20-methylcholanthrene, for 60 generations. They found that plasma lactate dehydrogenase activity, the number of

peripheral blood leukocytes and adrenal weight were increased abruptly during early transplant generations, and that the levels returned to near their normal values during the later generations of the tumour. Haemoglobin concentration, liver catalase activity, plasma iron levels and total iron-binding capacity were significantly decreased in rats bearing primary tumours and remained at these lowered levels throughout all of the transplant generations studied.

It also appears that the site of transplantation of the tumour is an important factor in the type of effects produced by the tumour. Kampschmidt and Upchurch (1966) implanted the Jensen sarcoma, the Walker carcinoma and the Novikoff and MDAB hepatomas into a series of rats. Each rat received an inoculum of cells from one of these tumours either intramuscularly, intraperitoneally or subcutaneously. They found that for each tumour, the intraperitoneal transplant grew the most rapidly, produced the largest adrenals, smallest thymuses, the lowest total iron-binding capacity, and the most severe anaemia. The intramuscular transplants caused the most carcass weight loss and the greatest lowering of plasma iron and liver catalase, whilst the subcutaneous tumour growth appeared to be associated with the most pronounced gains in liver and spleen weight.

Considerable differences also exist between the tumour-host relationships observed during the growth of different types of transplantable tumour. The responses of several types of tumour to alterations in dietary protein intake (Babson, 1954; Hilf, 1956; Voegtlin and Thompson, 1949; Green et al., 1950) have already been discussed (page 8), and give an indication of the variation which can be encountered. Even the very closely related Morris hepatomas exhibited considerable diversity in their ability to concentrate alpha-aminoisobutyric acid, and in the respons of the amino-acid transport system to hydrocortisone and glucagon treatment (Baril et al., 1969).

These results show quite clearly that data obtained by experiments on animals bearing transplantable tumours apply only to one species, one tumour, and one set of experimental conditions, and broad generalisations drawn from such work should be treated with caution.

(ii) The Walker 256 carcinosarcoma

The Walker 256 carcinosarcoma originated in the mammary gland of a Wistar rat at the Johns Hopkins Hospital in 1928, and has since been propagated by serial transplantation in many laboratories. It grows as a globular mass in epithelial or muscular tissues, and has a cellular

peripheral rind and a necrotic central core. Macroscopic metastases and bacterial infection are rarely observed under normal circumstances (Earle, 1934). This tumour has been used for all the studies described in this work.

Mider (1953) observed that the growth of the Walker tumour could be divided into three phases:

1. An initial period of comparatively slow increment in mass by both tumour and host.
2. A longer phase of greatly accelerated tumour growth, during which host tissues, with the exception of liver and spleen, lose weight, and dietary intake declines.
3. A brief final stage when the neoplasm grows little if at all, and all of the host tissues lose weight very rapidly.

Death may occur late in phase 2 or in phase 3, and the host normally dies in the third or fourth week of tumour growth.

Scope of the present work

In the present work, the study of the effects of the growth of the Walker 256 carcinoma upon some aspects of the protein metabolism of rat diaphragm muscle was undertaken.

The first part of the work involved studies on the microscopic appearance and protein content of diaphragm muscles of tumour-bearing rats, to verify that the depleting effects of tumour growth on the carcass (Mider et al., 1948) and skeletal muscle (Sherman et al., 1950) did extend to the diaphragm muscle.

The cachexia evident in the tissues of the tumour-bearing host involves depletion of the muscle proteins (Sherman et al., 1950) but it is not known whether this depletion arises from a decreased synthesis or an increased catabolism of protein within the muscle. Norberg and Greenberg (1951) showed that labelled glycine was incorporate less readily into skeletal muscle protein of mice bearing the 6 C_3H ED tumour than into the proteins of normal mice. Winnick et al, (1948) had previously found that the uptake of labelled tyrosine in the tissues of rats with transplanted tumours was less than that in the control. Both these studies had been performed in vivo, so that the results may have been obscured by other tumour effects, such as those on amino-acid transport (see page 4). In the present work, diaphragm muscles from normal rats, and from rats bearing a Walker 256 carcinoma were compared for their ability to incorporate ^3H-lysine into protein in an in vitro

incubation system. Variations of the composition and concentration of the amino-acids added to the incubation medium were tested for possible effects on the relative incorporation by the muscles from the healthy and diseased animals.

The action of insulin in stimulating protein synthesis in diaphragm muscle in the system used has been well-documented (Wool, 1969). Although it is known that tumour-bearing animals are not diabetic (Begg, 1958), they do show impaired glycogen deposition in liver when subjected to a glucose test load (Goranson, 1955; Young et al., 1947; Goldfelder, 1928), which can be restored to normal by administration of insulin (Begg, 1955). This finding could be taken to suggest that there is an impairment of production or a decreased sensitivity to insulin in the tumour-bearing animal, or else that the demands of the tumour for administered glucose are so high that the liver is unable to compete until the insulin level is raised. The influence of insulin administered both in vivo and in vitro upon incorporation of isotope into the muscle of normal and tumour-bearing animals was tested, to determine if the hormone could alleviate the interference of the tumour with muscle protein metabolism.

The systemic effects of toxohormone reported in the literature have been largely concerned with iron metabolism, but Obara and Ono (1965) reported an inhibitory effect upon rat lens and corneal protein synthesis. If toxohormone production is an important factor in the mechanism of action of the tumour, some effect upon protein metabolism should be apparent in the muscles of animals treated with toxohormone. Accordingly, a toxohormone fraction was prepared and purified, and tested for an inhibitory effect upon incorporation of labelled amino-acid into the muscle protein of healthy animals.

It was hoped that these investigations would throw some light upon the mechanisms involved in the action of the tumour upon the host's metabolism, and that the involvement of toxohormone in the production of the cachectic effect of the tumour might be clarified.

SECTION 1

The effect of growth of the Walker 256 carcinoma on the microscopic appearance, water content, extracellular space and protein content of diaphragm muscle.

INTRODUCTION

It has been pointed out (page 12) that the cachectic condition of tumour-bearing rats is most evident in the case of skeletal muscle tissue. This section is an account of changes observed in the morphology, water and protein content of the diaphragm muscle of rats bearing a Walker 256 carcinoma.

The muscle appeared by eye to be much thinner and more transparent in the tumour-bearing rat than in the normal animal and was more fragile and liable to damage during dissection. In view of these observations, diaphragms from healthy and tumour-bearing animals were examined microscopically, and an estimate made of muscle fibre diameter.

Studies were also carried out on possible alterations in extracellular space in diaphragms of tumour-bearing rats. In addition, since diaphragm preparations were to be used later to study the effect of the tumour on protein synthesis using labelled amino-acids, attempts were made to compare the accessibility of such preparations to small molecular weight compounds. Extracellular space estimations require the use of a marker which does not penetrate the muscle cell membrane. Inulin, a polysaccharide of approximately 5,000 molecular weight, has been reported as meeting this requirement (Davson, 1964). It is not, however, a suitable

compound to study the penetration of diaphragm preparations by small molecular weight compounds. The sugar alcohol, sorbitol, has similar diffusion characteristics to glucose, and does not penetrate the intact muscle cell (Morgan et al., 1961). Sorbitol was therefore chosen as a low molecular weight extracellular marker and also as a molecule to estimate the accessibility of diaphragm preparations.

The conditions of incubation of the muscle with the marker are critical to the accurate measurement of the extracellular space available to the marker. Sufficient time must be allowed for complete equilibration of the marker with the extracellular space. Kipnis and Cori (1957) found that the initial rate of penetration of inulin was lower than that of thiosulphate and raffinose, probably reflecting a slower rate of diffusion owing to its larger molecular weight and size. After 2 hours all markers tested by Kipnis and Cori gave constant results. Accordingly, this was the incubation period selected for these experiments.

The temperature of incubation also appears to be very important. Preliminary soaking of rat diaphragm in Krebs-Henseleit buffer at 0°C has been reported to result in an increased glucose uptake on subsequent incubation at 37°C (Brown et al., 1952). The same authors report that such treatment also results in a 30% increase in the extracellular space. Incubation at 12°C for periods up to 30 minutes

caused an average increase of 10% in wet weight, with concomitant increases in both extracellular and intracellular volumes, although incubation for the same length of time at 17°C did not alter the total water content (Brown et al., 1952). In the present work, diaphragms were incubated at 28°C as recommended by Kobayashi and Yonemura (1967).

Kipnis and Cori (1957) used two preparations of rat diaphragm muscle for their extracellular space determinations. These were the 'cut' or hemidiaphragm preparation, where the muscle is excised from the rib-cage and bisected, and the 'intact' preparation, where the diaphragm is dissected from the animal with all its insertions intact. These preparations are described on page 44 . Kipnis and Cori showed that the thiosulphate space of cut diaphragm incubated in vitro is consistently larger than that of the intact diaphragm preparation, and concluded that in the former, marker was penetrating the cut ends of the muscle cells. Both types of preparation were employed in the present work.

The work of Sherman et al. (1950) indicated that skeletal muscle as a whole was depleted of protein by tumour growth, but did not indicate whether all types of muscle were equally sensitive to the effects of the tumour. There is evidence that in rats bearing the Walker 256

carcinoma, the gastrocnemius muscle is depleted to a much greater extent than is the soleus (Clark and Goodlad, 1971). An investigation was therefore made of the effect of the tumour on the total protein and also on the relative amounts of contractile, sarcoplasmic and extracellular proteins of rat diaphragm.

The solubility characteristics of adult striated muscle were investigated by Robinson (1952). The intracellular proteins of adult tissue may be conveniently divided into the proteins of the sarcoplasm, such as myogen and myoalbumin, including the enzymes of the glycolytic cycle; and the proteins of the myofibril, such as actin and myosin. Extracellularly, connective tissue fibres of collagen and elastin ramify throughout the muscle (Bate Smith, 1937). Bate Smith (1934,1935) demonstrated that up to 85% of the intracellular protein was extracted by strong salt at neutral pH, and that subsequent dilution to lower ionic strengths selectively precipitated myofibrillar proteins, leaving the sarcoplasmic proteins in solution. Robinson (1952) extended this work by extracting the muscle with salt solutions of different ionic strengths and pH values. He found that a dilute KCl buffer at pH 7.1 would extract the sarcoplasmic proteins and the myofibrillar protein actomyosin. Actomyosin could be selectively precipitated by an ethanol

concentration of 7% (v/v) at 0°C. Dilute alkali extracted the myofibrillar proteins, leaving only collagen and elastin (Lowry et al., 1941). This procedure was used, as modified and described by Dickerson (1960), for the fractionation of proteins in this work.

EXPERIMENTAL

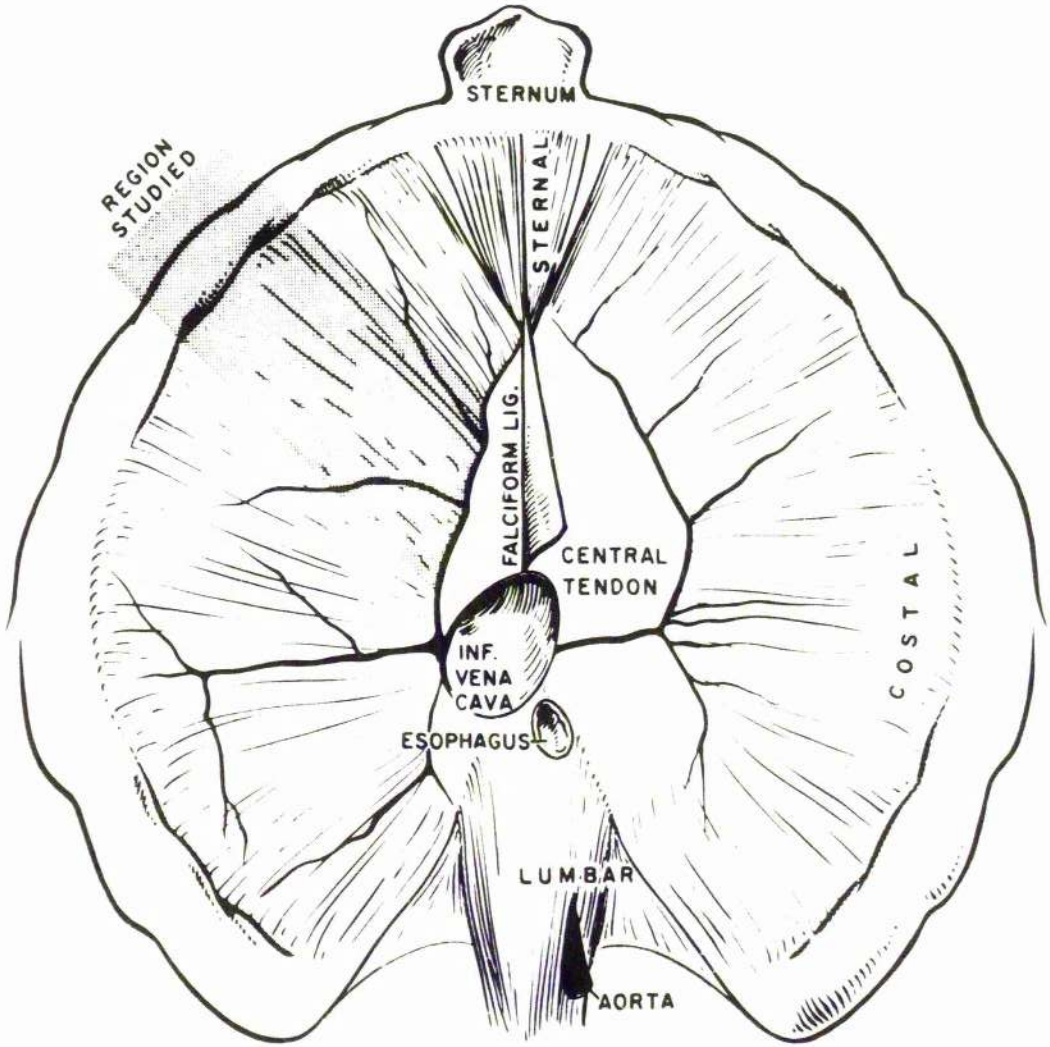
Microscopic examination of diaphragm muscle from normal rats and rats bearing a Walker 256 carcinoma.

A healthy rat and a tumour-bearing rat were sacrificed by stunning followed by exsanguination, and a section of diaphragm muscle, approximately 1 cm^2 in area, was excised. Figure 1 shows the area of the muscle from which the samples were obtained. These muscle sections were rinsed in 0.9% (w/v) NaCl and frozen onto a block of 12.5% (w/v) gelatine by blasting with CO_2. Longitudinal sections of 6 microns thickness were cut on a SLEE cryostat, the blade of which was maintained at -20°C. The sections were transferred to microscope slides and fixed for 30 minutes in a 90 : 10 (v/v) mixture of glacial acetic acid and 90% (v/v) ethanol. The slides were rinsed three times with 75% (v/v) ethanol, soaked in distilled water and stained with 1% (w/v) xylidene ponceau - 0.5% (w/v) acid fuchsin for 2 minutes. The stain solution was washed off with distilled water and the sections dehydrated by successive washings with 96%(w/v) ethanol and absolute ethanol. Finally the slides were cleared with xylol before mounting in styrene. The sections were viewed under a Zeiss photographic microscope, and representative fields from each section photographed.

The mean diameter of fibres from muscles from normal and tumour-bearing animals was estimated using the vernier attachment on the microscope. 3 sections from each type

FIGURE 1

Abdominal surface of the diaphragm muscle. The shaded region shows the area of the muscle studied in the microscopy experiments.



DIAPHRAGM - ABDOMINAL SURFACE

of muscle were examined, and within each section the width of 12 groups of 5 neighbouring fibres was measured, in randomly selected fields.

Measurement of the extracellular space of diaphragm muscle from normal rats and rats bearing a Walker 256 carcinoma

Preparation of animals

Male Wistar rats, weighing between 175-225 g., were used throughout this work. Animals were housed individually and fed a diet of a paste of 12 g. freshly-ground rat-cake (North Eastern Agricultural Co-operative Ltd.), moistened with water, given daily at 5 p.m. Water was given *ad libitum* throughout the experimental period. The animals were starved for 24 hours before commencing their diet, and their initial body weight noted.

A rat bearing a large Walker 256 carcinoma was killed by chloroform/ether anaesthesia. A portion of the rapidly-dividing tumour cortex was quickly excised and gently homogenised in an all-glass Potter homogeniser (Potter and Elvehjem, 1936), with 5 ml. of a sterile solution of 0.9% (w/v) NaCl and 1% (w/v) glucose. An 0.5 ml. aliquot of this homogenate was injected subcutaneously and aseptically into a shaved area on the side of the rat. It was found that the tumours took from ten to fourteen days to reach 20-40 g. Tumours grown from cells of the same donor tumour normally attained approximately the same size in different rats.

Dissection of muscles

Two muscle preparations were used in this work - the intact diaphragm preparation of Kipnis and Cori (1957) and the 'cut' diaphragm preparation, described by Manchester and Young (1958).

The intact diaphragm preparation was accomplished as follows: the sternum was divided horizontally at the level of the fourth costal cartilage, after which the ribs were transected superior to the insertions of the diaphragm. After the attachments of the abdominal and back muscles had been cut from the lower rib-cage, and the intrathoracic and abdominal structures adhering to or traversing the central tendon had been severed, the diaphragm was freed by transecting the spine above and below the twelfth vertebra. The insertions of the diaphragm to the xiphoid process, ribs, central tendon and spine were maintained, and the diaphragm thus prepared exhibited integrity of all its muscle fibres (Kipnis and Cori, 1957). Figure 2 shows the points of incision for this preparation.

In the dissection of the 'cut' diaphragm, the rats were killed by stunning followed by exsanguination. The diaphragm was rapidly excised from the rib-cage and transferred immediately to a Petri-dish containing ice-cold Krebs-Henseleit bicarbonate buffer (Krebs and Henseleit, 1932) (see page 45). Excess tendon and any adhering

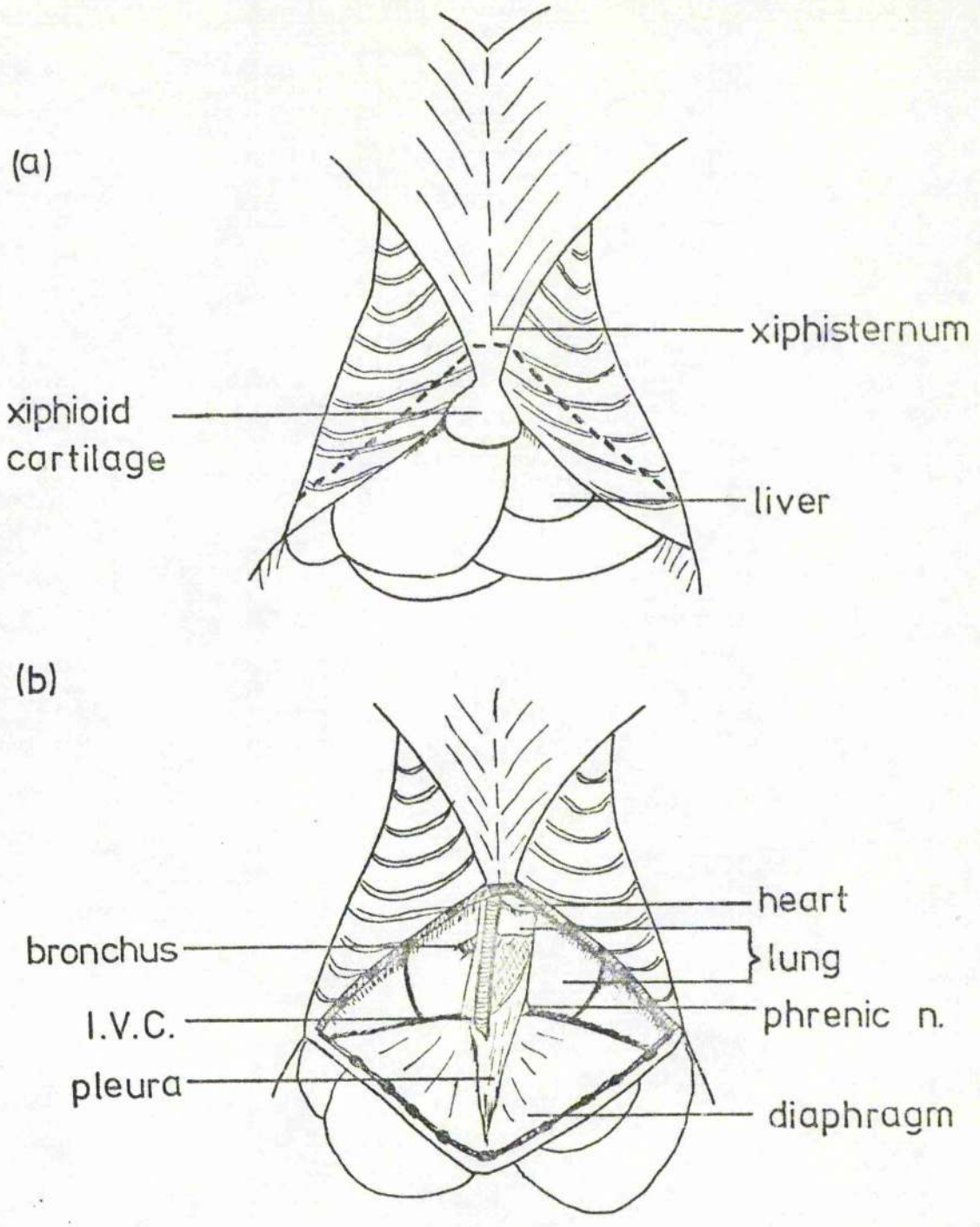


Figure 2.

(a) Dotted line shows the rib-cage incision made in the preparation of the intact diaphragm.

(b) View of the intrathoracic adhesions to diaphragm.

I.V.C. - inferior vena cava.

lung and liver tissue was trimmed off and the lateral and dorsal slips removed. The hemidiaphragms were then obtained by carefully cutting along the outer boundaries of the central tendon.

Determination of the inulin and sorbitol spaces of diaphragm muscle from normal rats and rats bearing a Walker 256 carcinoma

Animals were prepared as detailed on page 43 and the hemidiaphragms or intact diaphragms were excised. Krebs-Henseleit bicarbonate buffer pH 7.4 (Krebs and Henseleit, 1932) formed the basis of the incubation medium, used with both extracellular markers.

This buffer was prepared from the following six stock solutions:

(1) 0.9%(w/v) NaCl; (2) 1.15%(w/v) KCl; (3) 1.22%(w/v) $CaCl_2$; (4) 3.83%(w/v) $MgSO_4$; (5) 2.11%(w/v) KH_2PO_4; (6) 1.30%(w/v) $NaHCO_3$.

Solution (6) was prepared fresh every day and gassed with CO_2 for 1 hour before use. The six stock solutions were mixed in the correct proportions immediately before the medium was required, the requisite amounts being:

100 volumes (1) : 4 vols. (2) : 1.5 vols. (3) : 1 vol. (4) : 1 vol. (5) : 17.5 vols. (6).

The complete medium was gassed for 15 minutes with a 95:5 (v/v) mixture of O_2:CO_2, and extracellular marker added to make the final medium 1% (w/v) with respect to the marker.

For the determination of the inulin space of hemi-diaphragms, the muscles were soaked in 5 ml. of the inulin medium for 2 hours at a temperature of 28°C, in which time the inulin was considered to have diffused into the extra-cellular space (Kipnis and Cori, 1957). The muscles were then removed, blotted gently to remove any inulin-containing solution from the surface of the muscle, and soaked for another 2 hours in 5 ml. of inulin-free Krebs-Henseleit buffer. In this time the inulin contained in the extra-cellular space should diffuse into the inulin-free buffer (Kobayashi and Yonemura, 1967).

The amount of inulin which had diffused out into the inulin-free solution was measured spectrophotometrically, utilising a diphenylamine colour reaction (Harrison, 1942). Diphenylamine reagent was prepared by dissolving 3 g. diphenylamine in 100 ml. glacial acetic acid. 60 ml. concentrated HCl was added to this solution, and the resultant reagent could be stored at 6°C for 1 month in an amber bottle. The test solution was diluted to be within the concentration range of 2 - 12 ug. inulin/ml. 2 ml. of the test solution were mixed with 4 ml. diphenylamine reagent and incubated at 100°C for 30 minutes. Inulin is

a polysaccharide composed of fructose, and the hydrolysis with the concentrated acid of the diphenylamine reagent has the effect of breaking the molecule down to its consistent sugars, which are capable of reacting with the diphenylamine to form a colour complex. The extinction produced by the colour complex was measured at 620 nm, and the concentration of inulin in the test solution estimated from a standard curve, shown in Figure 3.

A tissue blank value was obtained by incubating a muscle in inulin-free medium for 2 hours, and transferring to fresh inulin-free medium as described above. The total water content was determined by heating to constant weight at 110°C. The extracellular space could then be calculated according to the formula:

$$\text{Extracellular space (\% total water content)} = \frac{N}{I} \times \frac{1}{W} \times 100$$

where

N = ug inulin diffused out from muscle

I = initial concentration of inulin in incubation medium expressed in ug/ul.

W = total water content of muscle in ul.

In the case of the sorbitol space determination, ^{14}C-U-sorbitol, 6.7uCi/umol. (Radiochemical Centre, Amersham) was used as the extracellular marker. The specific activity

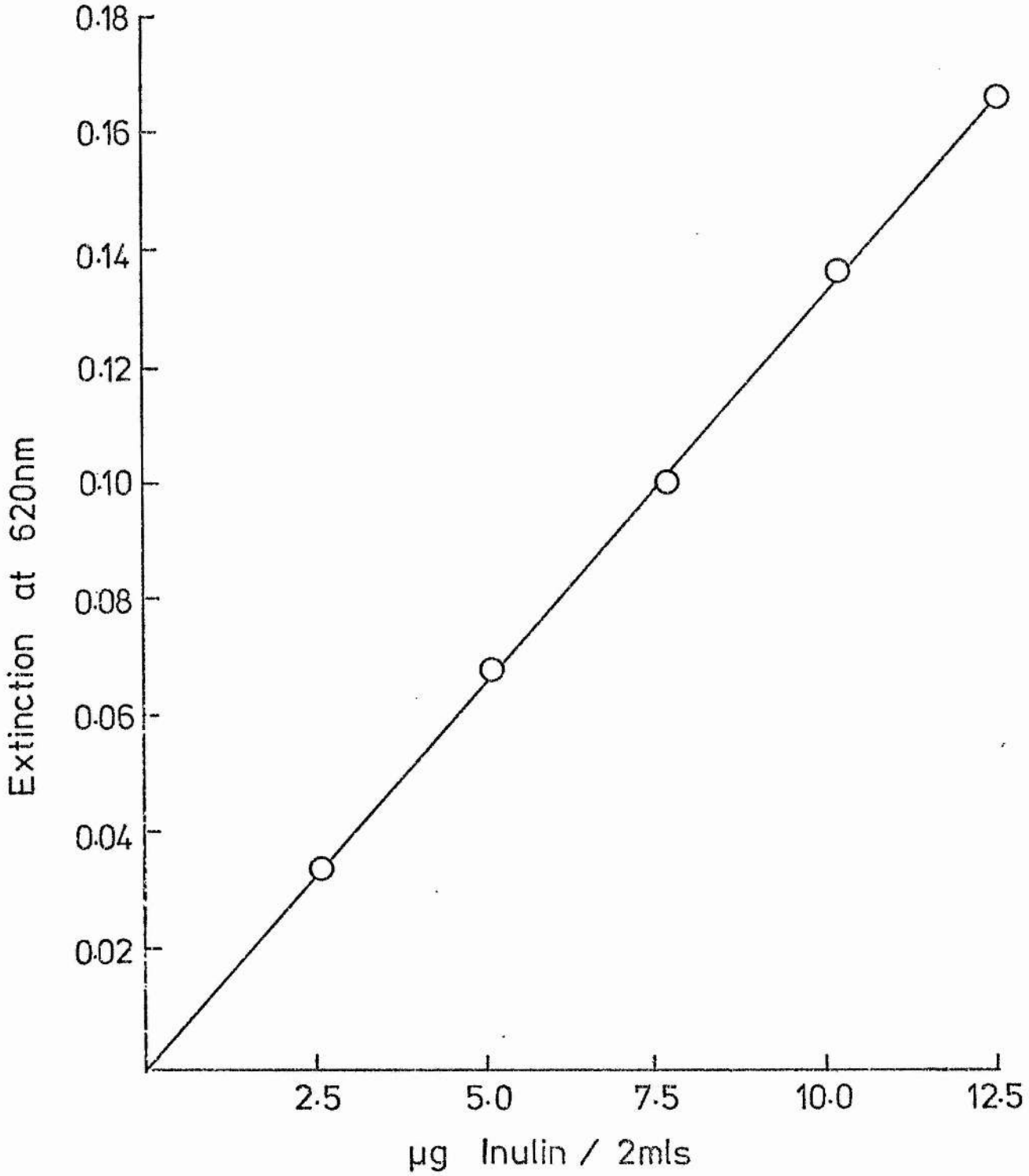


Figure 3. Standard curve for the estimation of inulin by the diphenylamine method (Harrison, 1942).

of the medium was adjusted to 0.1 uCi/ml. After the 2 hour incubation the muscles were blotted gently and immersed in boiling water for 15 minutes, during which time all the small molecular weight components of the muscle were assumed to have been extracted (Battaglia and Randle, 1960). The muscle was removed, the water allowed to cool, and the volume adjusted to exactly 5 ml. 0.1 ml. aliquots were counted in 5 ml. NE 220 scintillation cocktail (Nuclear Enterprises, Edinburgh). The radioactivity of the medium was similarly assayed, and the total water content of the muscle estimated by heating to constant weight at 110°C. The sorbitol space of the muscle was calculated by application of the formula:

$$\text{Extracellular space (\% total water content)} = \frac{N}{I} \times \frac{1}{W} \times 100$$

where

N = counts/minute in boiling water extract

I = counts/minute/microlitre in incubation medium

W = total water content of muscle in microlitres.

Isolation and estimation of proteins from diaphragm muscle of normal rats and rats bearing a Walker 256 carcinoma

Isolation of total intracellular protein

Frozen muscles were minced finely with scissors and homogenised in 2 ml. 0.3N NaOH with a tightly fitting motor-driven pestle, at 0°C. The homogenate was centrifuged at 600g. for 15 mins. at 2°C. The residue was rehomogenised twice, the supernatants being collected by centrifugation. The final homogenate was allowed to stand for 12 hours at 2°C before centrifugation. The residue, consisting of collagen and other metabolically inert proteins (Robinson, 1952), was discarded. A half-volume of 30% (w/v) trichloracetic acid (TCA) was added to the collected supernatants, and the precipitate allowed to stand for 30 minutes at 2°C, before collection by centrifugation at 600g for 15 mins. at 2°C. The protein was washed six times by suspension and centrifugation with 5 ml. 10% (w/v) TCA to remove any residual non-protein-bound nitrogenous material, twice with ethanol to remove TCA and water, and finally, once with ether to remove the ethanol. The proteins were dried and stored under vacuum.

Isolation of sarcoplasmic, myofibrillar and residual proteins

The flow chart of this extraction method is shown in Figure 4. The frozen muscles were minced finely and homogenised at 0°C with 1 ml. 'dilute salt' solution, which consisted of 0.066M NaH_2PO_4: K_2HPO_4; 0.1M KCl; pH 7.1 I 0.2 (Dickerson, 1960). The homogenate was centrifuged for 15 mins. at 600g. at 0°C, the extraction with dilute salt solution repeated twice and the supernatant pooled. One half-volume of 30% (v/v) ethanol was added to the collected supernatants, and then allowed to stand overnight at 0°C to precipitate actomyosin.

The residue from the dilute salt extractions was homogenised three times with 0.1N NaOH, the supernatants being collected by centrifugation. The final homogenate was left to stand for 12 hours at 2°C before centrifugation.

Proteins in all fractions were precipitated by the addition of one half-volume of 30% (w/v) TCA and washed and dried in the manner described on page 49 for the total proteins.

Estimation of muscle proteins

Dried muscle protein fractions prepared as described in the previous two sections were redissolved in 0.3N NaOH

and estimated by the Lowry method (Lowry et al., 1951). A standard curve is shown in Figure 5. The residual protein fraction, being insoluble in 0.3N NaOH, was dissolved in nitrogen-free concentrated sulphuric acid, and estimated by micro-kjeldahl nitrogen (Ma and Zuazaga, 1943).

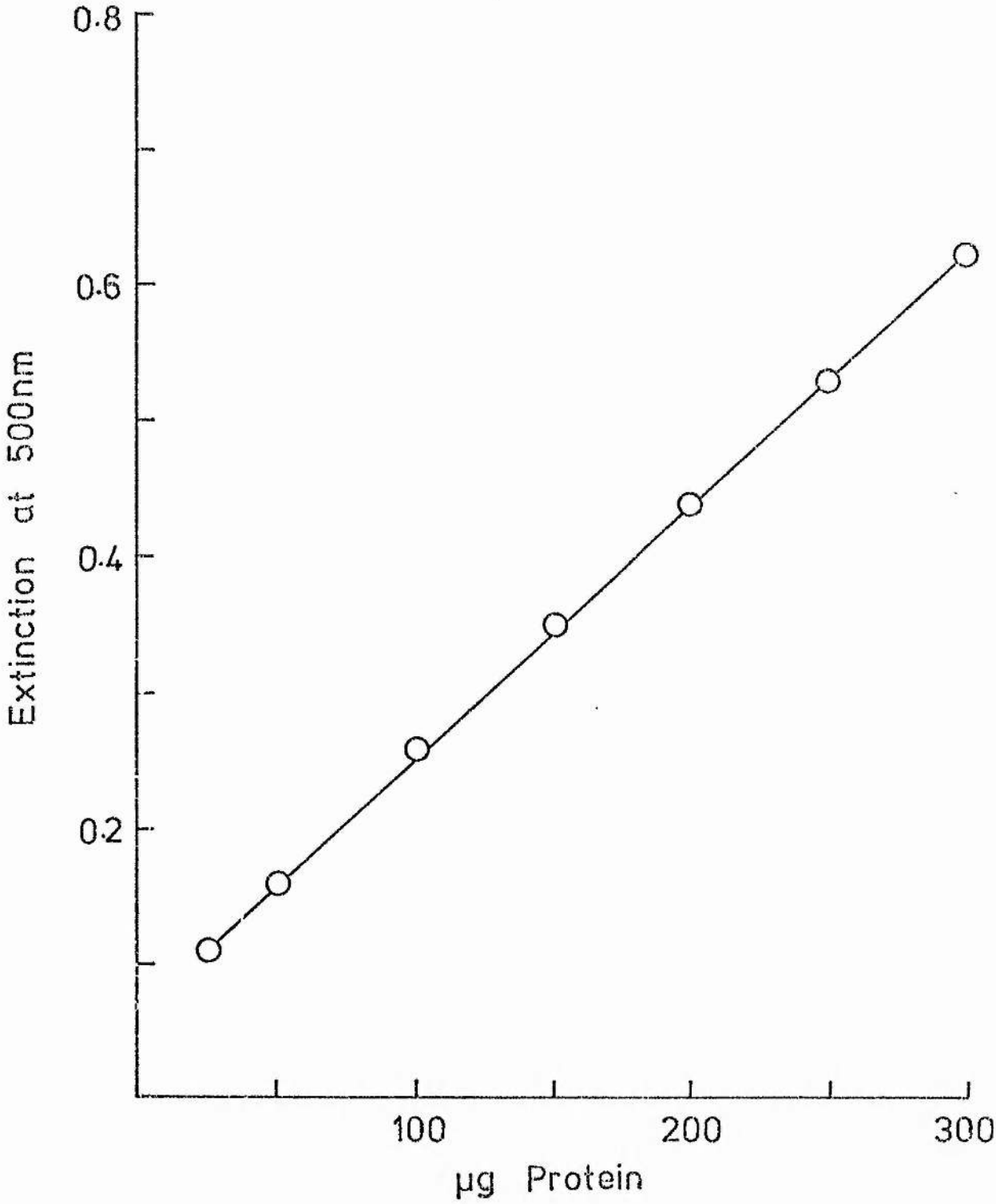
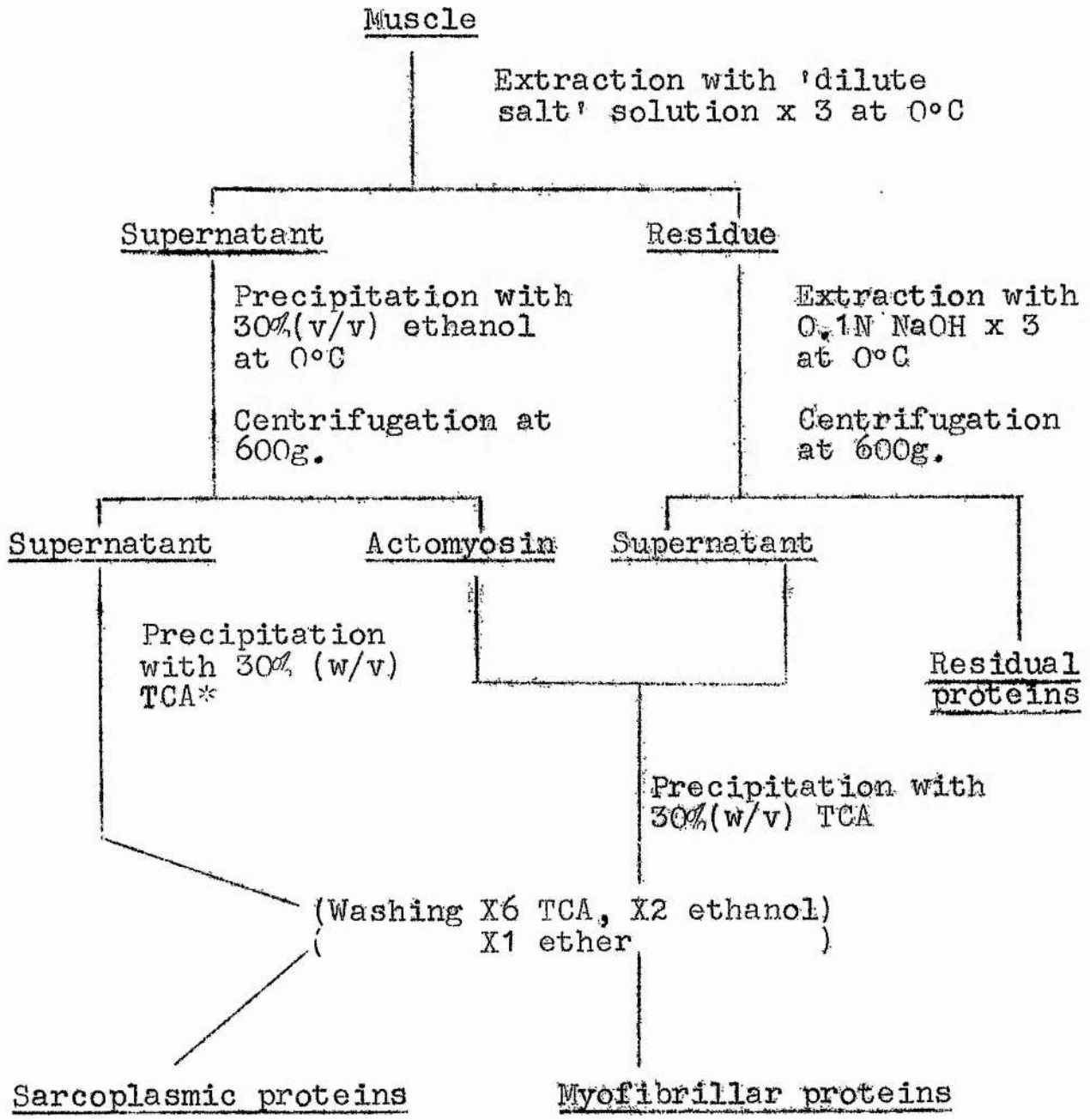


Figure 5. Standard curve for the estimation of muscle proteins by the Lowry method (Lowry et al., 1951). A solution of mixed muscle proteins was estimated by the microkjedahl nitrogen method, and subsequently used as a standard protein solution.

Figure 4 Fractionation procedure for muscle protein



* trichkoracetic acid

RESULTS AND DISCUSSION

The microscopic appearance of diaphragm muscle from normal rats and rats bearing a Walker 256 carcinoma

Plate 1 shows a typical field from a section of diaphragm muscle taken from a normal rat, whilst Plate 2 shows a field from a section of diaphragm muscle excised from a rat bearing a Walker 256 carcinoma. The magnification of both of these fields is x 100.

The fibres in the muscle from the tumour-bearing animal appear noticeably thinner than do the fibres from the normal rat, and this observation was borne out by the figures obtained for the mean diameter of the fibres from the two types of muscles. The results shown in Table 1 indicate that there is a highly significant decrease in the mean diameter of fibres from diaphragm muscle of tumour-bearing rats.

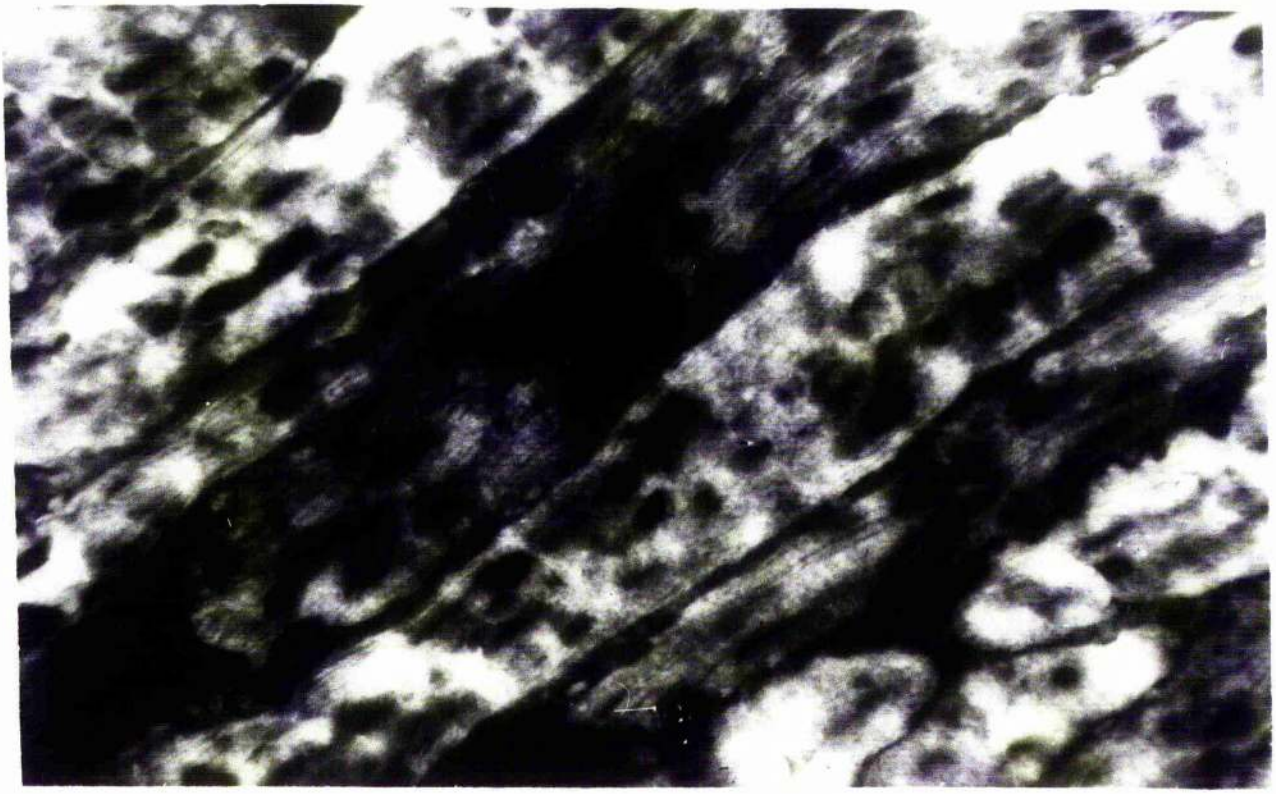
These figures for the mean fibre diameter are valuable only for comparison of the muscles from control and tumour-bearing animals and do not represent a true value for the diameter of the fibres. Since the sections were cut longitudinally, only a small proportion of the fibres will have been cut along the centre, showing their true diameter. The remainder, cut either above or below the centre line of the fibre, will appear to be smaller than their true diameter. Ideally, fibre diameter should be measured on

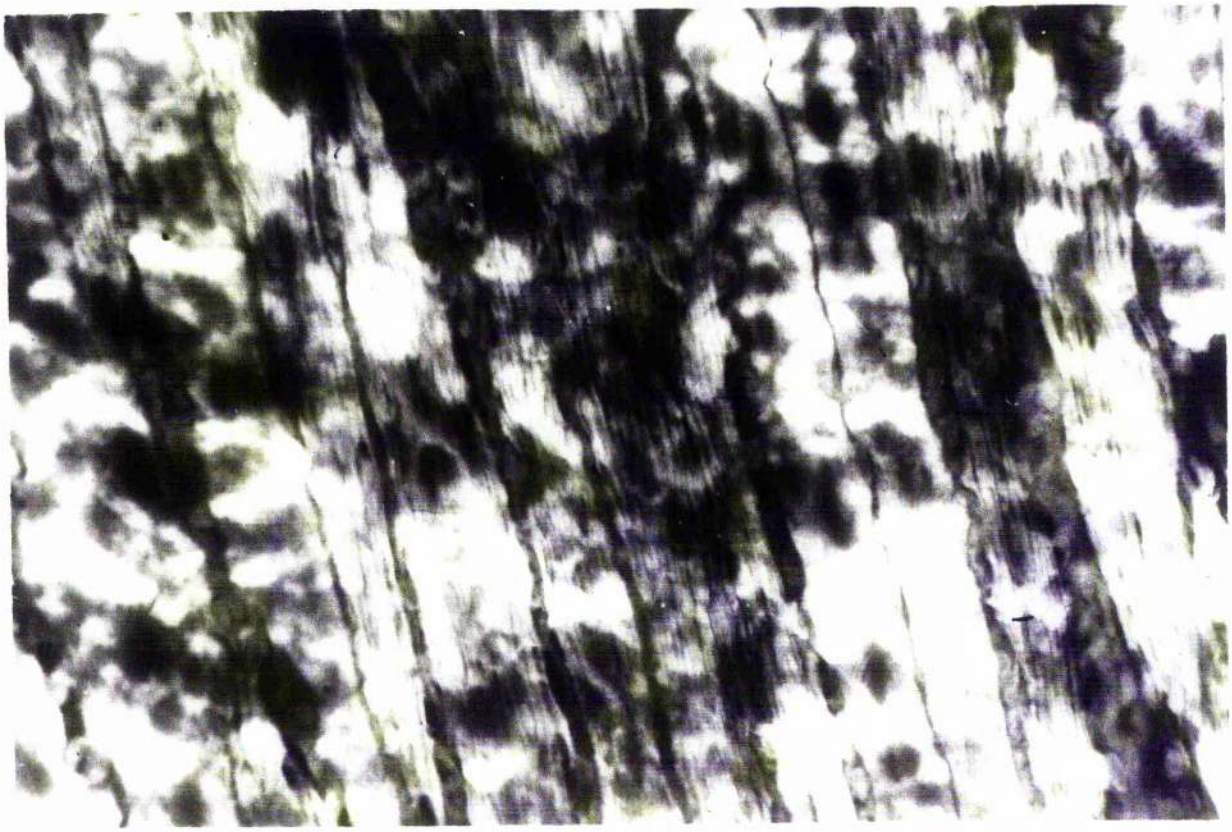
PLATE 1

Photomicrograph of a section of diaphragm muscle from a normal rat.
Magnification was x 100

PLATE 2

Photomicrograph of a section of diaphragm muscle from a rat bearing a Walker 256 carcinoma.
Magnification was x 100





a transverse section of the muscle, but the diaphragm does not lend itself well to this type of sectioning because of its very small cross-sectional area.

However, the value obtained for the control muscle is in close agreement with the mean diameter of 27 microns reported for the red muscle fibres, which constitutes 80% of the fibre population of adult rat diaphragm (Gauthier, 1969; Gauthier and Padykula, 1966; Padykula and Gauthier, 1963).

Table 1

The effect of the Walker 256 carcinoma upon the mean fibre diameter of rat diaphragm muscle

	Control	Tumour-bearing
Mean fibre diameter in microns	28.59 ± 0.83*	22.66 ± 0.59
P**	-	<0.01

* Results are expressed as the mean ± S.E. of 36 measurements.

** In this and the following tables in this section, P refers to the statistical significance of the differences between control and tumour-bearing rats, as estimated by Student's t-test.

The determination of the inulin and sorbitol spaces of diaphragm muscle from normal rats and rats bearing a Walker 256 carcinoma

The results obtained from this series of experiments are shown in Table 2.

Table 2

The effect of the Walker 256 carcinoma upon the extracellular space of rat diaphragm muscle.

Muscle preparation	Extracellular marker	Extracellular space (% total water content)		P
		Control	Tumour-bearing	
Hemidiaphragm	Sorbitol	63.6±1.93*	66.3±2.17	n.s.
Intact diaphragm	Sorbitol	21.8±1.65	23.3±1.35	n.s.
Hemidiaphragm	Inulin	40.3±1.66	23.4±1.09	<0.01

* Results are expressed as the mean ± S.E. of 12 rats except for the data on the sorbitol space of intact diaphragm, where the results are the mean ± S.E. of 4 rats.

The total water content of the intact diaphragm preparation and the hemidiaphragm preparation from both normal and tumour-bearing animals was estimated in the course of these experiments, and Table 3 shows the data obtained.

Table 3

The effect of the Walker 256 carcinoma upon the water content of rat diaphragm muscle

Muscle preparation	Total water content (ml per g wet weight muscle)		P
	Control	Tumour-bearing	
Hemidiaphragm	78.9±1.1*	76.2±0.6	n.s.
Intact diaphragm	77.6±2.2**	74.1±3.1	n.s.

* Results are expressed as mean ± S.E. of 24 rats

** Results are expressed as mean ± S.E. of 4 rats

These results do not show any significant differences between the total water contents of diaphragms from normal and tumour-bearing rats. This indicates that the diaphragm muscle does not share in the general carcass increase in water content demonstrated by Haven et al., (1961).

Published values for the extracellular space of diaphragm muscle vary considerably, and some of these are shown in Table 4.

From the figures shown it would appear that there is considerable penetration of the cut diaphragm preparation by both large and small extracellular markers, almost certainly via the cut ends of the fibres (Kipnis and Cori,

1957; Arvill and Ahren, 1966).

In the present work when sorbitol was used as a marker, the values obtained for both hemidiaphragm and intact diaphragm preparations were in close agreement with the published values (Tables 2 & 4). Furthermore, no differences were observed between the sorbitol spaces measured in the muscle preparations from normal and tumour-bearing animals, indicating that the muscle preparations in both cases have a similar extracellular space, and also that small molecular weight compounds have equal access to the muscle cells of the diaphragm preparations in normal and tumour-bearing rats.

Table 4

Some published values for the extracellular space of diaphragm preparations in vitro

Source	Muscle preparation	Extracellular marker	Extracellular space *
Kipnis and Cori(1957)	Hemidiaphragm	Inulin	53
ibid.	Hemidiaphragm	raffinose	64
ibid.	Hemidiaphragm	thiosulphate	62
ibid.	Intact	thiosulphate	25
Arvill and Ahren(1966)	Intact	inulin	24

* All extracellular space values have been expressed as % total water content.

The results obtained for the inulin space of hemidiaphragms appeared anomalous, in that the space in the muscle from the tumour-bearing animal was found to be 42% less than the space in the muscle from the normal rat (Table 2). If the true extracellular space of diaphragm muscle is approximately 25%, as evidenced by the figures presented in Tables 2 and 4 for intact diaphragm, then the larger apparent inulin spaces found with hemidiaphragms from normal rats must be due to penetration of the inulin to the inside of the muscle cell, probably through the cut ends of the fibres. In order for the inulin space of the hemidiaphragm from the tumour-bearing animal to be lower than that of the normal animal, the marker must either be failing to reach the inside of the cell or be failing to penetrate the extracellular space of the muscle. It would seem unlikely that the cut ends of the fibres in the hemidiaphragm from the tumour-bearing animal could be so much smaller than those in the normal animal as to prohibit the entry of a relatively small molecular weight compound such as inulin. It has been shown that protein polysaccharide or hyaluronic acid solutions possess a measurable exclusion volume to molecules such as haemoglobin and serum albumin, but not to small molecular weight compounds (Laurent, 1966; Schubert & Hamerman, 1968). These findings can be extended to connective tissue matrices in general (Gerber & Schubert, 1964). It could be envisaged that a proliferation or reorganisation of the connective tissue components of muscle, induced by the cancerous state of

the animal, might cause exclusion of a polysaccharide molecule such as inulin, whilst allowing free access to smaller markers such as sorbitol and raffinose. If this is the case, then the inulin space of the intact diaphragm from the tumour-bearing animal should be very small. Measurement of the inulin space of intact diaphragm is not feasible using a spectrophotometric method for the estimation of inulin. By its nature, the intact diaphragm preparation has present a large amount of extraneous tissue, such as costal cartilage, which releases diphenylamine-positive substances under the conditions of the extracellular space determination. The estimation could be performed successfully using suitably labelled inulin, such as ^{3}H-inulin (Marlow and Sheppard, 1971).

The protein content of diaphragm muscle from normal rats and rats bearing a Walker 256 carcinoma

Table 5 shows that the diaphragm muscle lost weight in the tumour-bearing animal, and that the total protein content of the diaphragm muscle was significantly reduced.

Table 5

The effect of the Walker 256 carcinoma upon the size and protein content of rat diaphragm muscle.

	Control	Tumour-bearing	P
wt diaphragm (wet weight in g) per 100 g initial body weight	0.308±0.154 *	0.234±0.146	<0.01
mg total protein per diaphragm per 100 g initial body weight	59.40±3.20	44.08±3.45	<0.01
mg myofibrillar protein per diaphragm per 100 g initial body weight	38.70±2.14	27.15±2.13	<0.01
mg sarcoplasmic protein per diaphragm per 100 g initial body weight	15.60±0.78	12.12±0.95	<0.01
mg residual protein per diaphragm per 100 g initial body weight	5.81±0.60	3.91±0.62	n.s.

* Results are expressed as mean ± S.E. of six rats.

On separation of the proteins of the diaphragm into myofibrillar, sarcoplasmic and residual fractions, it was found that in tumour-bearing rats the total amounts of myofibrillar

and sarcoplasmic proteins were significantly decreased, while there was no significant alteration in the quantities of residual protein.

It can be seen from Table 6 that no significant differences were found in the amounts of the protein fractions per g muscle in the presence of the tumour.

Table 6

The effect of the Walker 256 carcinoma upon the amounts of the protein fractions in rat diaphragm muscle.

	Control	Tumour-bearing	P
mg total protein per g wet weight diaphragm	195.5±1.7 *	190.2±6.4	n.s.
mg myofibrillar protein per g wet weight diaphragm	127.5±2.4	116.6±4.5	n.s.
mg sarcoplasmic protein per g wet weight diaphragm	49.4±1.0	52.3±1.9	n.s.
mg residual protein per g wet weight diaphragm	20.0±0.9	21.2±0.7	n.s.

* Results are expressed as the mean ± S.E. of six rats.

It is interesting to note that while the amount of myofibrillar protein was slightly reduced in the diaphragm from the tumour-bearing rat, the amount of sarcoplasmic protein was slightly increased. This tendency was reflected in the figures given in Table 7, which shows the relative amounts of the three protein fractions.

Table 7

The effect of the Walker 256 carcinoma upon the relative amounts of myofibrillar, sarcoplasmic and residual proteins in rat diaphragm muscle.

	mg protein per 100 mg total protein		
	Sarcoplasmic	myofibrillar	residual
Control	25.27±0.75*	64.89±0.40	10.30±0.34
Tumour-bearing	27.48±0.42	61.51±0.58	10.68±0.30
P	<0.01	<0.01	n.s.

* Results are expressed as mean ±S.E. of 6 rats

Thus it would appear that a general depletion of proteins occurs in the diaphragm muscle during the growth of the tumour, paralleling the loss in weight of the muscle. The data in Table 7, showing a significant decrease in the amount of myofibrillar protein in the tumour-bearing animal, with an increase in the amount of sarcoplasmic protein, indicates that there is a tendency for the contractile proteins of the muscle to be most affected. The residual or extracellular proteins are the least depleted. These findings are in agreement with those of Clark and Goodlad (1971), who showed that the total amount of myofibrillar protein per gastrocnemius muscle was significantly reduced in animals bearing a Walker 256 carcinoma. The total amounts of sarcoplasmic

and extracellular proteins were unaltered.

The diaphragm muscle in the rat and other small mammals consists of predominantly red muscle fibres. The finding of marked depletion of protein in this muscle in the tumour-bearing rat is therefore of interest in view of the fact that the soleus muscle, which is also a predominantly 'red' muscle, is relatively resistant to the depleting effects of the tumour (Clark and Goodlad, 1971). However, despite its high proportion of red fibres, the diaphragm differs from other such muscles in that it cannot be classed as a slow contracting muscle. Its speed of contraction appears intermediate between that of 'red' and 'white' muscles (Ritchie, 1954; Miledi and Slater, 1969). It is also unusual in that it has a low innervation ratio (number of contractile fibres/number of innervating motor neurons), 25, instead of the normal 100 - 1000 (Wuerker et al., 1965; Clark, 1931).

Thus the extent to which the muscle responds to the tumour is not simply dependent upon its fibre composition, even though the rate of protein synthesis in red and white muscle fibres is markedly different in the normal animal (Goldberg, 1967; Beatty et al., 1963).

SECTION 2

The effect of the growth of the Walker 256 carcinoma upon the uptake of ^{3}H-lysine into the protein of rat diaphragm muscle.

INTRODUCTION

Attention has already been drawn to the fact that the wasting of body tissues is one of the most obvious physical effects on a host animal of the growth of a tumour such as the Walker 256 carcinoma. Sherman *et al.* (1950) found that most of the tissues which relinquish protein during the course of simple caloric starvation also yield up a fraction of their nitrogen during the period of tumour growth. They further showed that quantitatively the most important contribution of protein precursors for utilisation by the tumour was made by skeletal muscle. Results in the present work (Section 1) have indicated that diaphragm muscle proteins are depleted in the tumour-bearing animal. This depletion could result from a decrease in the rate of protein synthesis within the muscle, or from an increase in the rate of protein catabolism, or from a combination of these two effects. This section describes experiments designed to investigate the comparative rates of incorporation of a radioactively labelled amino-acid into diaphragm muscle protein from normal and tumour-bearing rats.

There are advantages attached to performing *in vitro* experiments such as those described in this section, the principal one being that the labelled amino-acid has free access to the muscle cell, and does not have to pass through

the liver and plasma of the animal, thereby avoiding to a large extent the isotope re-utilisation and dilution inevitable under *in vivo* conditions. Further, when testing the action of a hormone such as insulin, the possibility of the effect being masked or enhanced by secondary metabolic interactions is eliminated.

Arvill and Ahren (1966) laid down that a tissue preparation suitable for the *in vitro* study of transport phenomena through the cell membrane must fulfil at least the following two conditions: the relation between the surface area and tissue mass must be such that good diffusion properties between the incubation medium and the extracellular fluid of the whole preparation are obtained; and it must also be possible to dissect out and incubate the preparation with intact and undamaged cells. It was recognised at an early stage that the rat diaphragm is sufficiently thin to fulfil the first of these conditions, and this muscle has been widely used for *in vitro* work. This muscle was first introduced as the hemi-diaphragm preparation (Meyerhof *et al.* 1925; Takane, 1926; Gemmill, 1940). This cut diaphragm preparation develops an abnormally large extracellular space *in vitro* (see Section 1), and its permeability to ions, carbohydrates and amino-acids is markedly changed as compared to the tissue *in vivo* (Norman *et al.*, 1959;

Menozzi et al., 1959; Kipnis, 1959). Kipnis and Cori (1957) described a technique for the dissection of an intact diaphragm preparation (see Section 1) which fulfils both the above-mentioned requirements for an in vitro system.

Although the validity of the first requirement laid down by Arvill and Ahren (1966) remains unquestioned, experiments using intact or cut diaphragm preparations have given similar results with respect to amino acid uptake into protein under a variety of conditions. Both these preparations have been used widely in studies on protein synthesis since Sinex et al., (1952) reported a stimulatory effect of insulin upon the incorporation of ^{14}C from carboxyl-labelled alanine into diaphragm muscle protein in vitro.

Manchester (1966) studied the effects of various factors upon the response of diaphragm muscle to insulin. He showed that incorporation of ^{14}C-glycine was less in the intact diaphragm preparation than in the hemidiaphragm preparation. This finding he attributed to the fact that accumulation of glycine occurs more slowly in the former preparation (Kipnis and Noall, 1958; Manchester and Young, 1960). On the other hand, the incorporation of leucine and phenylalanine, the rates of accumulation of which are more comparable in the two preparations

(Manchester and Young, 1960), is less in the isolated hemidiaphragm, i.e. the damaged preparation, than in the rib-cage preparation. This trend is perhaps the more normal, for the cutting of diaphragm into strips and small pieces was shown by Manchester (1966) to lead to a substantial diminution in the capacity of the muscle to incorporate amino-acids. As the tissue becomes increasingly damaged, the capacity to respond to insulin is also progressively lost, and this is in conformity with the generally held view that <u>in vitro</u> actions of insulin cannot be readily detected in homogenates (Hendler, 1962). Hendler (1962) has suggested that protein synthesis near the membrane may be a significant proportion of the total synthetic activity in muscle. If so, physical damage to the membranes may be the chief factor involved in the diminution of incorporation when the tissue is cut up. Manchester and Young (1960) studied the rate of penetration into isolated rat diaphragm preparations of certain ^{14}C-amino-acids, and the effect of insulin upon this rate. No important differences between the behaviour of cut and intact preparations was observed. With several amino-acids, including lysine, the amino-acid used in the present studies, the ratio of concentration of radioactivity in tissue water to that in the medium was greater than unity, indicating the involvement of some form of active transport

mechanism. These workers noted also that the addition of insulin to the medium significantly raised the ratio for glycine and α-aminoisobutyric acid, but not for any of the other amino-acids studied. Thus it was concluded that the action of the hormone in increasing muscle protein synthesis was not due to its increasing the uptake of amino-acids.

The results obtained in the present work (Section 1), while confirming that the extracellular space of the hemi-diaphragm preparation is abnormally large, show that there is no difference in the space available to small molecules in the muscles from normal and tumour-bearing animals. In view of the proven competence of the hemi-diaphragm preparation for in vitro studies of amino-acid incorporation, this preparation was largely employed in the present work, although some initial experiments were carried out using intact diaphragm.

The work of Manchester (1966) showed that the choice of incubation medium is highly significant with regard to the results obtained in in vitro studies with diaphragm muscle. Battacharya (1959a,b, 1961) found that replacement of much of the normal ionic composition of the incubation medium for diaphragm with sucrose interfered with the stimulation by insulin of glucose uptake. Mg^{2+} appeared to possess a unique role in the preservation of sensitivity to insulin.

Manchester (1966) tested the effect of the alteration of the ionic composition of the incubation medium on the incorporation of ^{14}C-glycine into the protein of isolated rat diaphragm, and the stimulation of this process by insulin. He found that there was little difference in either the incorporation of labelled amino-acid or in the sensitivity of the incorporation to stimulation by insulin, using either of the three commonly employed incubation media - namely Krebs-Ringer phosphate buffer, Krebs-Henseleit bicarbonate buffer (Krebs and Henseleit, 1932), or Stadie-Zapp buffer (Stadie and Zapp, 1947). It was found, however, that swelling or shrinkage of the muscle during incubation was minimised by the use of Krebs-Henseleit bicarbonate buffer, and this was the incubation medium employed throughout the present series of experiments.

Sinex et al. (1952) found that the transfer of ^{14}C from 1-^{14}C-alanine into protein of diaphragm muscle incubated in vitro was less if glucose or pyruvate were present in the medium when no insulin had been added, and that in the presence of glucose or pyruvate addition of insulin raised the incorporation of radioactive amino-acid into the muscle protein by only a very small amount. On the other hand, Krahl (1953) observed that the in vitro incorporation of ^{14}C from 1-^{14}C-glycine into protein of

diaphragms of normal fasting rats was raised by the addition of glucose to the medium, the presence of insulin in the medium in addition to glucose having no significant effect. Manchester and Young (1958) confirmed the finding of Sinex et al. (1952), and extended the data to include several other amino-acids, including lysine. In view of these findings no oxidisable substrate was added to the media used in the experiments described in this section.

Radioactively-labelled muscle proteins were counted as a solid suspension in a scintillator gel. This method was employed because of the tendency of an alkaline solution of muscle proteins to cause gradual precipitation in the scintillator fluid, producing a fall in the counting-rate of the sample. The earliest report of the use of suspension counting was that of Hayes et al. (1956), who resorted to the simple procedure of dispersing finely divided solids in the scintillator, and correcting for the loss of efficiency as the radio-active material settled. This method was obviously unsatisfactory where large numbers of samples were involved and Funt (1956) reported a method for prevention of settling by gelation. He utilised the thixotropic properties of aluminium soap solutions, described by Sheffer (1948), and showed that these properties extend

to the formation of gels in scintillation fluids. The mixing of 5% (w/v) aluminium stearate into a normal toluene or xylene based liquid scintillator solution produced a colloidal solution which gelled on heating to 70°C. With a ^{137}Cs source, a pulse height of 60% of that of the original scintillator solution was obtained. The gels were completely transparent and colourless, and exhibited the fluorescence properties of the original scintillator solution. Radioactive material could be incorporated in the gel without appreciable settling, and it has been shown that the count-rate of such a radioactive sample remains constant over a period of several weeks (White and Helf, 1956).

This type of method has since been shown to give reliable and reproducible results with the more energetic β-emitters (Greene, 1970). Tritium labelled samples, as used in the work, can also be counted, but a great deal of care in sample preparation is required. This is due to the relatively low energy of tritium emissions. The energy of the mean tritium beta emission is 9 keV, which corresponds to a range of 0.5 microns in media with a specific density of 1 (Cleaver, 1967). Particles produced for suspension counting techniques are generally several microns in diameter, so that only those β-particles emitted near the interface of the radioactive

material and the fluor will cause photon emission. For a tritiated material of given specific activity, the count-rate would be a function of the surface area. Any technique used for sample preparation must, therefore, ensure that the particle size produced in different samples does not vary.

EXPERIMENTAL

Scintillation counting of muscle proteins

Muscle proteins were counted in suspension using a scintillator gel. The size of the protein particles had to be such that the final preparation would suspend easily and uniformly, and so that the problem of self-absorption, especially important in the case of tritium labelling, would be minimised. This was achieved by the use of an agate ball-mill (RIIC Ltd., London), shown in Plates 3 and 4. The scintillator chosen was the commercially available NE 210 (Nuclear Enterprises, Sighthill, Edinburgh), which is a standard toluene-based scintillation cocktail, with a heat-gelling agent added.

The length of milling-time required to reduce the overall particle size to one below which the specific activity of the sample was constant, was determined. Successive portions of the same protein sample were treated in the agate ball-mill for increasing periods of time, and the specific activities of the resulting powders were plotted against milling-time. The resultant graph, displayed in Figure 6, shows that a plateau, indicating a constant minimum particle size,

was reached after ten minutes. This time was employed in all subsequent preparations. The optimum amount of protein which should be added to each scintillator vial was found by counting increasing amounts of a protein sample which had been milled for ten minutes. Counting rate was then plotted against weight of protein counted, and Figure 7 shows that linear conditions obtain up to a sample weight of 10-12 mg. after which the count-rate of the protein is not proportional to the amount. All samples counted subsequently weighed less than 10 mg.

In view of the small quantities of sarcoplasmic protein sometimes obtained from hemidiaphragms, the reproducibility of the method in counting samples of this magnitude was tested. Three samples of the same preparation of sarcoplasmic protein, each weighing less than 2 mg., were separately milled, weighed and counted. Table 8 shows that the agreement obtained between the three values falls within an acceptable range of error.

Table 8

Weight of sarcoplasmic protein counted (mg)	Specific activity (counts/minute/milligram)
1.43	486
0.78	471
1.01	477

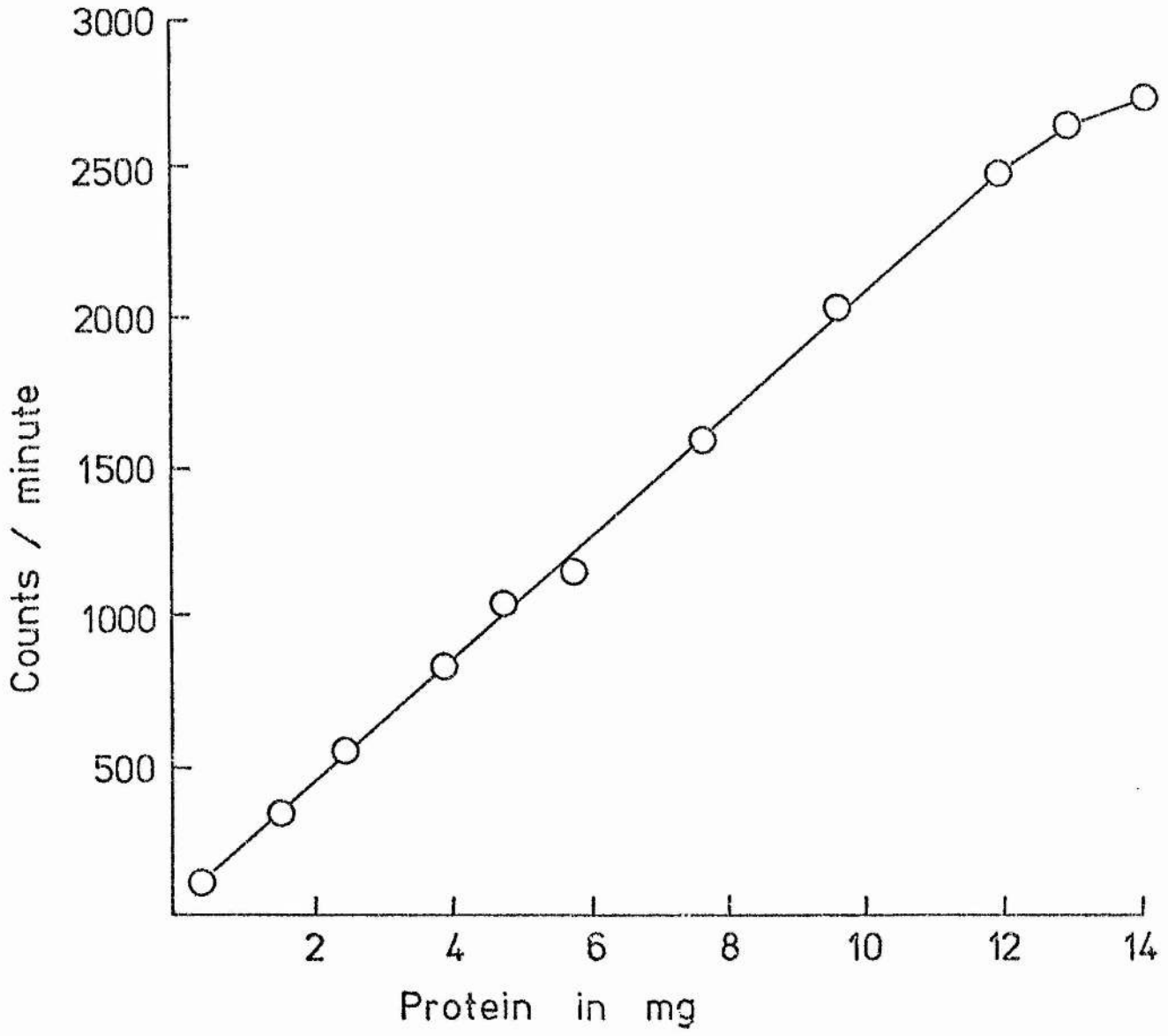


Figure 7. The counting rate of a protein sample, milled for 10 minutes, plotted against the weight of protein counted.

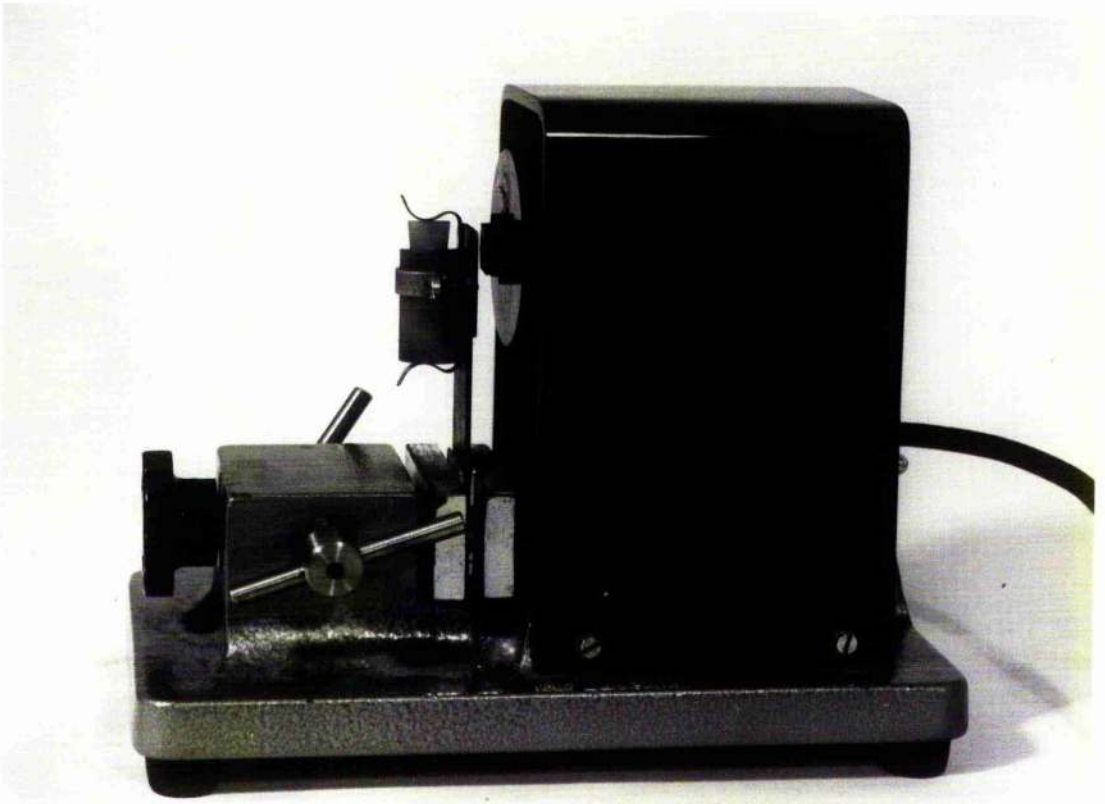


PLATE 3

The RIIC ball-mill used in the preparation of dried protein samples for scintillation counting.

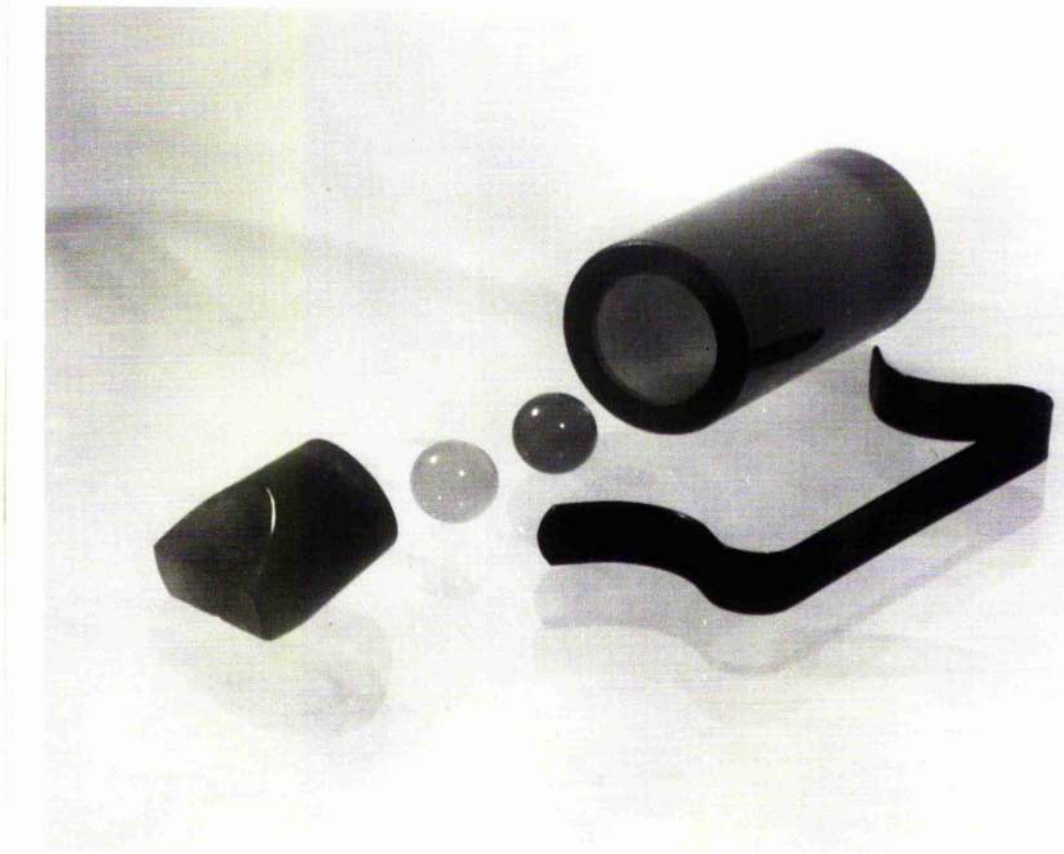


PLATE 4

The agate capsule and agate balls used in the preparation of dried protein samples for scintillation counting.

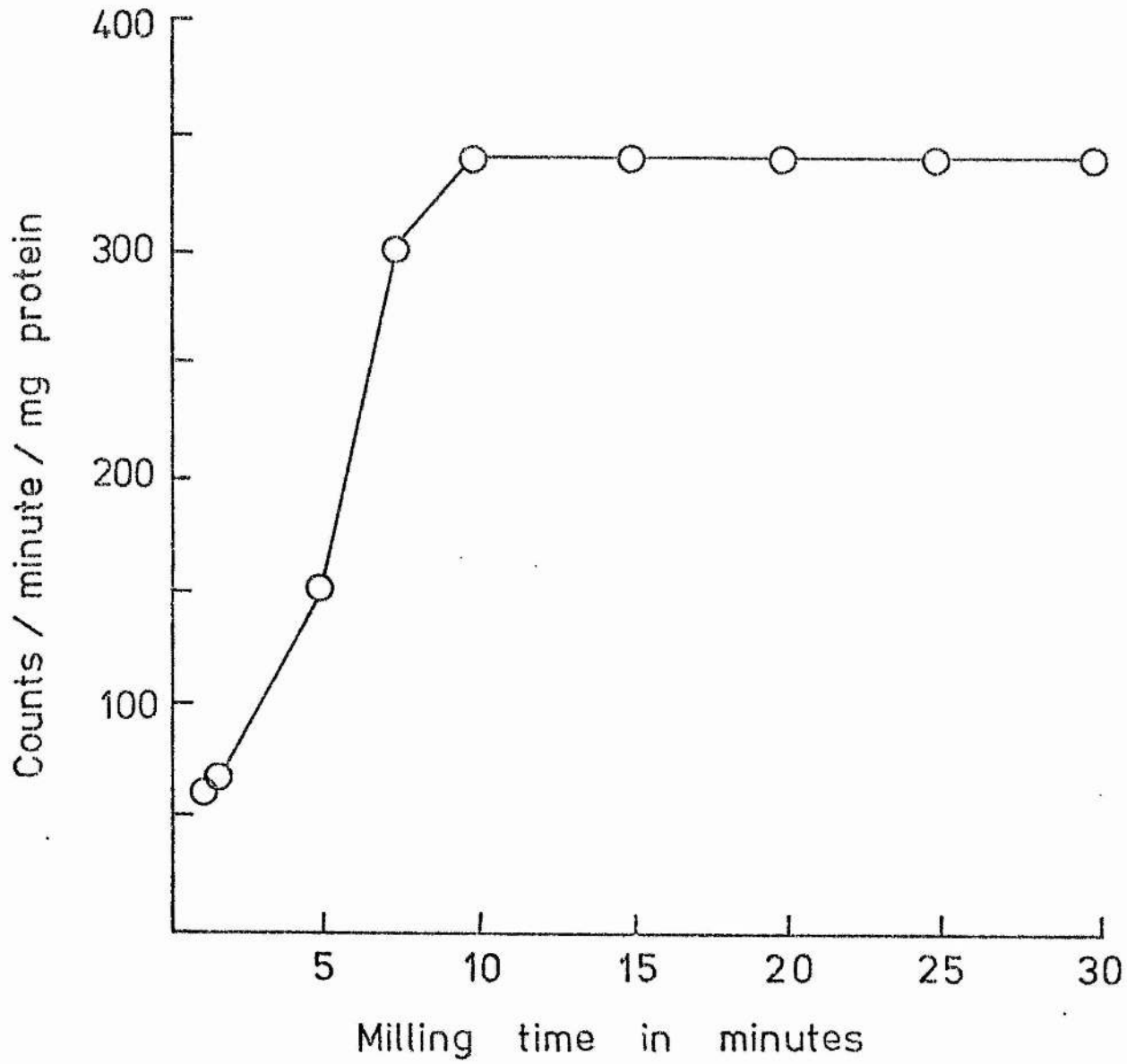


Figure 6. The specific activities of successive portions of the same protein sample, treated in the agate ball-mill for increasing periods of time.

In its final form, the procedure adopted was to mill each protein sample for ten minutes, after which the protein powder was equally divided between two pre-weighed empty scintillator vials. The vials were re-weighed on an Oertling R20 analytical balance, and the weight of the protein sample determined by subtraction, within an error range of ± 0.05 mg. 5 ml. NE 210 scintillator were measured into each vial, and after gassing with nitrogen, the vials were well-shaken and placed in a water-bath at 90°C for 20 minutes. After cooling for 1 hour, the outsides of the vials were carefully cleaned with acetone. Plate 5 shows the appearance of the scintillator before and after gelling. A vial of normal scintillator fluid is shown for comparison. The protein samples were counted using a Beckman LS 100 liquid scintillation counter, using the preset tritium channel, and a counting time of 20 minutes.

The absolute efficiency of the method was determined in the following manner. A weighed sample of milled protein was placed in a hydrolysis tube and hydrolysed in 1.5 ml. 6N HCl for 24 hours at 100°C. The HCl was subsequently removed by repeated evaporation under vacuum. The residue of amino-acids was taken up in 25 ml. distilled water, and 0.2 ml. aliquots were counted in NE 250 (Nuclear Enterprises, Edinburgh) scintillation cocktail, with and

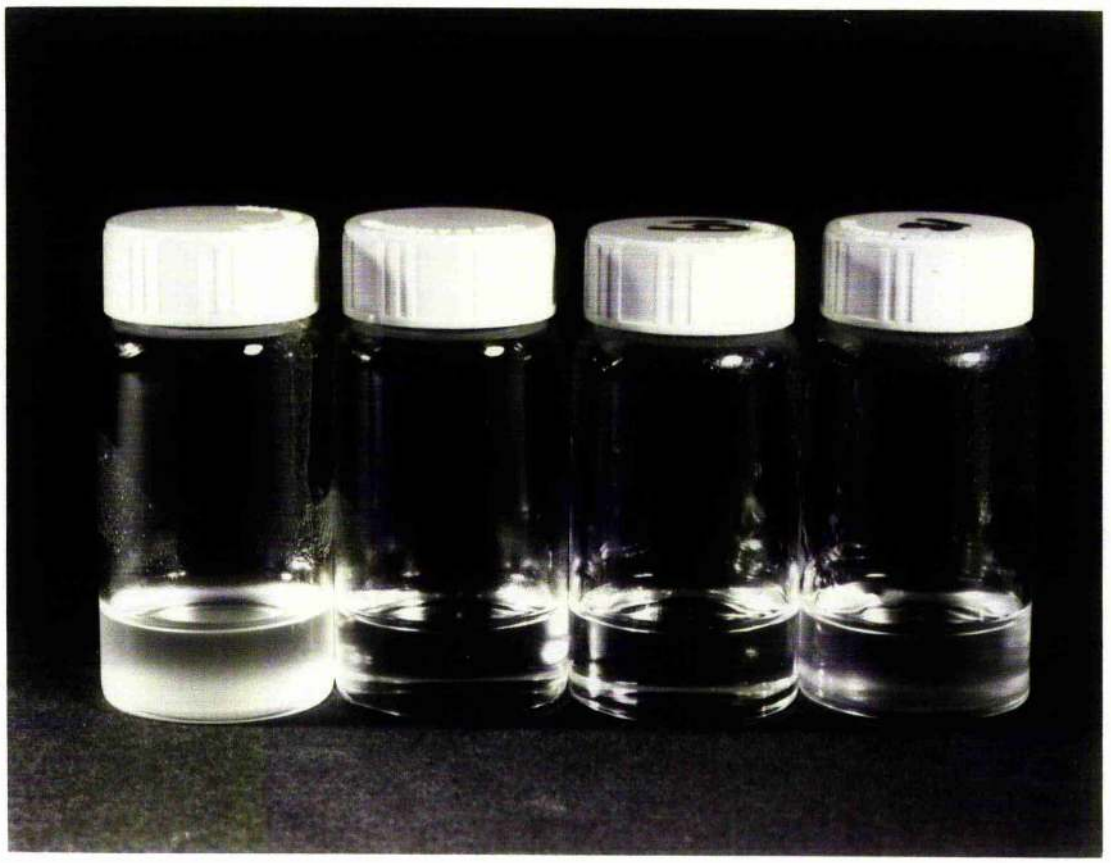


PLATE 5

The appearance of NE 210 scintillator showing, from left to right,

NE 210 scintillator before gelation
NE 210 after gelation
NE 210 liquid scintillator
10 mgs. protein suspended in a NE 210 scintillator gel.

without the addition of an internal standard of an accurate dilution of ^{3}H-lysine supplied by the Radiochemical Centre, Amersham. The inclusion of the internal standard allowed calculation of the efficiency with which the protein hydrolysate was being counted, and hence the calculation of the absolute specific activity of the original protein sample. A fresh sample of the same protein was now counted suspended in a gel as dedcribed above. Table 9 displays the results of these experiments.

Table 9

Scintill-ator	Internal standard added (dpm)	Protein added (mg)	cpm	dpm pro-tein	Effic-iency %
NE 250	11,100	0	3,167	0	28.53
NE 250	11,100	10.00	3,696	-	-
NE 250	0	10.00	1,214	5,430	22.36
NE 210	0	5.31	489	2,889	16.96

Conditions of incubation of diaphragm preparations

Krebs-Henseleit physiological buffer, pH 7.4 (Krebs and Henseleit, 1932) formed the basis of the incubation medium. The buffer was prepared as described in Section 1 (page 45).

^{3}H-(4,5)-lysine (Radiochemical Centre, Amersham) was added to the medium in sufficient amount to yield a final specific activity of 0.3 uCi/ml and the medium was divided into an appropriate number of aliquots. In the case of experiments involving the use of intact diaphragm preparations, 25 ml. of medium were added to each of a series of 50 ml. stoppered conical flasks, which were placed in a Gallenkamp metabolic shaker, and allowed to equilibrate to a temperature of 37°C. Freshly excised muscles were dropped into the preheated medium, the flasks were gassed out for 30 seconds with 95 : 5 (v/v) O_2 : CO_2, firmly stoppered and shaken at 110 cycles/minute, usually for 2 hours. In the case of the cut diaphragm preparations incubation was carried out in 5 ml. of medium. At the end of the incubation period, the muscles were removed from the medium, excised from the rib-cage if necessary, and carefully blotted. The tissues were frozen in liquid nitrogen before storage at -40°C. The muscles were immersed in liquid nitrogen no more than 30 seconds after being removed from the incubation medium.

The uptake of ^{3}H-lysine into total protein of diaphragm muscle from normal rats and rats bearing a Walker 256 carcinoma

The animals were prepared as described on page 43, and the intact diaphragms were excised. Diaphragms from control animals and tumour-bearing animals were incubated in Krebs-Henseleit medium containing ^{3}H-(4,5)-lysine at a specific activity of 0.3 uCi/ml. The incubation was stopped after 2 hours. The total protein was extracted from the frozen muscles by the method described on page 49, and the specific activities of the protein samples from the various muscles were determined.

The experiment was repeated using hemidiaphragms from the control and tumour-bearing animals. In this case the incubation medium was made 5 mM with respect to lysine, and the specific activity of the medium was raised to 3 uCi/ml.

Further experiments with hemidiaphragms were carried out where the incubation was supplemented with amino-acids in the amounts shown in Table 10 . These amino-acid concentrations are approximately double those found in the serum of normal rats (Scharff and Wool, 1964; Schimassek and Gerok, 1965; Clemens and Korner,1970). The activity of the medium was 3 uCi/ml.

Table 10.

Amino-acid	Concentration (uM/100 ml.)	Amino-acid	Concentration (uM/100 ml.)
Arginine	44	alanine	80
asparagine	8	valine	40
aspartic acid	8	methionine	14
threonine	58	isoleucine	19
serine	58	leucine	34
glutamic acid	38	tyrosine	18
glycine	43	phenylalanine	16
histidine	18	lysine	96
proline	48	cysteine	88
glutamine	38	tryptophan	14

The effect of incubation time upon the uptake of ^{3}H-lysine into protein of hemidiaphragms from normal rats and rats bearing a Walker 256 carcinoma

The animals were prepared as described on page . The hemidiaphragms were excised and incubated in medium containing ^{3}H-lysine at a specific activity of 0.3 uCi/ml. Hemidiaphragms from control and tumour-bearing animals were removed after ½-, 1-, 2- and 3-hour incubation periods. The muscles were frozen, and the sarcoplasmic and myofibrillar fractions were prepared, dried and counted.

The experiment was repeated upon a further group of control and tumour-bearing rats, the incubation in this case being carried for two hours with all muscles.

The uptake of ^{3}H-lysine into protein of hemidiaphragms from normal rats, incubated in serum from rats bearing a Walker 256 carcinoma

The animals were prepared as described on page 43 . The rats were anaesthetised by an intraperitoneal injection of 0.4 ml. 6% (w/v) sodium pentobarbitone (Abbott Laboratories Ltd., Kent). Blood was removed from the heart using a finely-drawn pasteur pipette and pooled until 30-40 ml. had been collected from both normal and tumour-bearing rats (usually 4 or 5 animals). The blood was allowed to clot for 1 hour, and the serum collected by centrifugation at 600g. for 30 minutes at 10°C. Sufficient ^{3}H-lysine was added to the serum to give an activity of 0.6 uCi/ml. serum. 1 ml. of serum was placed in each of a series of 10 ml. stoppered conical flasks, and equilibrated to 37°C in a Gallenkamp metabolic shaker. Further normal rats, which had been maintained under the same experimental conditions, were sacrificed, and the hemidiaphragms excised. One hemidiaphragm from each rat was placed in a conical flask containing serum from a normal rat, whilst the other was

placed in a flask containing serum from a tumour-bearing animal. The flasks were gassed for 30 seconds with a 95:5 (v/v) O_2:CO_2 mixture, tightly stoppered and incubated for two hours at 37°C. At the end of the incubation period, the muscles were removed, blotted and weighed. Sarcoplasmic and myofibrillar proteins were extracted from the muscles and counted.

The experiment was repeated, the serum being supplemented with amino-acids as described on page 80, and the activity of the serum being increased to 3 uCi/ml. serum.

The effect of insulin administered in vivo upon the uptake of ^{3}H-lysine into proteins of hemidiaphragms from normal rats and rats bearing a Walker 256 carcinoma

The animals were prepared as described on page 43. One hour before use, half of the rats were injected intraperitoneally with 0.5 ml. of an insulin solution, of activity of 2 units/ml. (Boots Pure Drug Co., Ltd., Nottingham). The remainder of the animals were given an injection of 0.9% (w/v) NaCl. The hemidiaphragms were excised and incubated in medium containing ^{3}H-lysine with a specific activity of 0.3 uCi/ml. for 2 hours. The protein fractions were prepared from the frozen muscles, dried and counted.

The effect of insulin administered in vitro upon the uptake of ^{3}H-lysine into proteins of hemidiaphragms from normal rats and rats bearing a Walker 256 carcinoma

The animals were prepared as described on page 43. The hemidiaphragms were excised and one hemidiaphragm from each animal was incubated in medium containing ^{3}H-lysine at a specific activity of 0.3 uCi/ml., whilst the other hemidiaphragm was incubated in a similar medium with the addition of 0.1 units/ml. soluble insulin. The incubation period was 2 hours, at the end of which time the muscles were frozen, and the fractions extracted and counted.

RESULTS and DISCUSSION

The uptake of ^{3}H-lysine into total protein of diaphragm muscle from normal rats and rats bearing a Walker 256 carcinoma

Table 11 shows that incorporation of ^{3}H-lysine into the total intracellular protein of diaphragm muscle from tumour-bearing rats was significantly decreased.

Table 11 - The uptake of ^{3}H-lysine into protein of intact diaphragms from normal and tumour-bearing rats

	counts/minute/mg protein	P**
Control	501 ± 11*	-
Tumour-bearing	254 ± 8	< 0.01

* Results are expressed as the mean ± standard error of 5 rats.

** In this and following tables in this section, P refers to the statistical significance of the differences between the values obtained for the control and tumour-bearing rats.

The specific activity of the medium was 0.3 uCi/ml., and the incubation time was 2 hours.

These results suggest that the tumour is exerting an inhibitory effect upon the incorporation of ^{3}H-lysine into muscle protein. This decrease in uptake could be due to a decrease in the specific activity of lysine in the case of the preparation from the tumour-bearing animal due to dilution by unlabelled lysine arising from an increased catabolism of muscle protein. An attempt was therefore made to eliminate this possibility by increasing the lysine concentration of the medium to 5mM, which is approximately 10 times the amount found in normal rat serum. Table 12 shows that there is still a significant decrease in incorporation under these conditions.

Table 12 - The effect of an increase in lysine content of the incubation medium upon the uptake of ^{3}H-lysine into protein of hemidiaphragm from normal and tumour-bearing rats.

	counts/min./mg. protein	P
Control	972 ± 22*	-
Tumour-bearing	555 ± 26	< 0.01

* Results are expressed as the mean ± standard error of 5 rats. The specific activity of the medium was 3 uCi/ml., the lysine concentration 5mM, and the incubation time 2 hours.

It was possible that the decrease evident in the muscle from tumour-bearing animals (Table 11) could be due to a lack of specific amino-acids, other than lysine, essential for continuing protein synthesis. This was tested for by incubating the muscles in a medium containing all of the amino-acids normally present in serum at twice the levels found in normal serum, and measuring the incorporation of ^{3}H-lysine into the total intracellular protein. Table 13 shows that the protein in the muscle from the tumour-bearing animal took up significantly less labelled lysine than did the control muscles, under these conditions of incubation, demonstrating that the inhibition observed (Tables 11,12,13) was not due to any lack of amino-acids in the tumour-bearing animal.

Table 13 - The effect of an amino-acid mixture upon the uptake of ^{3}H-lysine into protein from diaphragm muscle from normal and tumour-bearing rats.

	Counts/min./mg.protein	P
Control	962 ± 45*	-
Tumour-bearing	452 ± 14	< 0.01

* Results are expressed as the mean standard error of 5 rats. The specific activity of the medium was 3.0 uCi/ml. and the incubation time was 2 hours.

The effect of incubation time upon the uptake of ^{3}H-lysine into proteins of hemidiaphragms from normal rats and rats bearing a Walker 256 carcinoma

The results of this experiment are displayed graphically in Figures 8 and 9. Each point in these graphs represents the mean of the data from 5 rats. It can be seen that whereas the myofibrillar protein fraction exhibits the decrease in incorporation of isotope seen in the total protein experiments (Table 11), the sarcoplasmic proteins are virtually unaffected by the growth of the tumour. This experiment was designed to ascertain whether or not the incorporation of labelled amino-acid into the protein fractions from healthy and diseased muscles was proportional to time. That this should be so is important to the interpretation of the data on the relative incorporations. The possibility existed with such an *in vitro* system, that when the diaphragm was removed from the tumour-bearing animal, and hence from the direct effects of the tumour, the effect of the tumour on the protein metabolism might have begun to revert to normal. The proportionality of the uptake of ^{3}H-lysine with increasing time suggests that this is not the case, and that the system studied was truly exhibiting effects due solely to the growth of the tumour in the host animal.

On repetition of the experiment with a single incubation time of 2 hours, it was confirmed that the sarcoplasmic proteins of the muscle from the tumour-bearing animal exhibit

a normal uptake, whilst the myofibrillar fraction shows a greatly depressed level of radioactivity (Table 14).

Table 14 - Effect of the Walker 256 carcinoma on the uptake of ^{3}H-lysine into myofibrillar and sarcoplasmic proteins of rat diaphragm.

	Counts/minute/mg.protein		P
	sarcoplasmic	myofibrillar	
Control	466 ± 27*	509 ± 15	-
Tumour-bearing	458 ± 13	176 ± 14	<0.01

* Results are expressed as the mean ± standard error of 6 rats. The specific activity of the medium was 0.3 uCi/ml. and the incubation period was 2 hours.

Clark and Goodlad (1971) showed that while incorporation of ^{14}C-valine into both the myofibrillar and sarcoplasmic proteins of rat gastrocnemius muscle, essentially composed of white fibres, was significantly decreased in tumour-bearing animals, the proteins of the soleus muscle were relatively unaffected. The amino-acid incorporation into the medial head of the gastrocnemius muscle, which, like the soleus, is composed of red fibres, was however decreased. This suggestion that red muscle fibres vary in their ability to resist the action of the tumour is supported by the present

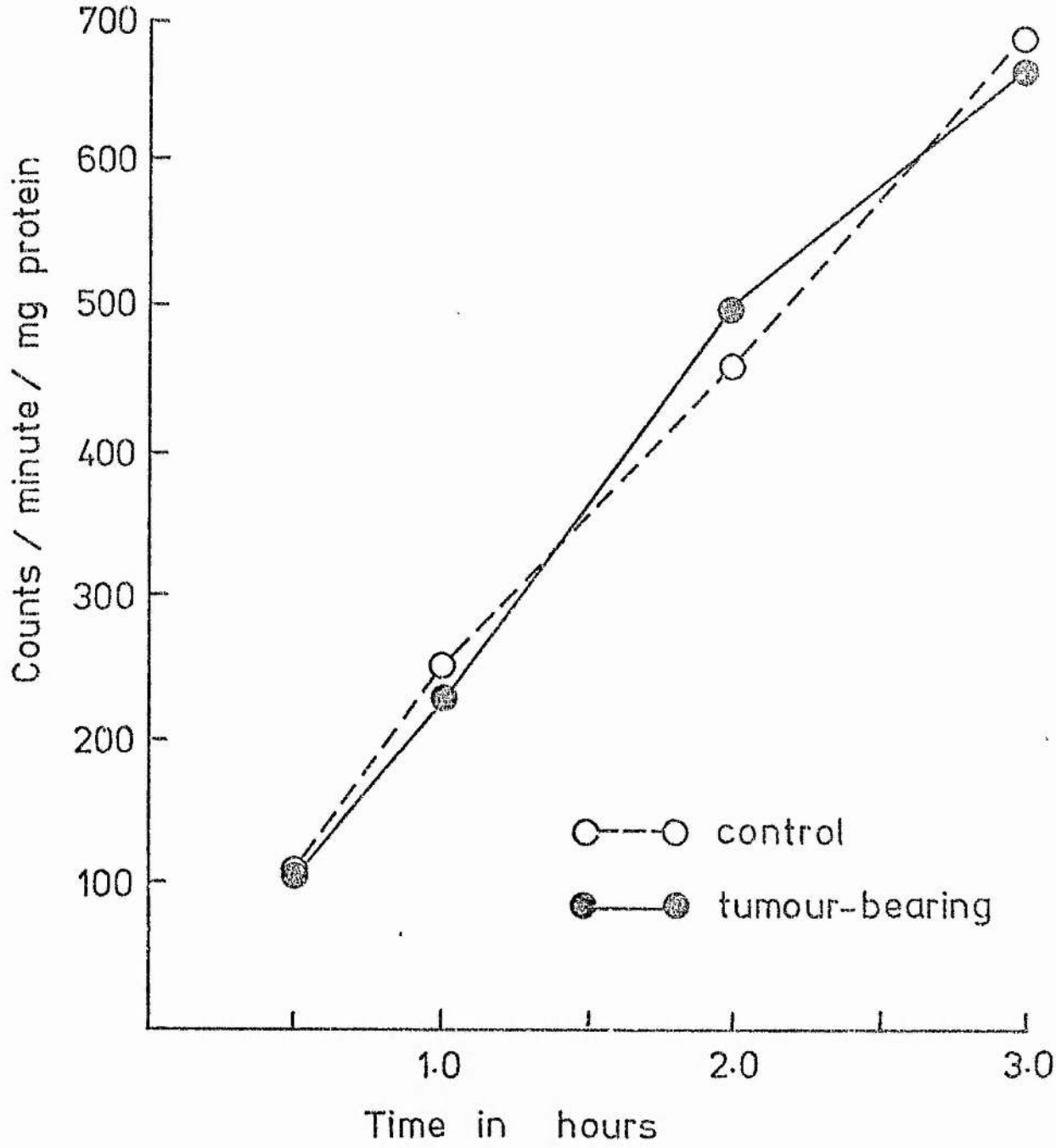


Figure 8. The uptake of ^{3}H-lysine into the sarcoplasmic proteins of diaphragm muscle from normal and tumour-bearing animals. The specific radioactivity of the medium was 0.3 uCi/ml.

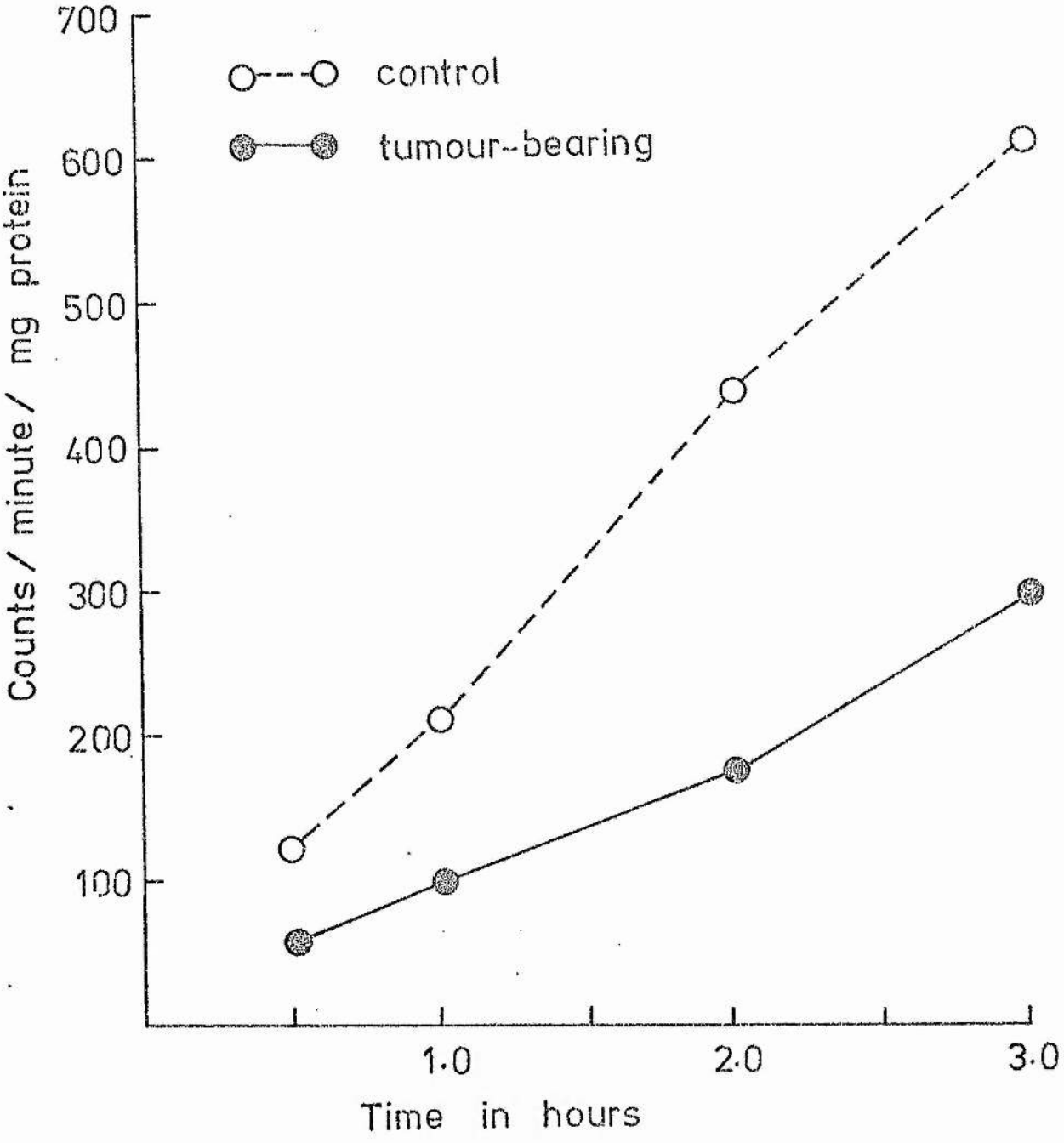


Figure 9. The uptake of ^{3}H-lysine into the myofibrillar proteins of diaphragm muscle from normal and tumour-bearing rats. The specific activity of the medium was 0.3 uCi/ml.

results, which show that although the sarcoplasmic proteins are unaffected, amino-acid incorporation into the myofibrillar protein fraction is considerably decreased in diaphragm muscle from the tumour-bearing animal.

The differential effect upon the contractile and sarcoplasmic protein must be attributed to some form of compartmentation within the muscle cell, either physical or chemical. The results could be explained in terms of separate amino-acid pools for the synthesis of myofibrillar and sarcoplasmic proteins. Velick (1956) interpreted his results on differential labelling of various muscle proteins to mean that H-meromyosin, actin and the glycolytic enzymes are synthesized from the same pool of protein precursors, while L-meromyosin is formed from a separate pool. Kipnis et al (1961) investigated the kinetics of tracer amino-acid appearance in the intracellular pool and in cellular protein under steady state conditions in the intact rat diaphragm preparation. The concentration ratio of tracer amino-acid between the extracellular and intracellular amino-acid pools approached equilibrium exponentially, whereas incorporation into cellular protein proceeded linearly from the earliest time studied. These workers presented a theoretical formulation predicting the kinetics of amino-acid incorporation into cellular protein if the total intracellular amino-acid pool were an obligatory

intermediate in protein synthesis. The marked deviation of experimental observations from those theoretically predicted was taken to indicate a functional heterogeneity of the intracellular amino-acid pool. Hider et al. (1969, 1971) claimed to have identified a discrete extracellular pool of amino-acids, with a different composition from the intracellular pool. Using high specific activities of tracer amino-acid, and a double-labelling technique, they detected a lag period before linear incorporation of ^{14}C-glycine into muscle protein, which they interpreted as showing that the extracellular pool is the direct source of amino-acids for protein synthesis. Thus there is considerable evidence to support the view that compartmentation exists in muscle cells, and that it may be responsible for differing rates of synthesis in different proteins.

It has been shown above that the effect of the tumour in decreasing the uptake of amino-acids into muscle protein is not due to a deficiency of amino-acids, but the effect could be due to an inhibition of the transport of amino-acids from the medium to the pool responsible for protein synthesis. Furthermore, it cannot be assumed that alteration in the rate of incorporation of labelled amino-acid into protein is necessarily due to an alteration in the rate of synthesis of that protein. Simon et al. (1962) have stated that the

relationship between the specific activity of a protein and the rate of protein synthesis is a direct one only provided that the specific activity of the tracer amino-acid in the free amino-acid pool responsible for protein synthesis is the same in all muscles under consideration, and that the level of tracer in the pool is also constant. If the tumour exerts any effect on amino-acid transport in the muscle, then these conditions would not be fulfilled. Thus the observed effect of the tumour could be due to an effect on the transport of the amino-acid.

Gan and Jeffay (1967) calculated that under steady state conditions, 30% of the lysine incorporated into rat muscle protein was derived directly from protein catabolism. In the present case, therefore, if the depleting effect of the tumour was mediated by increasing the rate of protein catabolism in muscle, the net result would be dilution of the tracer amino-acid in the free amino-acid pool. This would lead to a decreased incorporation of the tracer into protein even although the absolute rate of protein synthesis may be unaltered.

Clark and Goodlad (1971) did show that the ratio of RNA/DNA in gastrocnemius muscle from tumour-bearing animals was decreased, and suggested that this might indicate that the action of the tumour was directed at protein synthesis. However, until more data has been obtained on the nature of

the amino-acid pools responsible for the provision of precursors for protein synthesis in muscle, and the effect of the tumour growth upon these pools has been elucidated, it will be impossible to define the site of action of the tumour upon the muscle.

The uptake of ^{3}H-lysine into protein of hemidiaphragms from normal rats, incubated in serum from rats bearing a Walker 256 carcinoma

Table 15 shows that the uptake of ^{3}H-lysine into myofibrillar but not into sarcoplasmic proteins by isolated hemidiaphragms from normal rats is decreased when they are incubated in serum from a tumour-bearing rat compared to the activity they exhibit when incubated in serum from a normal rat.

Table 15 - The uptake of ^{3}H-lysine into protein of hemi-diaphragms from normal rats, incubated in serum from normal or tumour-bearing rats.

	Counts/minute/mg.protein	
	sarcoplasmic	myofibrillar
Control serum	82.3 ± 7.2*	77.0 ± 5.2
Tumour-bearing serum	93.5 ± 4.4	54.1 ± 1.9
P	n.s.	< 0.01

* Results are expressed as the mean ± standard error of 6 rats The specific activity of the serum was 0.6 uCi/ml. and the incubation time was 2 hours.

Again there is a possibility that this effect is due to an amino-acid abnormality in the serum of the tumour-bearing animals. El Mehairy (1950) found an increase in α-amino-nitrogen in the blood of tumour-bearing mice, and Wu and Bauer (1960) using a semi-quantitative assay method. concluded that the free amino-acids in the plasma of rats bearing large tumours were increased at the expense of muscle proteins as the wasting process progressed during tumour growth.

A higher level of amino-acids in the serum of tumour-bearing animals would serve to dilute the added isotope and consequently would result in a decreased incorporation of

tracer into the muscle protein. Equally, should there be a specific or non-specific deficiency of amino-acids in the serum, protein synthesis would be limited, leading to a decreased incorporation. However, although this could be a significant factor in vivo, it would be unlikely that such a decrease would be evident in the present design of experiment, since the normal muscle would have sufficient amino-acids present to function normally, even with a deficiency in its incubation medium.

In an attempt to eliminate possible alterations in serum amino-acid levels due to the tumour as the causative effect of observed results, the serum from normal and tumour-bearing animals was 'flooded' with an artificial mixture of the naturally occurring amino-acids, in sufficient amounts to raise the level of the amino-acids to at least three times the concentrations found in normal circulating rat serum. By this means it was hoped that the effect of any disparity in the amino-nitrogen concentrations would be reduced. In fact, Table 16 shows that the decrease in incorporation is still very apparent, so that it would appear that amino-acid concentration is not the cause of this inhibition of incorporation.

Table 16. - The effect of the addition of an amino-acid mixture on the uptake of ^{3}H-lysine by normal rat diaphragm muscle, incubated in serum from normal and tumour-bearing rats.

	counts/minute/mg.protein	P
Control rat serum	289 ± 12*	-
Tumour-bearing rat serum	176 ± 15	< 0.01

* Results are expressed as the mean ± standard error of 5 rats.

The effect of insulin upon the incorporation of ^{3}H-lysine into proteins of diaphragm muscle from normal rats and rats bearing a Walker 256 carcinoma

Normal blood plasma or serum stimulates both the uptake of glucose by the isolated rat diaphragm (Green et al., 1952) and the incorporation of ^{14}C-glycine into the protein of the diaphragm (Manchester and Young, 1959). This enhancement is abolished by the addition of guinea-pig antiserum to ox insulin to the incubation medium (Manchester and Young, 1959). This indicates that the enhancement noted on the addition of serum is due to the presence of a resting level of insulin in plasma and serum. Manchester and Young (1959) demonstrated that insulin was stimulatory to glucose uptake and protein synthesis upon addition to the incubation medium at levels as low as 0.5 milliunits/ml. Goodlad and Mitchell (unpublished results) showed that the average level of insulin in the plasma of tumour-bearing rats, maintained under the same dietary conditions as those described on page 43 , was reduced to 11.3 microunits/ml., as opposed to the control value of 16.5 microunits/ml. It is possible therefore that the decreased incorporation observed in the muscles incubated in the serum of tumour-oearing rats (Table 15) could be due to a deficiency of insulin in the serum.

In an attempt to obtain further information on this point some experiments were carried out to study the effect of an injection of insulin on the *in vitro* amino-acid incorporation by sarcoplasmic and contractile proteins of the diaphragm of tumour-bearing rats. Kurihara and Wool (1968) found that *in vitro* incorporation of labelled amino-acids into both sarcoplasmic and myofibrillar protein fractions of hemidiaphragm of diabetic rats was reduced by 50.3% and 65.7% respectively. If the diabetic rats were given insulin intraperitoneally 1 hour before they were killed the *in vitro* incorporation of amino-acid into both these fractions was restored to normal levels.

Tables 17 and 18 show the effect of an injection of 1 unit insulin on amino-acid uptake into the two muscle protein fractions of diaphragms of normal and tumour-bearing rats.

Table 17 - The effect of insulin administered in vivo upon the incorporation of ^{3}H-lysine into sarcoplasmic protein of diaphragm muscle from normal rats and tumour-bearing rats.

Insulin addition	counts/minute/mg.protein		P_1 **
	control	tumour-bearing	
-	431 ± 8 *	489 ± 22	< 0.05
+	476 ± 32	543 ± 22	n.s.
P_2 **	n.s.	n.s.	

* Results are expressed as the mean ± standard error of 5 rats.

** In this and following tables, P_1 refers to the statistical significance of the difference between control and tumour-bearing animals, while P_2 refers to the statistical difference between insulin-treated muscles and non-insulin-treated muscles.

The specific activity of the medium was 0.3 uCi/ml. and the incubation time was 2 hours. 1u of insulin was injected intraperitoneally into the rats 1 hour before they were killed.

Table 18 - The effect of insulin administered *in vivo* upon the incorporation of ^{3}H-lysine into myofibrillar protein from diaphragm muscle from normal rats and from tumour-bearing rats.

Insulin addition	counts/minute/mg.protein		P_1
	control	tumour-bearing	
-	430 ± 12 *	154 ± 16	< 0.01
+	621 ± 32	247 ± 30	< 0.01
P_2	< 0.01	< 0.05	

* Results are expressed as the mean ± standard error of 5 rats.

The data obtained for sarcoplasmic proteins and myofibrillar proteins was statistically treated according to the analysis of variance method (Colquhoun, 1971). The computer program written to accomplish this analysis is given in the Appendix. Table 19 shows the results of the analysis of variance. Comparison of the significance calculated by this method, and the significance calculated by Student's t-test, (Tables 17,18), enabled the following conclusions to be drawn from the experiment.

Table 19 - Analysis of variance of the in vivo effect of insulin on the incorporation of ^{3}H-lysine into diaphragm muscle protein fractions from normal and from tumour-bearing rats.

Source of variation	Degrees of freedom	F-value	P
Replicates	4	0.70	n.s.
Tumour-bearing - control	1	68.74	< 0.01
Insulin - saline	1	29.14	< 0.01
Muscle fraction	1	64.53	< 0.01
Interaction between tumour and muscle fraction	1	131.90	< 0.01
Interaction between tumour and insulin	1	0.15	n.s.
Interaction between insulin and muscle fraction	1	8.39	< 0.05
3-factor interaction	1	0.37	n.s.
Residual	28	-	-

The tumour, as has already been shown, has a greater effect upon the myofibrillar fraction than on the sarcoplasmic fraction of the muscle. Insulin also exerted a significantly greater effect upon the myofibrillar fraction of the muscle protein. The effect of insulin was not significantly different in the tumour-bearing animal than in the normal animal.

With the dose of insulin used in the present work, although the hormone tended to increase incorporation into the myofibrillar fraction, the extent of this increase was far from being of sufficient magnitude to restore the incorporation in the normal animal. The effect of insulin on protein synthesis in tumour-bearing animals is not therefore as marked as it is in the diabetic animal.

Upon testing the effect of insulin by in vitro administration, the data shown in Tables 20, 21, 22, were obtained.

Table 20 - The effect of insulin administered in vitro upon the uptake of ^{3}H-lysine into sarcoplasmic protein from diaphragm muscle of normal rats and tumour-bearing rats.

Insulin addition	counts/minute/mg.protein		P_1
	control	tumour-bearing	
-	463 ± 27 *	492 ± 18	n.s.
+	479 ± 18	571 ± 20	< 0.02
P_2	n.s.	< 0.02	

* Results are expressed as the mean ± standard error of 7 rats.

Table 21 - The effect of insulin administered *in vitro* upon the uptake of ^{3}H-lysine into myofibrillar proteins from diaphragm muscle of normal rats and tumour-bearing rats.

Insulin addition	counts/minute/mg.protein		P_1
	control	tumour-bearing	
-	447 ± 21*	175 ± 16	< 0.01
+	617 ± 10	187 ± 18	< 0.01
P_2	< 0.01	n.s.	

* Results are expressed as the mean ± standard error of 7 rats.

Table 22 - The analysis of variance of the effect of insulin *in vitro* on the incorporation of ^{3}H-lysine into diaphragm muscle protein fractions from normal and tumour-bearing animals.

Source of variation	Degrees of freedom	F-value	P
Replicates	6	1.30	n.s.
Tumour-bearing - control	1	143.46	< 0.01
Insulin - saline	1	20.80	< 0.01
Muscle fraction	1	147.37	< 0.01
Interaction between tumour - insulin	1	2.07	n.s.
Interaction between insulin - muscle fraction	1	2.21	n.s.
Interaction between tumour - muscle fraction	1	251.93	< 0.01
3-factor interaction	1	11.90	< 0.01
Residual	42	-	-

Examination of the data in Tables 20, 21 and 22 confirms that the effect of insulin is markedly reduced in the muscles from tumour-bearing animals. The tumour exerts its usual effect upon the myofibrillar fraction of the muscle protein, and the *in vitro* addition of insulin does not enhance the incorporation of label into this fraction in the tumour-bearing animal, as it does in the control. Throughout the experiments reported in this section, the sarcoplasmic fraction of tumour-bearing muscle tended to show a slight, but not significant, increase in incorporation, compared with the control. *In vitro* addition of insulin magnified this effect to the extent that a significant difference was noted ($P < 0.02$) (Table 20).

In no case was the level of incorporation into the myofibrillar fraction of protein from diaphragm muscle from a tumour-bearing animal restored to its normal level in the control animal by the action of insulin. This, together with the fact that the muscle from the tumour-bearing animal retains to a small degree the ability to respond to the stimulating action of insulin, indicates that the depression of the level of incorporation of tracer amino-acid into protein from the muscle of tumour-bearing animals is not due exclusively to a deficiency either in insulin level or in the mechanism of action of insulin on the muscle.

SECTION 3

The preparation, purification and bioassay of a toxohormone fraction from the Walker 256 carcinoma.

The effect of toxohormone upon the uptake of ^{3}H-lysine into proteins of diaphragms from normal rats.

INTRODUCTION

The concept of a toxin secreted by the tumour, producing the biochemical injuries characteristic of cancer-bearing hosts, has already been discussed (page 23). In the experiments described in this section, a toxohormone fraction was prepared and purified, and its influence upon the uptake of a labelled amino-acid into rat-diaphragm muscle protein was investigated.

The observation that liver catalase activity is markedly reduced in cancer patients and in tumour-bearing animals is of long-standing (Blumenthal and Brahn, 1910; Rosenthal, 1912; Brahn, 1914, 1916). Various methods were tried in the attempt to isolate a hypothetical liver catalase reducing factor from cancerous tissues in a sufficiently concentrated state to be measured in a biological assay system.

A polysaccharide fraction was isolated from tumour tissue by extraction with dilute trichloracetic acid, and a lipid fraction was obtained by extraction with ether. Both of these extracts were highly toxic to animals into which they were injected, but they did not possess any liver catalase depressing activity (Nakahara and Fukuoka, 1961).

A fraction was obtained by extracting various human malignant tissues with hot water, followed by ethanol precipitation (Nakahara and Fukuoka, 1948). This fraction

proved to be active in the depression of mouse liver catalase upon intraperitoneal injection in doses of 100 mg. and more (Nakahara and Fukuoka, 1948, 1949, 1950). Toxohormone fractions isolated by this method were proteins, but contained nucleic acids, often amounting to as much as 30% by weight. There are conflicting reports on the activity of the nucleic acid fraction; Nagagawa et al. (1955) separated a crude toxohormone fraction into protein and nucleic acid components. The fractionation was accomplished by treatment with hot trichloracetic acid, which removed the nucleic acids, leaving the protein component. The nucleic acid fraction was prepared by alcohol precipitation of the supernatant from the hot trichloracetic acid treatment. Nagagawa et al. (1955) claimed that of these two fractions, the nucleic acid was the more active in causing a depression of liver catalase activity. It would seem likely, however, that hot trichloracetic acid treatment would degrade the nucleic acids originally present, leaving these results open to some doubt. An active toxohormone fraction was isolated from human gastric carcinoma which showed a maximal absorption at 260 nm and contained no free amino-groups (Kuzin et al., 1955), although this fraction was not positively identified as nucleic acid.

On the other hand, Nakahara and Fukuoka (1949) found no activity in a 'nucleoprotein' component of their toxohormone

preparation. Endo (1954) isolated nucleic acids from tumour tissue by the method of Clark and Schryver, and found them to be without toxohormone activity.

It now seems probable that toxohormone is a polypeptide, and the fact that nucleic acid seems to be closely adherent to many toxohormone preparations suggests that toxohormone may be a basic polypeptide. This view is substantiated by the findings that methanol-acetic acid and picric acid extracts of tumours containing polypeptide material were active in depressing liver catalase activity (Ono et al. 1955; Fujii et al., 1960). These basic protein preparations were more active than any previously described, producing highly significant depression of liver catalase activity in doses of between 5 and 10 mg.

Various attempts have been made to further purify these crude fractions. Trojanowski et al. (1969) attempted to purify material prepared according to Nakahara's original method (Nakahara and Fukuoka, 1948), using ammonium sulphate precipitation followed by Sephadex gel filtration, and obtained five fractions, the most active of which depressed the level of mouse liver catalase in doses of 100 ug. This level of purification was not as good as that achieved earlier by Yunoki and Griffin (1960, 1961) using cation-exchange chromatography, and Nixon and Zinman (1966) using anion-exchange chromatography

on DEAE-cellulose. These workers obtained preparations active at doses of 10 ug. per mouse.

Toxohormone production appears to be a general property of cancer cells. There has been no report of a malignant tumour, whether spontaneous, transplanted or chemically induced, which did not yield active toxohormone. Greenfield and Meister (1951) isolated an active toxohormone fraction from the Walker carcinosarcoma, which, upon injection in doses of 50 mg., reduced the level of mouse liver catalase. The methods used in the present work for the isolation of toxohormone were based upon those of Yunoki and Griffin (1960). Like the method of Ono et al. (1955), this procedure was designed to extract a basic polypeptide fraction from the tumour tissue. The technique was originally designed for the isolation of adrenocorticotrophic hormone from pituitary tissue by Payne et al. (1950). This particular method was chosen because when it had been applied to human malignant tissue, the toxohormone isolated was very active (Yunoki and Griffin, 1960). The toxohormone fraction isolated and purified by this technique was shown to contain 20% lipid (Yunoki and Griffin, 1961), but an absolute requirement of lipid for toxohormone activity was not demonstrated.

A great deal of the research into toxohormone has been devoted to establishing that it is a product of living tumour

cells, and that it is not related either to the products of tissue necrosis or to bacterial contamination of the tumour. Nakahara and Fukuoka (1949) showed that necrotic and non-necrotic portions of cancerous tissue do not differ significantly with respect to the yield or potency of toxohormone. The possible involvement of bacteria in causing toxohormone production was suggested by Kampschmidt et al. (1963) who claimed that contaminating Salmonella typhimmurium was the most potent source of toxohormone in tumour tissue. Nakahara and Fukuoka (1949) divided a sample of tumour tissue into two portions without aseptic precautions, isolated the toxohormone fraction from one portion immediately, and from the other portion after incubation overnight at 37°C. Similar amounts of toxohormone of approximately the same activity were obtained in both cases, and Nakahara and Fukuoka interpreted these results to mean that not only is toxohormone unrelated to the tissue decomposition, but also that the amount of micro-organisms which may be contained in tumour tissue cannot be of material importance.

Further evidence against micro-organisms present in the tumour being the main source of toxohormone was presented by Matsuoko et al (1964) and Nakahara et al. (1966). The former group of workers found that the addition of 0.1% of Salmonella typhimmurium to 55 g. of sterile sarcoma tissue had

little effect on the amount of potency of toxohormone isolated from the tumour. The amount of bacteria they added was in fact approximately 100 times that obtained from 1 ml of pure culture under conditions of maximal growth. The latter group showed that bacteria-free tumours transplanted and maintained in germ-free mice were as rich a source of toxohormone as tumours obtained from conventional mice. It is also probable that viruses have no ability to produce or stimulate the production of toxohormone in the neoplastic cell (Ohashi, 1961).

Although these experiments would appear to preclude the possibility that toxohormone production in tumours is entirely due to bacterial activity, there is evidence that toxohormone-like materials may be isolated from bacteria or bacterial cultures. Thus Matsuoko et al. (1964) found that 1g of S. typhimurium yielded 33mg of such a factor, and Kampschmidt et al. (1963) isolated and purified lipo-polysaccharide endotoxins from several bacteria, which were shown to cause marked depression of liver catalase activity when injected into rats at a level of 100ug per 200g body weight. A great deal of the confusion with regard to the role of bacterial contamination in being a prime factor in the effect of the tumour on its host is undoubtedly due to the concentration of workers on the phenomenon of liver catalase depression. The Japanese workers have defined

toxohormone as a substance present in high concentrations in tumours, which possesses this property (Nakahara and Fukuoka, 1948). The relevance of liver catalase depression to the cachectic effect of the tumour remains obscure. Some tumours cause very little depression of the activity of the enzyme (Kampschmidt et al., 1963) and some tumours contain a high level of catalase activity though they are rich sources of toxohormone (Rechcigl and Sidransky, 1962). In contrast to liver catalase depression, other systemic effects of the tumour on its host appear to be independent of whether the tumour is contaminated by bacteria or not. Thus Kampschmidt and Upchurch (1963) found that liver, spleen and adrenal weight are increased and thymus weight decreased in rats bearing either a bacteria-free Walker tumour or one contaminated by Salmonella typhimurium. Bacterial endotoxins have, however, been shown to have the property of lowering plasma iron levels (Kampschmidt and Schultz, 1961; Kampschmidt and Upchurch, 1962).

The methods available for the assay of liver catalase activity are all based on the method of von Euler and Josephson (1927), in which the enzyme reaction is carried out at 0°C in a buffer containing hydrogen peroxide. The extent of the reaction is then estimated by measurement of the remaining hydrogen peroxide, either spectrophotometrically

(Chantranne, 1955), titrimetrically using $KMnO_4$, or by measurement of the evolved oxygen (Greenstein, 1942).

Although the measurement of the depression of liver catalase activity has been the most widely used method for the bioassay of toxohormone (Nakahara and Fukuoka, 1961; Nakahara, 1967), several of the other reported effects of toxohormone, listed in Table 23, have been employed. In particular, the depression of the level of plasma-bound iron has been found to give a rapid and sensitive measurement of the activity of toxohormone fractions (Kampschmidt et al., 1959). These workers showed that the plasma bound iron level was 250-500 times as sensitive to toxohormone as liver catalase and that the maximum effect was noted after 10 hours, as opposed to 24 hours for the liver catalase effect. The draw-back to the use of this technique on a routine basis was that the methods available for the estimation of plasma iron were very tedious and potentially more inaccurate than those described for liver catalase.

Iron is carried in the plasma linked to the protein transferrin, and dissociation of the iron-protein linkage is a necessary first step in any method for the determination of plasma iron. Most of the early methods accomplished this by means of dilute acid hydrolysis and precipitation of the plasma proteins (Kitzes et al., 1944; Kaldor, 1953; Schade et al., 1954; Trinder, 1956). Barkan and Walker (1940)

accomplished the dissociation by reducing the pH of the plasma to about 6 with a concentrated buffer, so that the plasma proteins stayed in solution. Schade et al. (1949) showed that all the iron is not released from the protein complex at this pH, and it is unlikely that all the iron could be released by this method without causing precipitation of the serum proteins.

Sanford (1963) introduced a method for the estimation of plasma iron based on previous work by Webster (1960), who used a detergent in the measurement of iron-dextran in blood after intravenous injection. Sanford (1963) found that this detergent (Teepol 610) effectively liberated iron from its plasma-protein complex. This release was achieved by the combined action of Teepol and ascorbic acid. Teepol alone slowly liberated the iron, and the addition of the ascorbic acid, with consequent lowering of the pH value, accelerated liberation. The iron is completely released at pH 6.3. Teepol also acts as an anionic detergent, and prevents precipitation of the plasma proteins. The released iron is complexed with bathophenanthroline (Trinder, 1956) after reduction to the ferrous state by ascorbic acid, and estimated colorimetrically.

One of the main disadvantages of the acid precipitation method is that haemoglobin iron is also released to some extent by the action of the acid. Schade et al. (1954) and Ramsay

(1953) stated that the iron released from 1uM of haemoglobin-iron complex in 100 ml. serum interferes significantly with the estimation of plasma iron. Teepol does not remove iron from its haem complex, and Sanford (1963) demonstrated that up to 4 uM/100 ml. haemoglobin-iron complex has no effect upon the measured value of plasma-bound iron, although the value of the internal blank is altered (Beale et al., 1961).

Nakahara (1967) points out that doubt still exists as to the identity of the substance(s) responsible for the effect upon plasma bound iron and toxohormone in the strict sense, which brings about the decrease of liver catalase activity. In the present work, liver catalase depression was followed in the preliminary experiments, but in later studies the ability of the toxohormone fractions to depress the level of plasma-bound iron was assayed.

Due to the lack of knowledge of the degree of purification of toxohormone fractions which have been isolated, there are few reports of physical characterisation of toxohormone. Several workers have performed amino-acid analysis on their partially purified preparations (Ono et al., 1957; Yunoki and Griffin, 1961). Molecular weight estimations have been restricted to those based upon end-terminal analysis (Yunoki and Griffin, 1961) or Sephadex gel filtration (Trojanowski, 1969). Amino-acid analysis and an ultracentrifugal molecular weight determination were therefore performed on the highly

purified toxohormone preparation obtained in the present work.

In view of the striking cachectic effect of the growth of the Walker 256 carcinoma in rats, emphasized in the previous sections, and the depression in the uptake of radioactive amino-acids by diaphragm muscle protein from tumour-bearing rats, reported in Section 2, the effect of toxohormone, administered *in vivo* and *in vitro*, upon the uptake of a labelled amino-acid into diaphragm muscle protein was investigated.

A possible influence of a toxohormone preparation upon protein synthetic processes has been reported only once, by Obara and Ono (1965). These workers prepared toxohormone using a modification of Ono's method for the basic protein toxohormone fraction (Ono *et al.*, 1955), and further purified the preparation using CM-cellulose. Their 'refined' toxohormone was effective in reducing the uptake of ^{14}C-leucine into the proteins of rat lens and cornea by a factor of 10, in an *in vitro* incubation system. The doses employed were 40 - 200 ug/ml medium, although there appeared to be little increased effect with increased dose, after the initial reduction due to the addition of 40 ug/ml toxohormone.

For the *in vivo* experiment in the present work, the dose was selected on the basis of the dose required to produce an

effective depression in the level of plasma iron in mice, the amount being increased to allow for the greater weight of the rat. The time lag between the injection and the sacrifice of the animals was chosen on the basis of the reported effects of toxohormone listed in Table 23 . Those effects such as thymus involution and adrenal hypertrophy which might be expected to involve alterations in protein metabolism do not become significant until at least 24 hours has elapsed since the first injection. The experiment was therefore designed so that one group of animals were killed 3 hours after their second injection of toxohormone, whilst a second group were killed 27 hours after their second injection. In this way, it was hoped that any short term transitory effect of the toxohormone would be detected, as would a longer term cumulative effect.

In the in vitro experiment, the toxohormone was added to the incubation medium so as to render the final concentration of toxohormone in the medium in excess of the concentration which could be expected in rat plasma during the in vivo experiment. Obara and Ono (1965) claimed that a toxohormone concentration of 40 ug/ml medium was sufficient to cause a significant effect on the synthesis of corneal and lens protein, whilst the injection of 1600 ug over a period of 4 days was necessary to cause the depression of

Table 23 - Reported effects of toxohormone following *in vivo* administration

	Effect	Time after administration (hrs.)	Source
Liver weight	increased	24	Kampschmidt *et al.* (1959)
Thymus weight	decreased	48	"
Spleen weight	increased	24	"
Adrenals weight	increased	10	"
Liver catalase activity	decreased	24	"
Kidney catalase activity	decreased	24	"
Haemoglobin level	decreased	72	"
Plasma-bound iron level	decreased	10	"
Unesterified iron binding capacity	increased	10	"
Liver protoporphyrin level	increased	24	Nakahara (1960)
Liver ferritin level	decreased	20	"
NAD synthesis	decreased	24	"
Liver ascorbic acid level	decreased	24	Hoshizima (1958)

mouse liver catalase. Although this indicates that toxohormone is more potent *in vitro* than *in vivo*, the dose administered in the present experiment was greater than that required to produce a significant depression in mouse plasma iron content.

EXPERIMENTAL

Preparation of toxohormone

The methods used for the preparation and purification of toxohormone were based upon those of Yunoki and Griffin (1960).

Preparation of a crude toxohormone fraction

Tumours were excised from rats bearing large Walker 256 carcinomas and stored at -40°C. 500 g of tumour tissue, which represented the accumulation from twelve to fifteen rats, were normally processed at one time. The frozen tumours were weighed and homogenised with approximately 3 volumes of acetone in an MSE Ato-Mix blender for 10 minutes. A further 2 volumes of acetone were added with stirring, and the homogenate allowed to stand for 12 hours at 4°C. The homogenate was filtered and the acetone powder washed with a further volume of acetone. The powder was dried overnight under vacuum. 90 g of acetone powder were generally obtained from 500 g of tumour.

Two volumes of acetone were added to the acetone-dried powder and sixteen volumes of glacial acetic acid were added with stirring. The solution was heated to 70°C over the course of 30-60 minutes with constant stirring, and allowed to cool. The insoluble residue was removed by centrifugation

and traces of supernatant collected by washing the residue with glacial acetic acid. To the clear supernatant one two-hundredth volume of 5M NaCl and one half-volume of acetone were added, and, after vigorous stirring, the mixture was allowed to stand for 12 hours at 4°C. The precipitate was removed by centrifugation, and an equal volume of ether was added to the clear supernatant. A large precipitate formed quickly and after two hours the mixture was centrifuged. The residue was thoroughly washed with acetone to remove acetic acid, dried under vacuum, and stored at -40°C. This fraction was designated crude toxohormone; 6g were generally obtained from 90g acetone-dried powder.

Purification of toxohormone

(i) Ammonium sulphate fractionation

1g of crude toxohormone was dissolved in distilled water, and the ammonium sulphate concentration of the solution raised by drop-wise addition of 3.9M $(NH_4)_2SO_4$ at 0°C. Precipitates were collected by centrifugation at 20,000g for 10 minutes, at ammonium sulphate concentrations of 0.39M, 0.78M, 1.17M, 1.56M, 1.95M, 2.34M and 2.73M. The fractions were redissolved in distilled water, and residual ammonium sulphate removed by ion-exchange chromatography (Hirs et al. 1953; Moore et al., 1958). A short column (3.0 x 8.0 cm) of Amberlite IR-120

(400 mesh) (Rohm and Haas, Ltd.) was washed with 500 ml 3N HCl, followed by distilled water until the pH of the eluate dropped to 5. The solution to be desalted was loaded, and the column was washed with distilled water until the eluate was neutral, and no precipitation was produced by the addition of a single drop of a saturated solution of barium hydroxide. The toxohormone fraction was eluted with 5N NH_4OH. The contaminating ammonia was evaporated under vacuum, and the solution lyophilised.

(ii) Cation-exchange chromatography

Amberlite CR-50 (200 mesh) (Rohm and Haas, Ltd.) was cycled once through the sodium form before use. To 500g of the resin suspended in 2 litres of water, 240g NaOH were added over a period of 1 hour. The pH of the suspension increased to about 11. Stirring was continued until the evolution of heat subsided, and the sodium salt of the resin was washed by decantation with five 2 litre portions of water. The resin was further washed with water on a filter until the pH was reduced to 10. The resin was converted to the acid form by passing 10 litres of 3N HCl through the filter over a 4-hour period, and it was finally washed with 6 litres of water. The acid form of the resin was stirred with 2 litres of 0.1M glycine-NaOH buffer, pH 9.00. The pH of the suspension was adjusted to pH 9.00 by titration

with 40% (w/v) NaOH. The buffer was removed by decantation and the process repeated until the pH of the resin suspension remained constant over two successive buffer changes.

A 2,5 x 40 cm column was packed with resin and washed with buffer at pH 9,00 until the pH of the effluent became equal to the pH of the buffer. 2g crude toxohormone was stirred with 200ml 0,1M glycine-NaOH buffer pH 9,00, for 2 hours at room temperature. Approximately 300mg remained insoluble and this was removed by filtration, dried and weighed. The clear filtrate was applied to the column, and the column developed with 0,1M glycine buffer, pH 9,00, at a flow-rate of 300 ml/hour. The eluate was monitored at 282nm with an LKB Uvicord MK II. Once the first fraction had been completely removed from the column, the pH of the eluant was gradually increased by the addition of 0,1M glycine buffer pH 11,00, using a 500 ml mixing chamber.

The fractions thus eluted from the cation-exchange resin were lyophilised and redissolved in the minimum volume of distilled water. Glycine and NaOH were removed by passing the solutions through a 1,5 x 90 cm column of G-15 Sephadex (Pharmacia Ltd., Uppsala), eluting with distilled water at a flow-rate of 30ml/hour.

The concentration of the toxohormone fractions eluted from the Sephadex column was determined by the method of Itzhaki and Gill (1964).

The micro-biuret method developed by Itzhaki and Gill (1964) is based upon the measurement of the ultraviolet absorption of the complex formed between protein and copper in strongly alkaline copper sulphate solutions.

The colour reagent consists of 0.21% (w/v) $CuSO_4.5H_2O$ in 30% (w/v) NaOH. 1 ml. of this reagent was added to 2 ml of the protein solution, and the colour allowed to develop for 5 minutes at room temperature before measuring the extinction at 310 nm.

A standard solution of bovine serum albumin was prepared, and the protein concentration of this solution determined by the microkjeldahl nitrogen method of Ma and Zuazaga (1942). This solution was subsequently used for the preparation of standard curves. A standard curve is shown in Figure 10 .

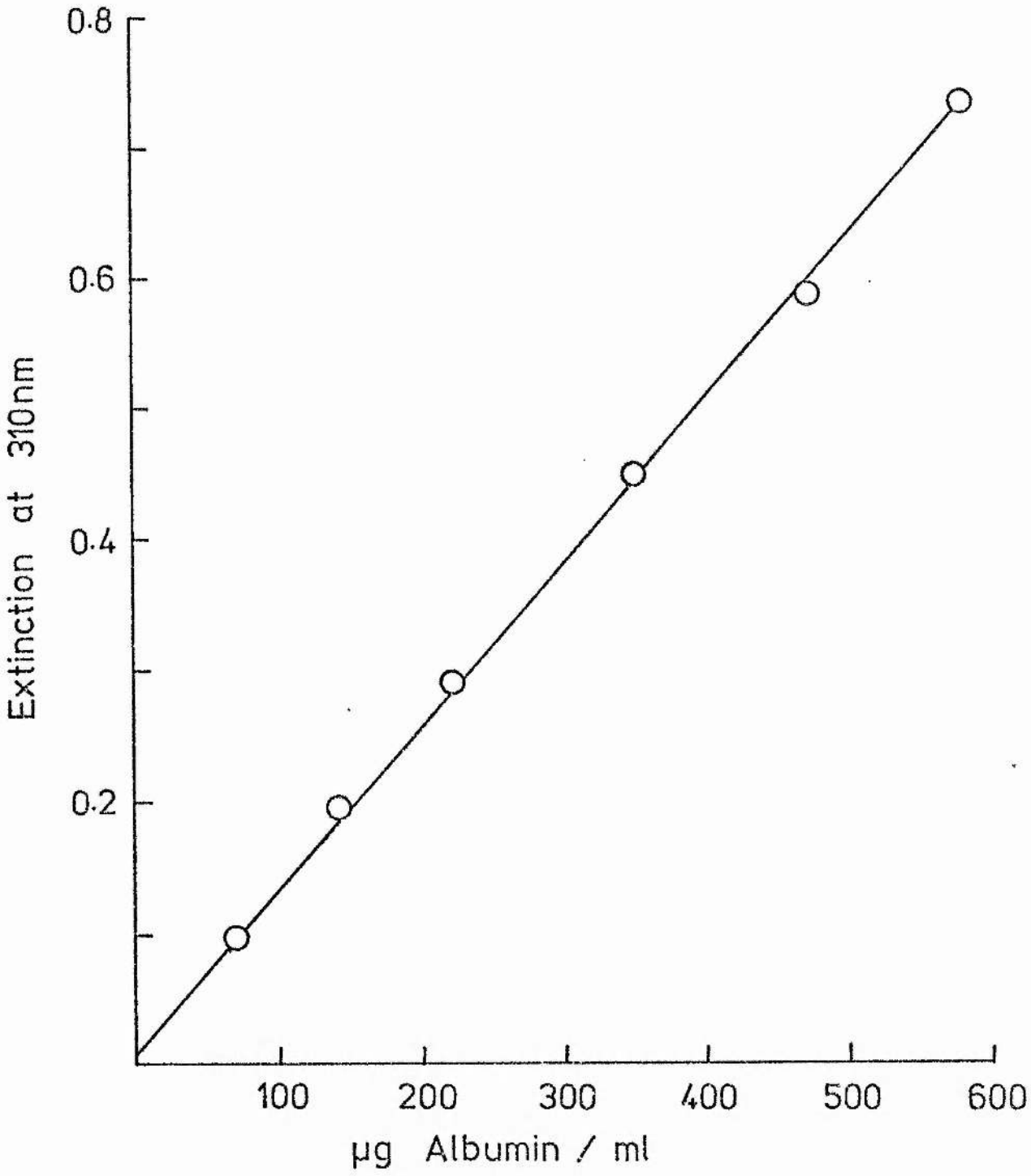


Figure 10. Standard curve for the estimation of protein by the micro-biuret method of Itzhaki and Gill (1964). Bovine serum albumin was used to prepare the standard protein solution.

The liver catalase depressing ability of toxohormone

The method used for the measurement of the depression of liver catalase activity was a modification of the method described by Adams (1950).

Four mice were injected intraperitoneally with 1 ml of a solution of the toxohormone fraction in water. A further four mice were injected with distilled water and served as controls. 24 hours later the mice were sacrificed by stunning followed by exsanguination, and the liver rapidly removed. After weighing, the liver was immediately homogenised in 20 ml ice-cold water using an M.S.E. overhead homogeniser fitted with an ice-cold jacket. The volume of the homogenate was adjusted with ice-cold water so that the final concentration was 2 mg original wet weight of liver/ml homogenate.

1 ml of the diluted homogenate was pipetted into 50 ml ice-cold 0.01N hydrogen peroxide solution (in M/150 phosphate buffer pH 6.8). A 5 ml sample was removed from the mixture after 2 minutes and dropped into 5 ml 2N sulphuric acid to stop the reaction. 10 ml 10% (w/v) potassium iodide were added along with 1 drop 1% (w/v) ammonium molybdate solution, and the iodine liberated from the iodide by any peroxide not decomposed under catalase action was titrated with 0.005N sodium thiosulphate solution, starch being added near the end-point tc act as indicator.

A reaction constant was now calculated by application of the equation:

$$K = \log \frac{a}{(a - x)}$$

where a = initial substrate concentration

a-x = final substrate concentration

The value of K obtained serves to provide a comparison between the catalase activity of two liver samples which have been diluted to the same concentration and incubated with substrate under identical conditions, provided that liver catalase activity is not affected by dilution of the homogenate. This possibility was tested by successively diluting a liver homogenate and estimating the amount of substrate used under the incubation conditions described above. The estimation at each homogenate dilution was performed in quadruplicate. The resulting reciprocal plot of enzyme concentration and volume of hydrogen peroxide destroyed is shown in Figure 11 , and the straight line obtained shows that the K values calculated as described above can be compared with confidence.

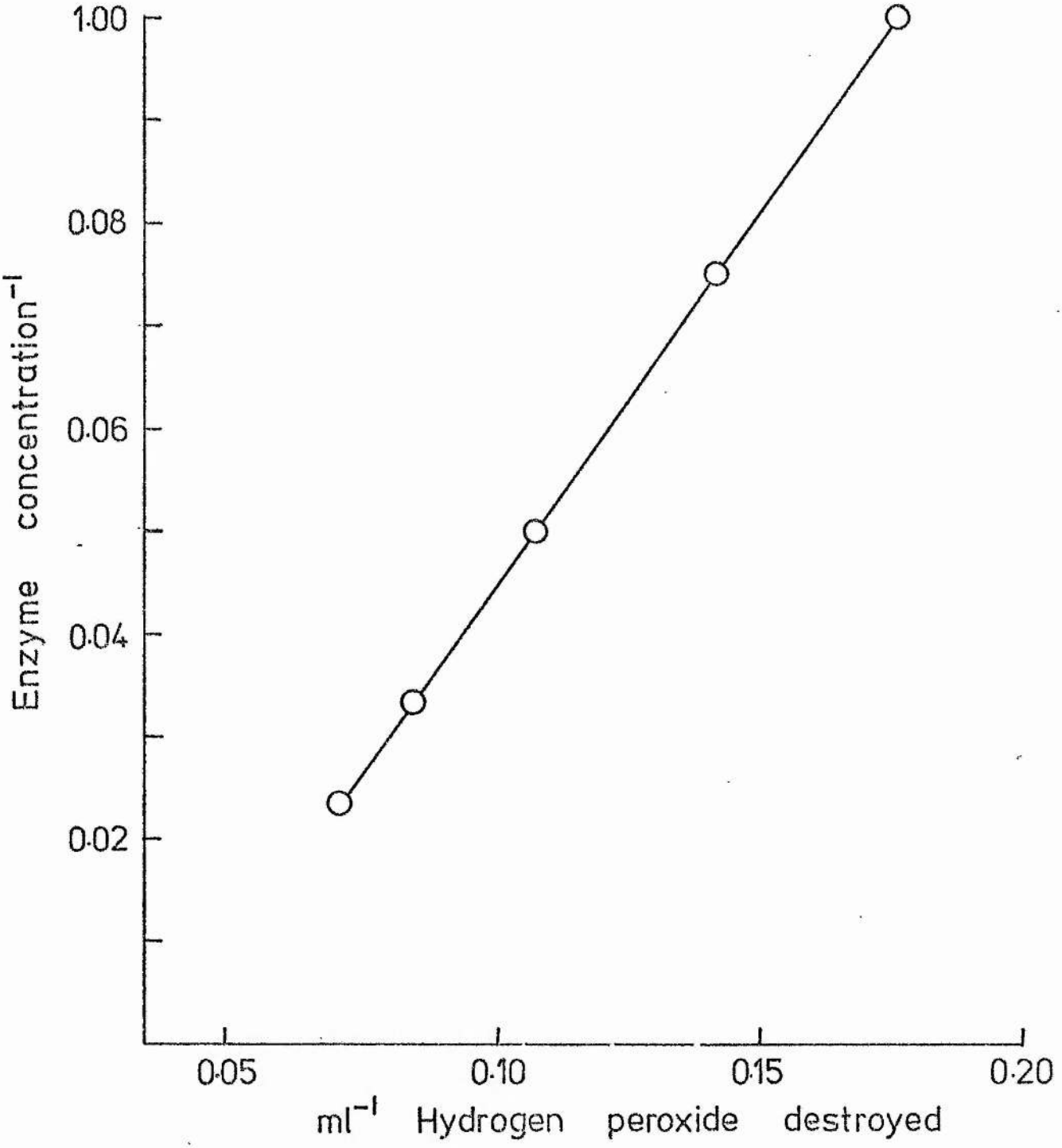


Figure 11. Reciprocal plot of liver catalase enzyme concentration versus substrate destroyed.

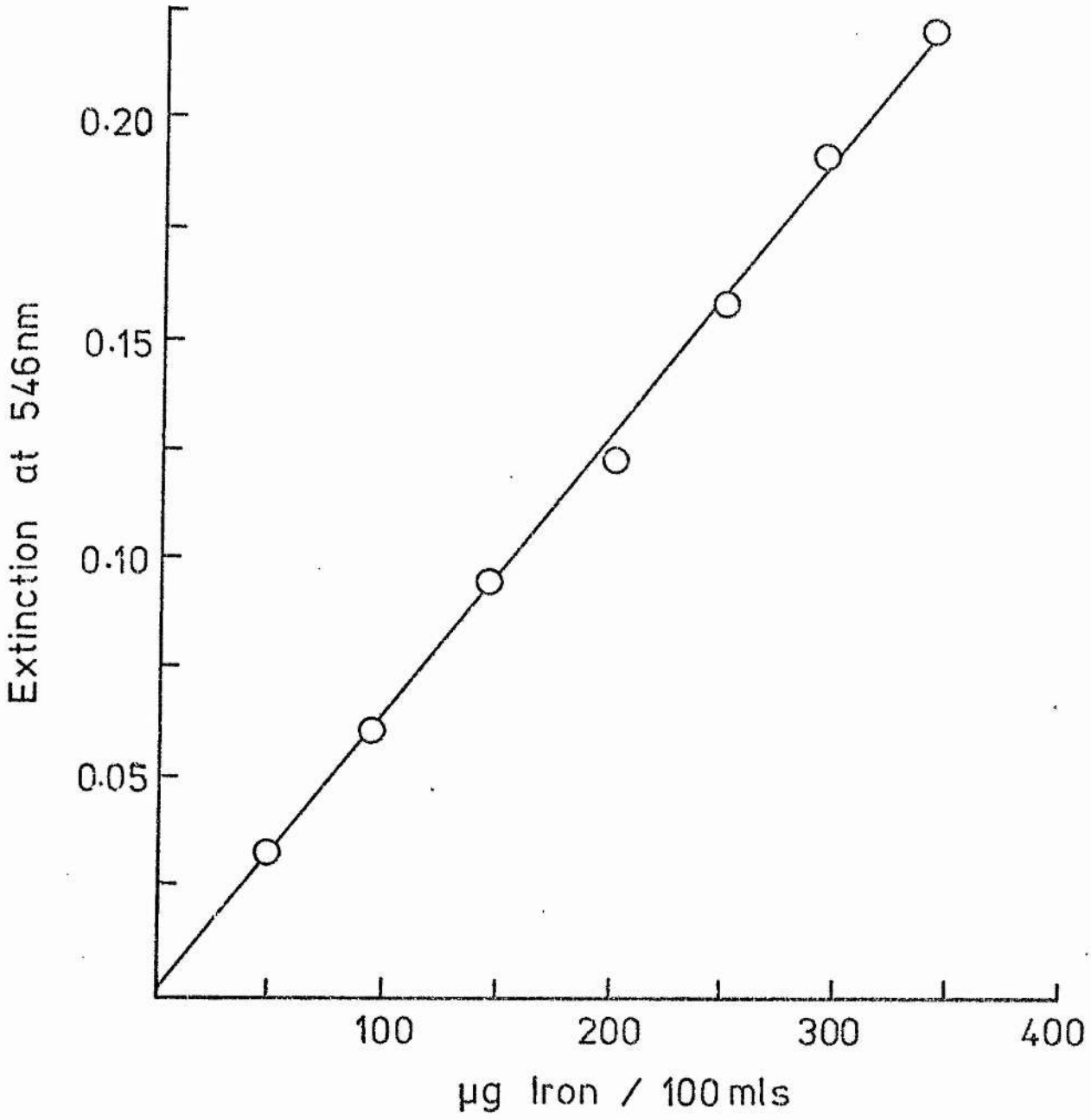


Figure 12 Standard curve for the estimation of iron by the method of Sanford (1963).

The plasma iron depressing ability of toxohormone

The activity of a toxohormone fraction was assayed by its ability to depress the level of plasma-bound iron in mouse blood. Plasma-bound iron was estimated by a modification of the method described by Sanford (1963).

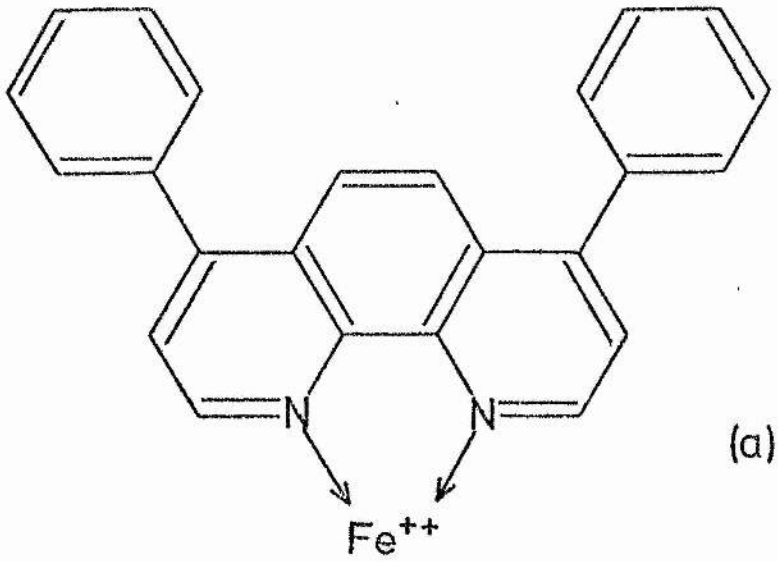
Six mice were injected intraperitoneally with 1 ml of a solution of the toxohormone fraction in water. A further six mice were injected with distilled water, and served as controls. All injections were carried out between 2100 and 2200 hours to minimise the influence of diurnal variation. Twelve hours later, the mice were anaesthetised by an intraperitoneal injection of 0.15 ml 6% (w/v) sodium pentobarbitone, and blood removed from the heart using a finely-drawn heparinised Pasteur pipette. Plasma was collected by centrifugation at 600g for 30 minutes.

Iron-free Teepol 610 reagent was prepared by adding an equal volume of water to Teepol 610 detergent (Shell Chemical Co.). The dilute detergent was passed through a 3.0 x 60 cm column of Zeo-Karb 225 (Permutit Co. Ltd.) in the sodium form. The Teepol reagent could be stored for several months at room temperature. Immediately before use, ascorbic acid was added to the detergent to make a final concentration of 0.75% (w/v) ascorbic acid. 0.4 ml of the acidified Teepol solution was added to 0.1 ml mouse plasma and the extinction, E_p, measured

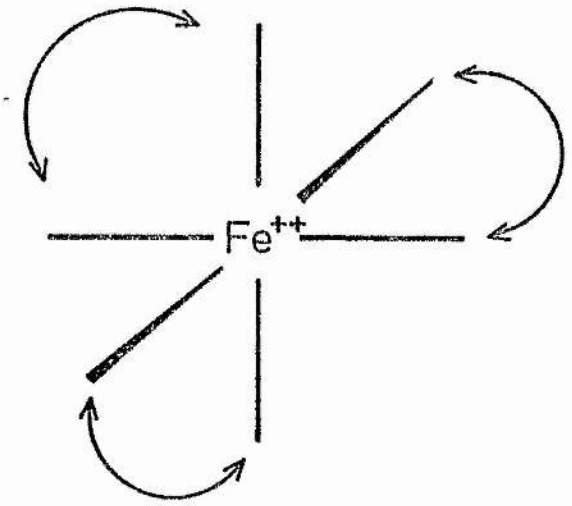
at 546 nm. 0.1 ml of a 0.1% (w/v) 4,7 diphenyl 1,10 - phenanthroline disulphonate (bathophenanthroline) solution (British Drug Houses, Ltd.) was added, mixed well, and the extinction, E_t, measured, again at 546 nm. The extinction, E_b, of a mixture of 0.1 ml water, 0.1 ml bathophenanthroline and 0.4 ml Teepol reagent provided a reagent blank. The extinction due to the colour complex formed between ferrous iron and bathophenanthroline was calculated according to the formula:

$$E_o = E_t - E_b - (E_p \times 0.833)$$

Calibration curves were prepared using a standardised $FeCl_2$ solution, and such a curve is shown in Figure 12 .
The chemical structure of the complex formed by Fe^{2+} and bathophenanthroline is shown in Figure 13.



(a)



(b)

Figure 13.

(a) The chemical formulation of the points of chelation of the bathophenanthroline molecule and the ferrous ion.

(b) The spatial configuration of the bathophenanthroline iron complex. The arrows refer to the electron-charing by two nitrogen atoms from each bathophenanthroline molecule.

Physical properties and chemical composition of the purified toxohormone fraction

Amino-acid analysis

Approximately 60 ug purified toxohormone was hydrolysed for 24 hours with 10 ml 6N HCl at 100°C. Hydrochloric acid was removed from the hydrolysate by successive evaporation under vacuum. The residue of amino-acids were taken up in 0.5 ml distilled water, and analysed on a Locarte amino-acid analyser.

Presence of RNA and DNA

The toxohormone preparation was tested for the presence of ribose by the orcinol method (Bial et al., 1902; Schneider, 1957) and for the presence of deoxyribose by the Ceriotti method (Ceriotti, 1952, 1955).

Absorption spectrum

The ultraviolet absorption properties of the toxohormone dissolved in distilled water were monitored on a Unicam SP 800 double beam spectrophotometer.

Ultracentrifugal analysis

Ultracentrifugal analysis was carried out in a Spinco Model E analytical ultracentrifuge. The meniscus depletion equilibrium method of Yphantis (1964) was used.

900 ug of purified toxohormone were dissolved in 1 ml 2% (w/v) KCl, and the solution and solute placed in a 12mm capillary type synthetic boundary double sector cell, with sapphire end windows. Rotor speed was 44,770 rpm, and the depletion of the meniscus was monitored using the Rayleigh interference optical system.

The slope of the plot of $\log_{10}$ fringe displacement versus the square of the distance from the centre of the rotor was calculated by linear regression. υ was assumed to be 0.725 and ρ to be 1.011. The weight average molecular weight was calculated by application of the equation:

$$M_w = 2RT \times \frac{2.303}{(1 - \rho\upsilon)\omega^2} \times \text{slope}$$

Where υ = partial specific volume

ρ = density

ω = angular velocity of the rotor

This equation is due to Van Holde and Baldwin (1958).

The effect of toxohormone administered in vivo upon the uptake of ^{3}H-lysine into proteins of hemidiaphragms from normal rats and rats bearing a Walker 256 carcinoma

Twelve normal rats were maintained under experimental conditions as described on page 43. Eight of these animals were injected intraperitoneally with 150 ug of toxohormone purified by ion-exchange chromatography as described on page 123. The remaining four animals were given injections of distilled water. After 24 hours these injections were repeated. 3 hours later, four of the toxohormone-treated animals and two of the control animals were killed. The hemidiaphragms were excised and incubated in medium containing ^{3}H-lysine with a specific activity of 0.3 uCi/ml, for 2 hours, (as described on page 79). 51 hours after the initial injections, the remaining animals were killed and the hemidiaphragms similarly incubated. The total protein fraction was prepared from the frozen muscles, dried and counted, as described previously (page 49).

The effect of toxohormone administered in vitro upon the uptake of ^{3}H-lysine into proteins of hemidiaphragms from normal rats and from rats bearing a Walker 256 carcinoma

Normal male rats were maintained under the experimental conditions as described on page 43. The animals were sacrificed and the hemidiaphragms excised. One hemidiaphragm from each animal was incubated in medium containing ^{3}H-lysine at a specific activity of 0,3 uCi/ml, (page 79), whilst the other hemidiaphragm was incubated in a similar medium with the addition of 30 ug/ml toxohormone. The incubation period was 2 hours. At the end of the incubation the muscles were frozen, and the total protein fractions were extracted and counted (page 49).

RESULTS and DISCUSSION

Preparation of toxohormone

Crude toxohormone (TH), prepared as described on page 119, was assayed for its ability to depress the activity of liver catalase in mice. The results are shown in Table 24.

Table 24. - The depression of mouse liver catalase activity by a crude toxohormone fraction (TH)

Dose of TH/mouse (mg)	K x 10^3	Percentage decrease in catalase activity	P**
0	426 ± 12*	-	-
100	173 ± 9	59.3	< 0.01
50	286 ± 17	32.9	< 0.01
25	331 ± 13	22.2	< 0.01
10	402 ± 6	4.5	n.s

* Results are expressed as mean ± standard error of 4 mice

** In this table, and subsequent tables describing the bioassay of toxohormone, P represents the statistical significance of the percentage depression of the parameter under examination.

A significant effect is maintained with doses down to 25 mg (Table 24), but a dose of 10 mg produced no significant depression in the liver catalase activity. Yunoki and Griffin (1961) claimed that their toxohormone sample was active in doses as low as 10 mg - although the percentage decrease which they obtained with this dose was only 27%. The depression produced by a 20 mg dose of their preparation was 38.5%, so that their preparation appeared to be considerably purer than that obtained in the present work by the same method.

Nevertheless, the fact that the material prepared from the Walker 256 carcinoma did produce a depression in activity of liver catalase qualifies the extract as toxohormone, as defined by Nakahara and Fukuoka (1948).

The crude toxohormone fraction was next assayed for its ability to depress the plasma-bound iron level in mice, and the results of these assays are displayed in Table 25.

Table 25. - The depression of the level of plasma-bound iron in mice by a crude toxohormone fraction (TH).

Dose of TH/mouse (mg)	Plasma iron level (ug/100ml)	Percentage depression of plasma iron level	P
0	184 ± 15*	-	-
10	100 ± 6	45.4	< 0.01
5	140 ± 12	23.9	< 0.05

* Results are expressed as the mean ± standard error of 6 mice.

With the plasma iron bioassay a considerable depression is produced by a dose of 10 mg crude toxohormone, and a significant effect can be detected from a dose of 5 mg. These findings bear out the results of Kampschmidt et al. (1959), although the depression in the level of plasma iron produced in this instance was not nearly so precipitous as that reported by Kampschmidt, who claimed that injection of 100 mg of a crude toxohormone preparation resulted in a 90% depression of the level of plasma iron. It is likely that the greater effect obtained by Kampschmidt was not due entirely to the larger dose used, for the depression which he reported seemed to many workers out of proportion to

what might be expected from the relatively mild degree of plasma iron depression in cancer-bearing patients and tumour-bearing animals (Nakahara, 1967), and bacterial contamination of Kampschmidt's toxohormone preparation was suspected. The subsequent work of Ono et al. (1960) and Nixon and Zinman (1966) showed, however, that a decrease of plasma iron does take place after the injection of toxohormone prepared from sterile tumours, although the depression is not as dramatic as first claimed by Kampschmidt.

The results obtained in the bioassay of the crude toxohormone preparation confirm that the plasma iron bioassay is more sensitive than the liver catalase assay (Tables 24 and 25).

A 'toxohormone' fraction was extracted from foetal and placental tissue obtained from 20 day pregnant rats, in exactly the manner described for tumour tissue (page 122). The crude fraction thus obtained was subjected to bioassay, and the results are shown in Table 26. Bovine serum albumin was also assayed for toxohormone activity.

These tests were performed as a check that the depression of the level of plasma iron was in fact due uniquely to a tumour product. Foetal and placental tissue was chosen because like the tumour it is composed of

rapidly dividing relatively undifferentiated cells. The fact that no alterations in plasma iron levels were induced by either foetal and placental tissue extract or bovine serum albumin supports the view that the toxohormone fraction studied was a tumour cell product.

Table 26. - The depression of the level of plasma iron in mice by an extract prepared according to Yunoki and Griffin (1960), from foetal and placental tissue, and by bovine serum albumin.

Injected material (dose in mg)	Plasma iron level (ug/100mg)	Percentage depression of plasma iron level	P
distilled water	204 ± 15*	-	-
foetal and placental extract (11.3)	213 ± 9	-	-
bovine serum albumin (10.0)	193 ± 12	5.3	n.s.

* Results are expressed as the mean ± standard error of 6 mice.

Ammonium sulphate fractionation of crude toxohormone

Several ammonium sulphate fractions were prepared and desalted as described on page 122, and each fraction was subjected to bioassay for toxohormone activity. The results of these assays are shown in Table 27, and indicate that considerable purification of the crude toxohormone can be achieved by use of ammonium sulphate fractional precipitation.

Trojanowski (1969) employed ammonium sulphate precipitation coupled with Sephadex chromatography, and obtained five fractions, the most active of which significantly lowered the level of liver catalase activity in doses of 100 ug. Table 27 shows that the most active fraction obtained in the present work was that precipitated by 2.73M ammonium sulphate, and that this significantly depressed the level of plasma-bound iron in doses of 100 ug. The resolution of the ammonium sulphate fractional precipitation method did not appear to be sufficiently great to separate a more active fraction.

Table 27 - The depression of the level of plasma iron in mice by toxohormone fractions prepared by ammonium sulphate precipitation.

Ammonium sulphate concn.	Dose/ mouse (ug)	Plasma iron level (ug/100ml)	Percentage depression	P
water	-	203 ± 6*	-	-
0.39M	1000	197 ± 15	-	-
0.78M	1000	217 ± 12	-	-
1.17M	1000	212 ± 8	-	-
1.56M	1000	136 ± 12	33.0	< 0.01
	500	182 ± 10	10.2	n.s.
1.95M	1000	83 ± 16	59.2	< 0.01
	500	143 ± 8	29.6	< 0.01
	200	189 ± 13	6.8	n.s.
2.34M	500	89 ± 13	56.3	< 0.01
	200	158 ± 10	22.2	< 0.01
	100	173 ± 14	14.8	n.s.
2.73M	200	74 ± 17	63.5	< 0.01
	100	131 ± 12	35.3	< 0.01
	33	192 ± 12	-	-

* Results are expressed as the mean ± standard error of 6 mice

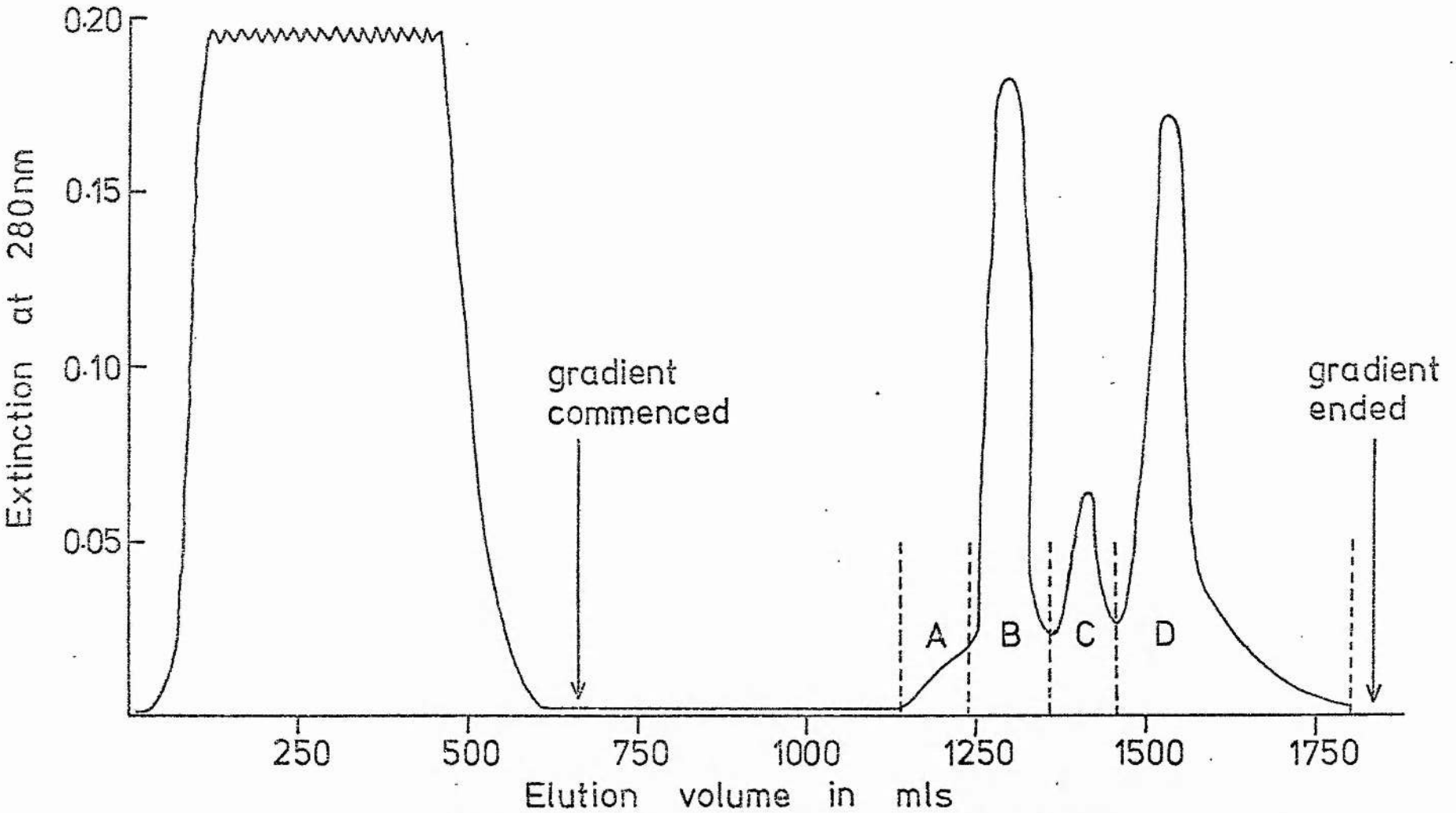


Figure 14. Elution pattern of crude toxohormone from Amberlite CR.50. The starting buffer was 0.1M glycine-NaOH, pH 9.00, and the gradient was produced by the addition of 500 mils. 0.1M glycine-NaOH, pH 11.00. through a 500 ml. mixing chamber. The flow rate was 300 mls.

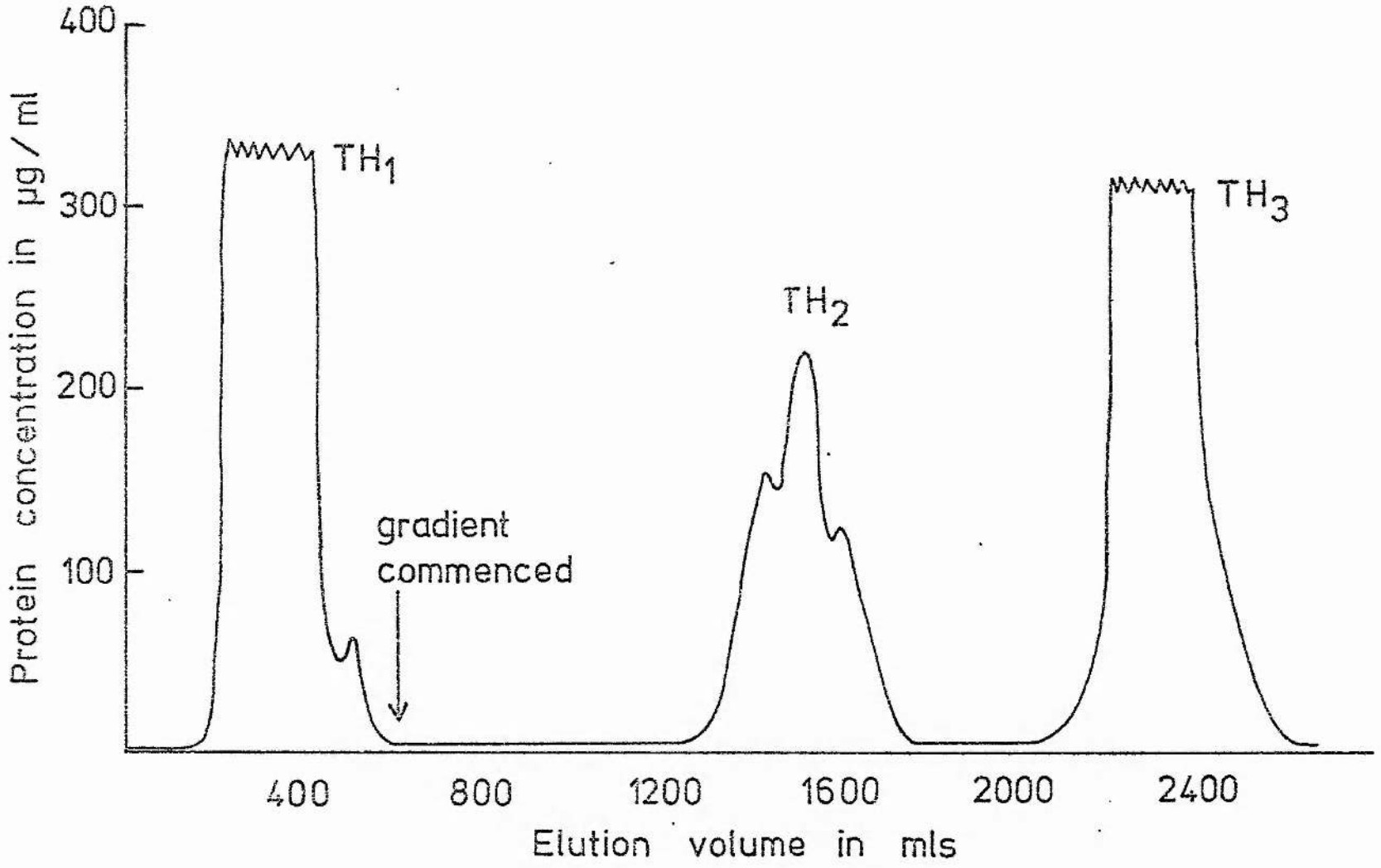


Figure 15. From Yunoki and Griffin (1961). Elution of crude toxohormone from Amberlite XE-64. Starting buffer was 0.1M glycine-NaOH, pH 9.40 and the gradient was produced by the addition of 0.1M NaOH through a 1000 ml. mixing-chamber. The flow rate was 10 mls./hour.

Cation-exchange chromatography of crude toxohormone

The elution pattern obtained by chromatography of crude toxohormone on Amberlite CR-50 is shown in Figure 14 . Fraction A started to be eluted at pH 10.3 and the gradient ended at pH 11.0. The starting buffer was 0.1M glycine-NaOH buffer, pH 9.0.

Yunoki and Griffin (1960,1961), in a similar experiment, used a starting buffer of pH 9.40, and their column was eluted with a gradient to higher pH, obtained by the addition of 0.1M NaOH through a 1000 ml mixing chamber. Figure 15 presents the elution pattern published by these workers. Yunoki and Griffin claimed that the TH_2 peak, their most active fraction, was eluted between pH 9.47 and 9.52, and their third peak, TH_3, at pH 9.60. However, using their data, calculation of the volume of NaOH which would have passed through the mixing chamber at the elution volumes shown for the TH_2 and TH_3 peaks suggested that the pH of the eluant at the point of elution of TH_2 was about 10.6 and that at the point of elution of TH_3, the eluant would have had no effective buffering capacity, and a pH value approaching 14.

In the present experiment, no peak corresponding to TH_3 of Yunoki and Griffin was eluted, probably because the pH was not increased above 11.0. The second group of

fractions, (A-D, Figure 14), to be eluted appeared to be comparable to the TH_2 fraction of Yunoki and Griffin.

Fractions A, B, C and D (Figure 14) were desalted on Sephadex G-15, and the elution profiles obtained are shown in Figure 16. Sephadex G-15 excludes molecules whose molecular weight is greater than 1500, and therefore peaks eluted with the void volume (50 mls) of the column were tested for toxohormone activity. The results of the bio-assay are displayed in Table 28.

Table 28. - The depression of the plasma iron level of mice by toxohormone fractions purified by column chromatography.

Toxo-hormone fraction	Dose/ mouse (ug)	Plasma iron level (ug/100ml)	Percentage depression	P
water	-	195 ± 5*	-	-
A	35	69 ± 9	64.6	< 0.01
	5	115 ± 15	41.0	< 0.01
B	25	88 ± 7	54.9	< 0.01
	5	122 ± 9	37.4	< 0.01
C	30	72 ± 10	63.2	< 0.01
	5	131 ± 7	32.8	< 0.01
D	40	60 ± 5	71.1	< 0.01
	5	117 ± 6	40.0	< 0.01

* Results are expressed as the mean ± standard error of 6 mice.

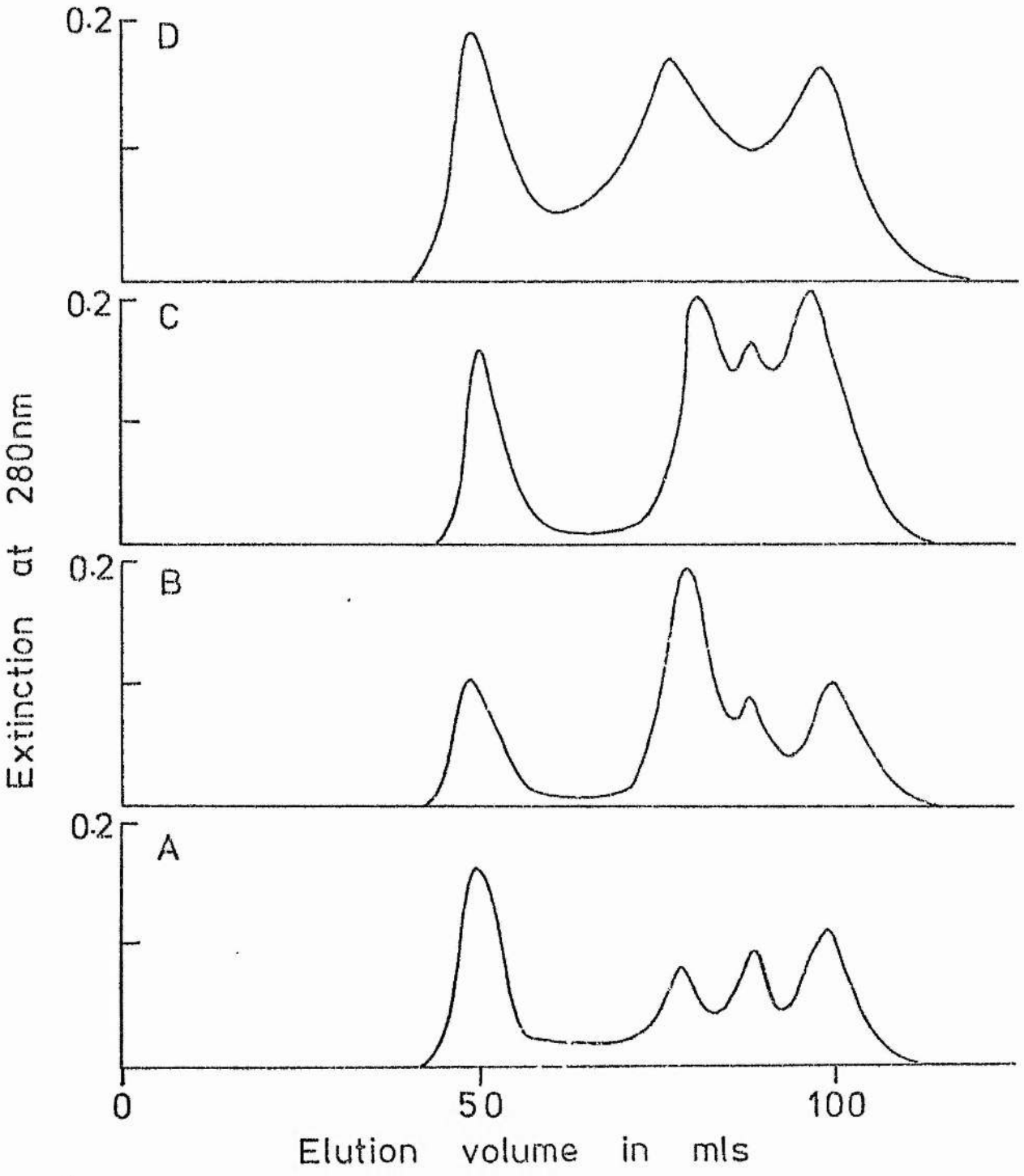


Figure 16. Elution of crude toxohormone fractions chromatographically separated on Amberlite CR-50, from Sephadex G-15. Elution was with distilled water, and the flow rate was 30 ml./hour. The column size was 1.5 x 85 cm. and the void volume 51 mls.

Yunoki and Griffin (1961) obtained three sub-fractions of their TH_2 peak (Figure 15), and did not attempt to further purify these sub-fractions. Bioassay by the liver catalase method revealed that all three sub-fractions were highly active, and Yunoki and Griffin concluded that they were dealing with a group of closely related substances, all possessing toxohormone activity. The large molecular weight fractions ($\geq$1500) obtained by Sephadex filtration of the four peaks from the cation-exchange chromatography all exhibited very similar activity in the depression of plasma iron (Table 28), and therefore a test was made to determine whether or not these fractions contained identical material.

The large molecular weight fractions from the four Sephadex separations was pooled and rechromatographed on Amberlite CR-50. In this case, a single low-profile peak was obtained, extending over the elution volume previously occupied by peaks A - D (Figure 14). Desalting on Sephadex G-15 produced a single peak in the excluded volume. It would therefore appear likely that the biologically active material is a single molecular species, eluted from the cation-exchange resin over a broad pH range. The presence of peaks B - D could then be due to the elution of the several small molecular weight compounds, subsequently separated by the Sephadex gel filtration.

A dose-response curve was drawn for the purified toxohormone sample and is shown in Figure 17. The curve shows excellent linearity over a dose range of 1 - 50 ug, beyond which the extent of the effect diminishes, indicating that toxohormone cannot reduce the level of plasma iron below a definite minimum (about 69% for this preparation).

The toxohormone fraction obtained by pooling the large molecular weight fractions from the Sephadex separations, referred to as the purified toxohormone fraction, was used in all subsequent studies in this work.

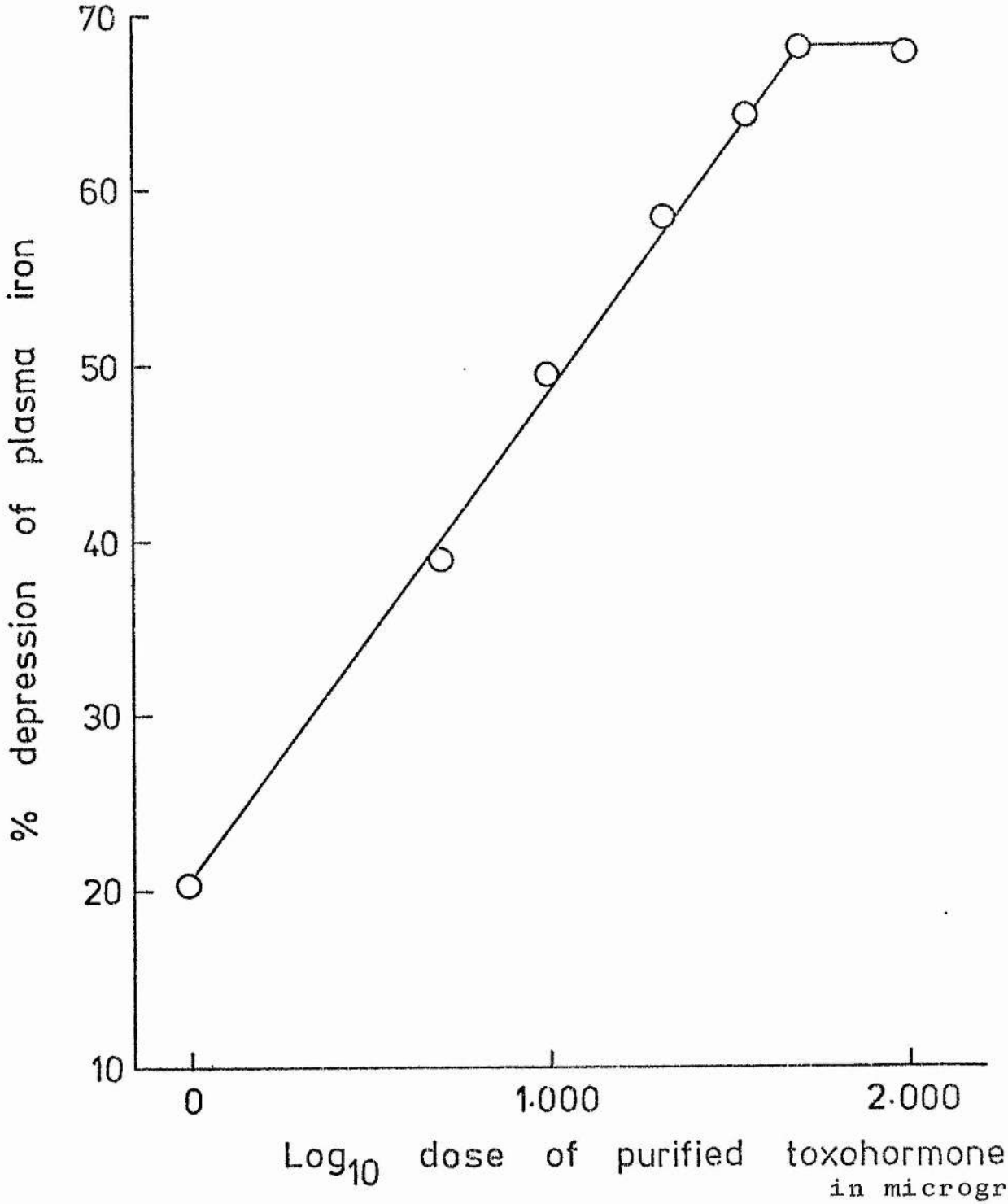


Figure 17. The dose-response curve of the purified toxohormone fraction.

Some studies on the physical properties and chemical composition of toxohormone

Ultracentrifugal analysis

The fringe displacement obtained in the present experiment is shown in Plate 6. The plot of $\log_{10}$ fringe displacement versus the square of the distance from the centre of the rotor is shown in Figure 18.

The fact that two slopes can be obtained from the graph indicates that the original solution of the purified toxohormone subjected to sedimentation was not homogeneous containing two molecular species of differing molecular weights.

The weight average molecular weight for each species was calculated by substitution of the relevant slope obtained from the plot into the equation of Van Holde and Baldwin (1958), given on page 131. In this case the molecular weights obtained for the low and high molecular weight compounds were 8,000 and 14,000 respectively. These estimations of molecular weight are approximately because of several assumptions made in the calculation, namely, the values selected for the density of the solution and for the partial specific volume of the polypeptide were chosen on the assumption that toxohormone has typical protein characteristics. The solution was also assumed to have

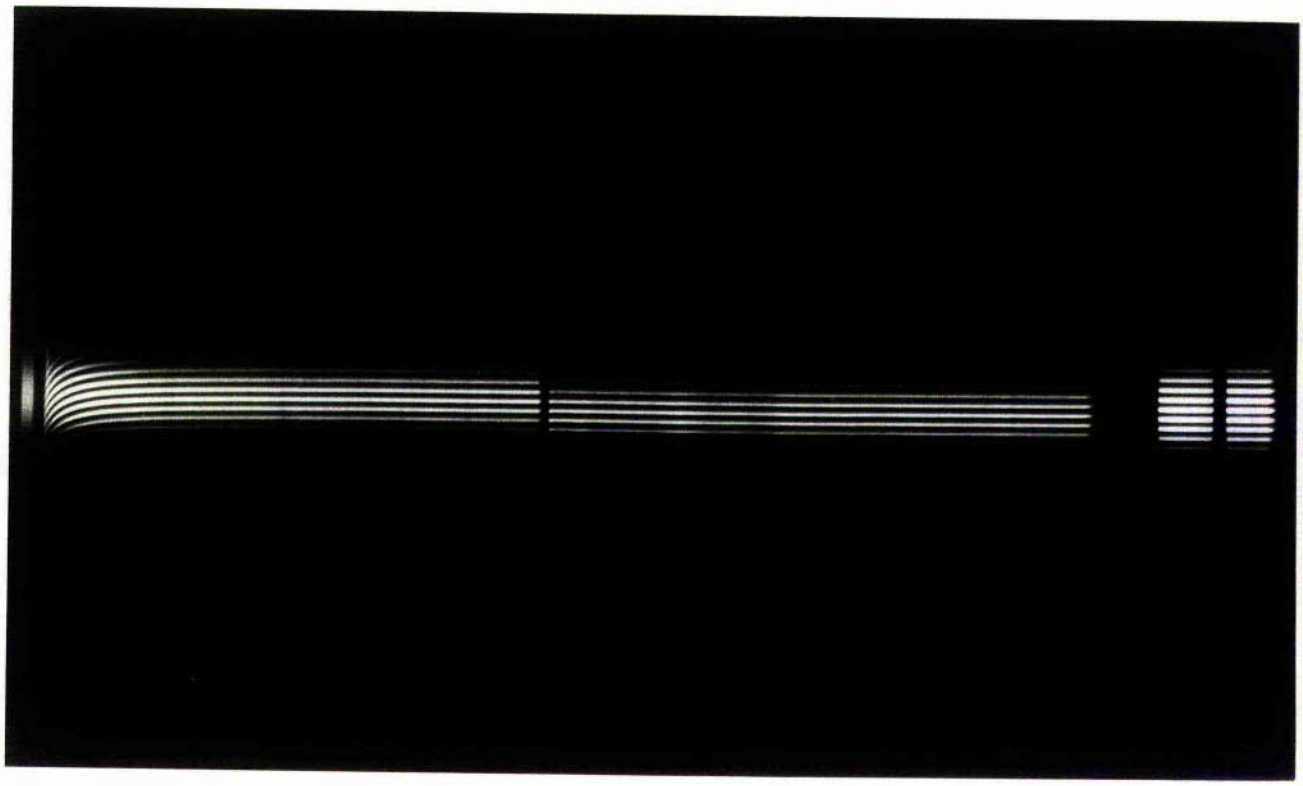


PLATE 6

The fringe displacement obtained in the ultracentrifugal analysis of the purified toxohormone fraction.

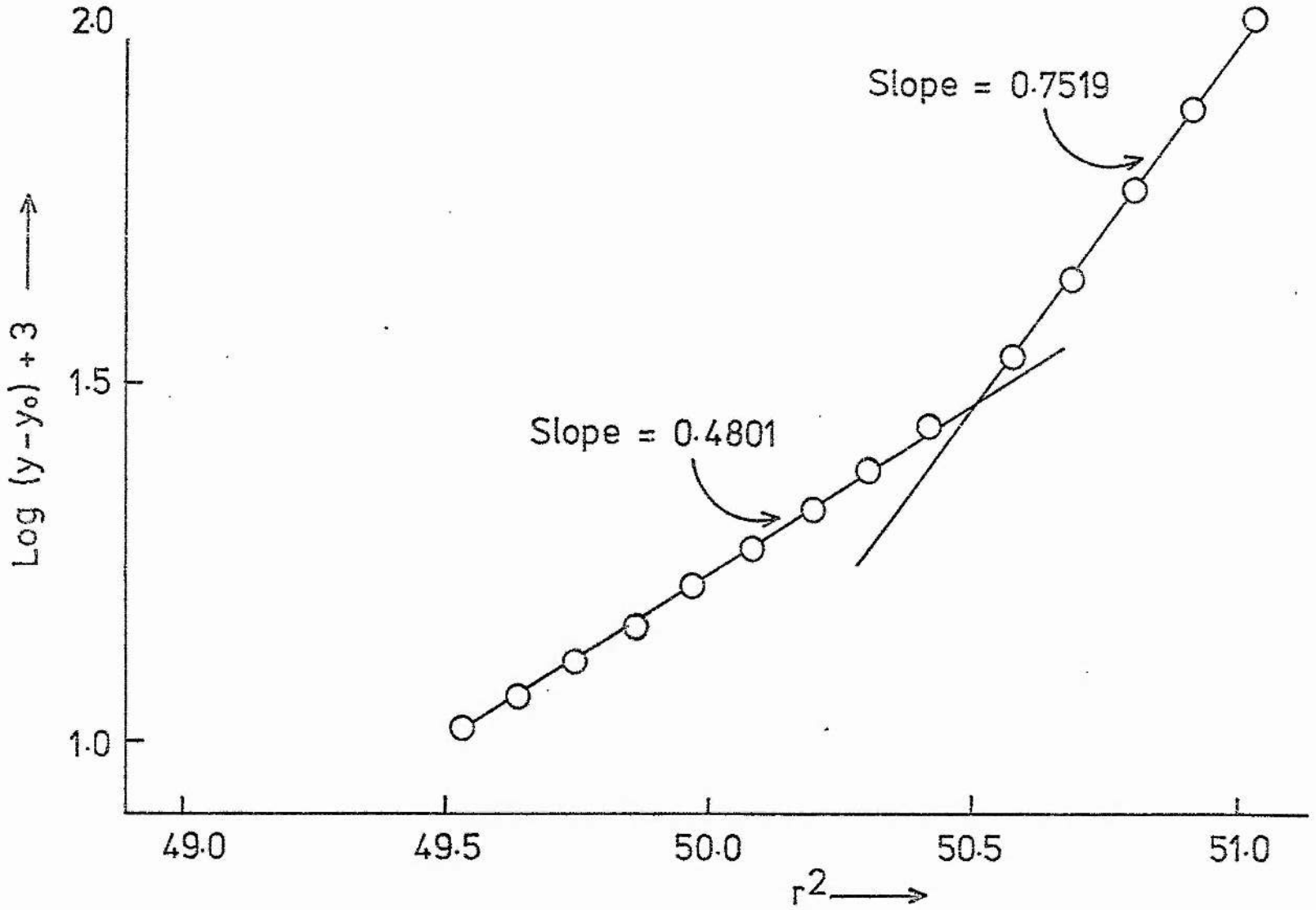


Figure 18. Ultracentrifugal molecular weight determination by the method of Yphantis (1964). Plot of $\log_{10}$fringe displacement versus the square of the distance from the centre of the rotor.

ideal behaviour because of the dilute conditions of the experiment.

The only other ultracentrifugal analysis of toxohormone described in the literature was reported by Yunoki and Griffin (1961), who performed a sedimentation analysis on their TH_2 fraction, using the Schlieren optical system. The sedimentation pattern obtained was diffuse at 59,780 rpm, and the only conclusion that they could derive was that TH_2 had a relatively small molecular weight. They also calculated minimum molecular weights from the N-terminal arginine content, and found these to be around 6,000 (Yunoki and Griffin, 1961).

Trojanowski et al. (1969) determined the molecular weights of their purified toxohormone fractions by chromatography on a calibrated column of Sephadex G-25, utilising the fact that the elution volumes of globular proteins from Sephadex are largely dependent on their molecular weights (Whitaker, 1963). The molecular weights thus determined were 5,000, 9,000 and 12,000. Although Trojanowski's preparation was impure (page 109), these results show that there was no high molecular weight component present in any significant amount in his preparation.

The present results confirm that biologically active toxohormone is a relatively small polypeptide, whose

molecular weight is in the order of 10,000.

Toxohormone, as originally isolated by Nakahara and Fukuoka (1948), or by Greenfield and Meister (1951), is non-dialyzable. It is interesting to note that subjection of their crude toxohormone preparations to proteolytic enzyme digestion results in a dialyzable substance, potent in vivo, and not precipitable by alcohol (Nakahara and Fukuoka, 1954). Nakahara and Fukuoka (1958) have since interpreted these results to mean that toxohormone in its elementary form may be a relatively small molecule, and that it may occur in tumour tissue for the most part in aggregate form, or as part of a larger molecule, constituting the non-dialyzable form. They further suggest that an association may occur with nucleic acids, because of the basic nature of many toxohormone fractions (page109). Nagagawa (1952) showed that a substance with toxohormone activity in the urine of cancer patients is precipitated by alcohol in spite of the absence of nucleic acid in the same fraction, so that the possibility that toxohormone normally exists as aggregates of smaller molecular forms has not yet been excluded.

Amino-acid analysis

The results of the amino-acid analysis of the purified toxohormone are shown in Table 29.

Table 29. - The amino-acid composition of toxohormone.

Amino-acid	Residues/ 100 residues	Amino-acid	Residues/ 100 residues
Aspartic acid	10.1	Threonine	3.8
Serine	4.6	Glutamic acid	10.1
Proline	8.6	Glycine	30.4
Alanine	7.4	Valine	6.3
Cysteine	1.6	Methionine	Trace
Isoleucine	3.9	Leucine	7.2
Tyrosine	Trace	Phenylalanine	2.7
Lysine	Trace	Histidine	Trace
Arginine	1.7		

Yunoki and Griffin (1961) performed an amino-acid analysis on their TH_2 fraction from human malignant tissues, and noted a very high proportion of aspartic acid and glutamic acid, and, in common with the present results, very small quantities of the aromatic amino-acids.

Ono et al. (1957) purified a toxohormone extract from Ehrlich carcinoma by adsorption on cellulose powder, and analysed the amino-acid content of the peptide after hydrolysis by paper chromatography of the dinitrophenyl derivatives of the amino-acids. Here again, the proportion of aspartate and glutamate was high, and the aromatic amino-acids virtually absent. These workers did not detect any cysteine or methionine in their hydrolysate, and the sulphur-containing amino-acids were present only in very small amounts in the toxohormone fraction prepared in this work (Table 29). It is of interest that the same tendencies in amino-acid composition should be noted in two toxohormone fractions prepared from different tumours by different extraction techniques. It must be pointed out, however, that Ono's preparation was active only at a minimum dose of 100ug and probably contained a high proportion of biologically inactive material, so that the data on amino-acid composition must be treated with caution.

Absorption spectrum

The absorption spectrum of the purified toxohormone fraction dissolved in distilled water is shown in Figure 19. The lack of a definitive maximum at 280 nm, normally expected in the absorption spectrum of a protein or peptide, may be due to the lack of aromatic amino-acids (Table 29), which are responsible for the absorption in this region. The high absorption at 260 nm led to the suspicion that nucleic acids might be present, possibly in association with the peptide. Warburg and Christian (1941) showed that the percentage of nucleic acid present in a nucleic acid - protein mixture can be ascertained from the E_{260}: E_{280} ratio. Using the present data nucleic acids could form an impurity to the extent of 2% by weight.

The presence of nucleic acids was tested for by the orcinol method for the estimation of ribose, and the Ceriotti method for the estimation of deoxyribose.

No colour formation was detected in either of these tests, using a 4 mg sample of the purified toxohormone in each case. This leads to the conclusion that if either deoxyribose or ribose were present, the quantities involved were insufficient to be detected by the methods employed. This being the case, any nucleic acid contamination of the toxohormone preparation would constitute less than 2% by weight.

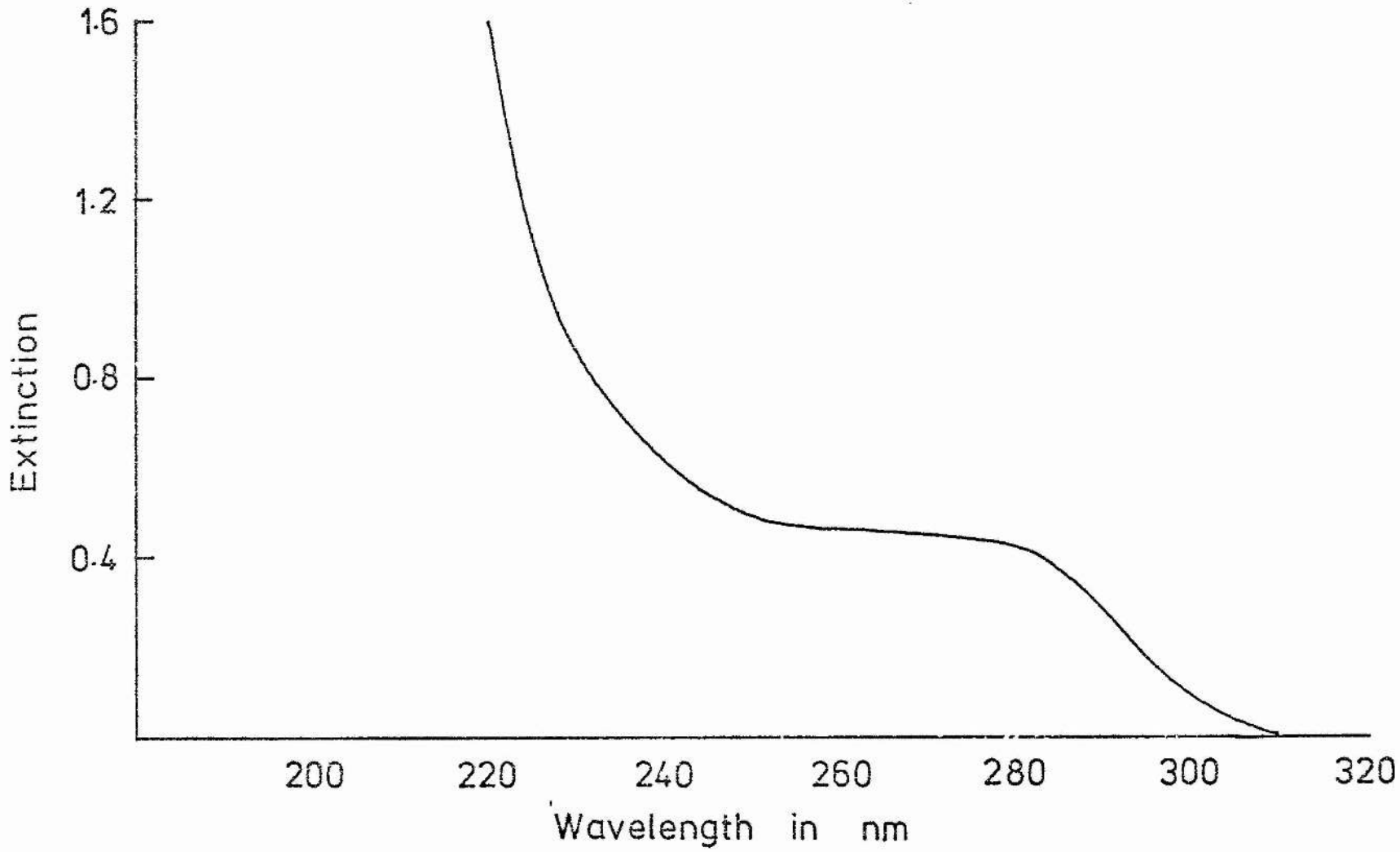


Figure 19. The absorption spectrum of the purified toxohormone fraction dissolved in distilled water.

The effect of toxohormone upon the uptake of ^{3}H-lysine into total protein of diaphragm muscle from normal rats

In view of the significant effects of the growth of the Walker 256 carcinoma upon the uptake of ^{3}H-lysine into the proteins of rat diaphragm muscle, reported in Section 2, the effect of the purified toxohormone prepared from Walker tumour upon protein metabolism in the muscle was investigated. The results shown in Table 30 show that the toxohormone, in common with the tumour, exerts an inhibitory effect upon incorporation of label into muscle protein.

Table 30. - The effect of 300ug toxohormone administered *in vivo* upon the uptake of ^{3}H-lysine into total protein of diaphragm muscle from normal rats.

Time after injection of toxohormone (hr)	Counts/minute/mg protein	P**
0	486 ± 9*	-
27	394 ± 13	< 0.01
51	143 ± 7	< 0.01

* Results are expressed as the mean ± standard error of 4 rats.

** In this and the following table, P refers to the statistical significance of the difference between the values obtained for the control and for the toxohormone treated rats.

Toxohormone was also tested for an effect upon the uptake of ^{3}H-lysine into muscle protein when added directly to the incubation medium. The results displayed in Table 31 indicate that the inhibitory action of toxohormone on protein synthesis is not confined to the in vivo situation.

Table 31. - The effect of toxohormone administered in vitro upon the uptake of ^{3}H-lysine into total protein of normal rat diaphragm muscle.

	Counts/minute/mg protein	P
Control	384 ± 23*	-
Toxohormone	198 ± 12	< 0.01

* Results are expressed as the mean ± standard error of 5 rats.

Toxohormone has been shown to interfere principally with the metabolism of iron-containing proteins (See general introduction). Nakahara and Fukuoka (1958) suggested that because of the fundamental biological importance of many of these proteins, it would not be unreasonable to suggest that cancer cachexia might ultimately be related to toxohormone activity. The same authors suggested that toxohormone

might be responsible for the induction of a hormonal imbalance in the tumour-bearing host, which could contribute to the cachectic state (Nakahara and Fukuoka, 1961).

The present results have confirmed that toxohormone has an inhibitory effect upon protein synthesis in muscle, and the fact that this activity is also evident in vitro, Table 31, indicates that the muscle itself could be a site of action of toxohormone, and that its effect on muscle protein need not be a consequence of a general disturbance of the metabolism of the host.

GENERAL DISCUSSION

It was shown in Section 1 that diaphragm muscle was subject to both loss of weight and loss of protein during the growth of the Walker 256 carcinoma. The percentage distribution of the intracellular proteins was altered, the loss of contactile proteins being significantly greater than the loss of sarcoplasmic proteins. This finding, similar to the results obtained for the gastrocnemius and soleus muscles by Clark and Goodlad (1971), confirmed that the diaphragm is one of the muscles affected by tumour growth.

The incorporation of labelled lysine into mixed muscle proteins from tumour-bearing animals was greatly decreased (Section 2), and, as in the experiments on the quantitative depletion of muscle proteins, a differential effect was observed on the two protein fractions examined. The sarcoplasmic proteins showed a slight but persistent increase in incorporation, whilst the uptake of radioactive amino-acid into the myofibrillar fraction was greatly reduced. There has been much evidence recently that reutilisation of labelled amino-acids, following a single injection, can obscure the true processes of protein synthesis and catabolism (Henshaw et al., 1968; Gan and Jeffay, 1967; Waterlow and Stephen, 1968; Klevecz, 1971). It seems probable, however, that the influence of the size

of the intracellular amino-acid pool may be less *in vitro* than *in vivo* (Hider *et al.*, 1969, 1971; Waterlow and Stephen, 1968; Millward, 1970a) and Clark and Goodlad (unpublished results) have shown that polysomes isolated from the gastrocnemius muscle of tumour-bearing rats show 50% less activity in a cell-free incorporating system than polysomes from normal muscle. It would appear, therefore, that the decreased incorporation of label into the protein fractions noted in this work can be attributed to a true inhibition of protein synthesis in the muscle from the tumour-bearing animal. The methods used in these experiments would not detect any alteration in the rate of protein catabolism which might result from the action of the tumour, and this factor cannot be eliminated as a possible contributory element in the present results.

Atrophy of skeletal muscle occurs as a result of a number of pathological conditions other than tumour growth, such as muscular dystrophy (Milhorat, 1967), denervation (Zak, 1962), starvation (Millward 1970b) or glucocorticoid treatment (Kochakian and Robertson, 1951).

Millward (1970b) showed that the rate of synthesis of sarcoplasmic protein in the muscles of starved animals fell to about 50% of the control level, but that the catabolic rate remained unchanged. With the myofibrillar

fraction, there was again a profound drop in the synthesis rate during starvation, accompanied by an increase in the rate of catabolism. Waterlow and Stephen (1966) and Millward (1970b) have shown that over long periods of starvation or protein deficiency, the loss from both fractions of protein is equal.

In genetically dystrophic mice, various investigators have observed a normal or increased incorporation of radioactivity into muscle protein following the *in vivo* injection of labelled amino-acids (Kruh *et al.*, 1960; Simon *et al.*, 1962; Coleman and Ashworth, 1959; Srivatava and Berlinguet, 1966; Weinstock, 1966), indicating that the synthesis of muscle proteins in maintained or even increased in dystrophic muscles. This finding would imply an increased catabolism in such muscles, to account for the observed atrophy, and increased catheptic activity has in fact been reported in nutritional muscular dystrophy (Weinstock, 1966). Berlinguet and Srivatava (1966) have suggested the presence of a specific muscle protease in dystrophic muscles.

That innervation plays an important role in the functional and metabolic activity of skeletal muscle can be shown by reversal of the nerve supply of tonic and phasic muscles, which results in a corresponding reversal of both

the functional and chemical characteristics of the two muscle types (Guth et al, 1968). There are conflicting ideas concerning the influence of denervation upon muscle protein metabolism (Schapira et al., 1953; Padieu, 1959; Pater and Kohn, 1967), but the work of Goldberg (1969) suggests that there is an increased catabolism of muscle protein, especially affecting the myofibrillar fraction, accompanied by a decrease in protein synthesis. In vitro studies on frog leg muscle have tended to support this theory (Muscatello et al., 1965; Margreth et al., 1966), although Hollosi and Balogh (1969) found the protein response to denervation to be genus characteristic.

Furthermore, there is evidence to suggest that the response of the diaphragm to severance of the phrenic nerve is probably unique. Harris and Manchester (1966) noted a transitory hypertrophy, accompanied by an elevation of incorporation into diaphragm muscle proteins, following denervation. The increased catabolism in the denervated muscle has been associated with increased lysosomal activity (Pellegrino and Franzini, 1963; Pollack and Bird, 1968).

Glucocorticoid-induced atrophy has also been shown to be due to increased catabolism, but in this case both myofibrillar and sarcoplasmic fractions are decreased by the

same amount (Goldberg, 1969), and the increase in catabolism is probably not due to increased lysosomal activity. (Weissman and Thomas, 1964). It was pointed out in the Introduction that tumour-bearing rats are not diabetic, although they appear to show a decreased response to a glucose test-load. It was shown in Section 2 that insulin has a roduced stimulatory effect upon the incorporation of label into muscle proteins from tumour-bearing animals, indicating that there may be some interference with the mechanism of insulin action. Wool et al(1968) have shown that injection of insulin into normal rats appears to cause a qualitative rather than a quantitative change in the protein synthetic capacity of normal muscle polysomes, and Young et al. (1968) showed that in this situation the proportion of large polysomes in muscle is increased. This is consistent with the observation in the present work of an increase in the incorporation of label into the myofibrillar proteins of normal muscle, with relatively little effect on the sarcoplasmic fraction. The response of polysome aggregation to insulin has been shown to be diminished by fasting for 2 days (Young et al., 1968), and it is possible that any condition of the animal which tends to suppress protein synthesis such as fasting or tumour growth will suppress the response to insulin.

Thus the systemic effects of the tumour do not appear to be explicable in terms of any interference with the nutritional or hormonal state of the animal. The atrophies produced by glucocorticoids, muscular dystrophy and denervation have been attributed, either wholly or partly, to increased catabolism within the muscle cell. While the role of protein catabolism in carcinomatous cachexia has not been investigated, there is evidence that there is no increase in the lysosomal population or the free acid phosphatase activity in muscles from tumour-bearing animals (Goodlad and Clark, unpublished results). The atrophy of the denervated muscle appears to result from mechanisms which are in direct contrast to those operative in work-induced hypertrophy (Goldberg 1969), and the role of muscular paralysis in denervation requires to be elucidated.

Most important, however, is the conclusion from the present results that the effects of the tumour upon muscle protein metabolism are not consistent with the 'Nitrogen trap' theory, proposed by many early workers (see Introduction). The patterns of protein depletion and protein synthesis in muscles from starved and tumour-bearing rats are quite different (Sections 1,2; Sobel,1966; Waterlow and Stephen, 1968; Millward, 1970b). This

conclusion was supported by the evidence that supplementation of the incubation medium in the present experiments with either high concentrations of lysine, or with a mixture of amino-acids, does not alleviate the inhibition of incorporation of labelled lysine into muscle proteins of the tumour-bearing rat. This finding rules out the possibility that the observed inhibition is due to a deficiency of one or several amino-acids essential for protein synthesis.

Although imbalance induced in both circulating amino-acid levels and hormone levels will undoubtedly contribute to the gross effect observed in the tumour-bearing animal, it seems improbable on the basis of the evidence presented in the Introduction and in the body of this work that the primary mechanism of tumour action could be attributed to such an imbalance.

It is now well-documented that depression of liver catalase activity and the level of plasma-bound iron are inevitable corollaries of tumour growth in an animal (see Introduction). These effects have been attributed to the action of toxohormone, a toxin secreted by the tumour (Nakahara and Fukuoka, 1948). The history of toxohormone and the evidence for its humoral nature are presented in the Introduction (page 23).

To evaluate the role of toxohormone in tumour-host relations it is necessary to establish the type of biological effects which can be produced in normal animals by the injection of an isolated toxohormone fraction. The first, and most often described effect was that of the depression of the activity of the enzyme liver catalase (Nakahara and Fukuoka, 1948). Several other effects on iron metabolism have been reported, notably a decrease in the level of liver ferritin (Hoshizima, 1957) and an increase in liver protoporphyrin (Ono et al., 1956). The latter finding reflects the reduced ability of the liver of toxohormone-treated animals to synthesis catalase (Hozumi and Sugimura, 1952).

As reported in Section 3 of this work, toxohormone was found to reduce the incorporation of labelled amino-acid into the protein of normal rat muscle quite dramatically both in vivo and in vitro. In the in vivo experiments, the muscles were exposed to the toxohormone in circulation in the rat, and incubated in a medium free of toxohormone. The fact that an inhibition of uptake of label was observed under these condition indicates that the effect of toxo-hormone was not readily reversible, and could support the hypothesis that toxohormone was acting directly upon the muscle, rather than through any intermediate metabolic

processes. That this was the case was demonstrated by the direct *in vitro* action of toxohormone upon the incorporation of label into normal muscle protein.

The apparent irreversibility of the effect on muscle protein is interesting in view of the fact that Kampschmidt *et al*,(1959) showed that the effect of toxohormone upon liver catalase and plasma iron were transitory, these parameters being restored to their normal levels 96 and 48 hours respectively after a single injection of toxohormone. However, the same workers showed that the alterations in organ weights displayed no signs of recovery at the longest time interval studied.

The question of the normal function of toxohormone in the non-neoplastic cell has not been discussed to a great extent in the literature, yet there seems little doubt that a toxohormone-like substance can be isolated, admittedly in small amounts, from certain normal tissues. Spleen appears to be a particularly good source of such material (Day *et al*.,1954; Nixon and Zinman, 1966). A recurring idea has been that toxohormone is toxic only when produced in abnormal amounts by the tumour, and that in normal cells it functions either specifically as a regulator of liver catalase synthesis (Nakahara and Fukuoka, 1958) or, perhaps more reasonably in view of the

wide range of effects, as a general regulator of metabolic activity (Greenstein, 1955).

The evidence presented in this work suggests that toxohormone does play an important part in the mediation of the effects of the tumour, but much work remains both in the further purification and characterisation of the material, and in the elucidation of the mechanism of its action. Of immediate interest would be the investigation, using the methods described in this work, of the effect of toxohormone upon the incorporation of label into the sarcoplasmic and myofibrillar protein fractions of normal muscle, in order to discover if the characteristic effect of the tumour upon the myofibrillar fraction alone is repeated. Another useful line of research would be the interaction of toxohormone and insulin, in view of the fact that insulin appears to have a greatly reduced effect in the tumour-bearing animal. Insulin has no stimulatory effect when added directly to a cell-free system from muscle (Wool et al, 1968), indicating that its mechanism of action could be primarily due to an effect upon the cell membrane. Similar studies with toxohormone could throw a great deal of light upon its site of action and the mechanism of its operation.

SUMMARY

1. The effect of the growth of the Walker 256 carcinoma upon various aspects of rat diaphragm protein metabolism was investigated.

2. Microscopic examination of diaphragm muscle from tumour-bearing rats revealed that the mean muscle fibre diameter was reduced compared with the normal animal.

3. The extracellular space of both 'cut' and 'intact' diaphragm preparations was measured, using sorbitol and inulin as extracellular markers. No differences were observed in the extracellular space of both types of diaphragm preparation from normal and tumour-bearing rats, using sorbitol as the marker. With inulin as marker, the extracellular space was much smaller in the 'cut' diaphragm preparation from the tumour-bearing animal.

4. Quantitative analysis of diaphragm muscle showed that the muscle lost weight during the growth of the tumour and that part of the weight loss was due to the loss of protein. Further, the loss of contractile proteins appeared to be more extensive than the loss of sarcoplasmic proteins.

5. The *in vitro* incorporation of ^{3}H-lysine into mixed proteins of rat diaphragm was shown to be greatly

reduced by the growth of the tumour. This effect seemed to be directed principally at the myofibrillar proteins. The inhibition was not alleviated by the addition of either high concentrations of lysine or a mixture of amino-acids to the incubation medium.

6. Normal diaphragm muscles incubated in serum from a tumour-bearing rat exhibited a greatly decreased uptake of labelled lysine into the myofibrillar proteins, and this pattern was not altered by supplementation of the medium with a mixture of amino-acids.

7. The stimulatory effect of insulin upon muscle protein synthesis was greatly reduced by the growth of the tumour. In the normal animals studied, the effect of insulin seemed to be directed principally towards the myofibrillar proteins

8. A toxohormone fraction was prepared from Walker tumour tissue by an acetic acid extraction method, and purified by cation-exchange chromatography and gel-filtration. The biological activity of the preparation was assayed by measurement of its effect upon the plasma iron level in mice.

9. The effect of the purified toxohormone fraction upon

muscle protein synthesis was investigated both *in vivo* and *in vitro*, and the toxohormone was observed to exert a profound inhibitory effect upon the incorporation of labelled lysine into mixed intracellular muscle proteins.

APPENDIX

This appendix contains the data-processing programme written i
in Fortran 1V for the IBM 360/44 computer. The programme was stored on RAX disc, and input data could be read from punch· cards, paper tape or a remote teletype terminal.

The programme is designed to calculate the specific activities of muscle protein fractions, incubated in medium with and without the addition of insulin. The programme also performs a three factor analysis of variance upon the calculated date.

The abbreviations used are as follows:

TMI: tumour-bearing animal, myofibrillar protein fraction, with insulin added to the medium.

TSI: tumour, sarcoplasmic, insulin added.

TMS: tumour, myofibrillar, no insulin added.

TSS: tumour, sarcoplasmic, no insulin added.

CMI: control, myofibrillar, insulin added.

CSI: control, sarcoplasmic, insulin added.

CMS: control, myofibrillar, no insulin added.

CSS: control, sarcoplasmic, no insulin added.

Data is read in as follows:

Card 1: number of muscles in each group (GP); background counts on scintillations counters (BGD).

Card 2: counts/minute TMI 1; weight in mgs. TMI 1.

Card 3: counts/minute TMI 2; weight in mgs. TMI 2.

.
.

Card n: counts/minute CSS (GP); weight in mgs. CSS (GP)

Output provides computed values for the specific activities of each of the protein samples, the mean and the standard error of the mean for each group, and an analysis of the variance between each of the eight groups.

```
      IMPLICIT INTEGER (A-R), REAL*8 (S-Z)
      DIMENSION CPM(15,8), W(15,8), D(15,8), A(8), AA(8),
     1AM(8), VE(8), SE(8), SEP(20), Z(15,8), X(8)
      READ (5,200) GP,BGD
      READ (5,210)   ((CPM(I,J),W(I,J),I=1,GP),J=1,8)
      G=0
      ZS=0.0
      R=0
      RS=0
      DO 51 J=1,8
      A(J)=0
      X(J)=0.0
   51 AA(J)=0
      DO 55 I=1,GP
      DO 55 J=1,8
      D(I,J)=(CPM(I,J)-BGD)/W(I,J)
   55 Z(I,J)=DSQRT(DFLOAT(D(I,J)))
      DO 60 J=1,8
      DO 65 I=1,GP
      A(J)=A(J)+D(I,J)
      X(J)=X(J)+Z(I,J)
   65 AA(J)=AA(J)+D(I,J)*D(I,J)
      AM(J)=A(J)/GP
      VE(J)=AA(J)/GP-AM(J)*AM(J)
      SE(J)=DSQRT(VE(J))/GP**0.5
      G=G+A(J)
   60 ZS=ZS+X(J)
      ZCF=ZS*ZS/(8*GP)
      TSS=DFLOAT(G)-ZCF
      DO 75 I=1,GP
```

```
      DO 70 J=1,8
   70 SEP(I)=SEP(I)+Z(I,J)
   75 ZR=ZR+SEP(I)*SEP(I)
      SRS=ZR/8.0-ZCF
      DO 80 J=1,8
   80 ZRS=ZRS+X(J)*X(J)
      TRS=ZRS/GP-ZCF
      SER=TSS-SRS-TRS
      XT=X(1)+X(2)+X(3)+X(4)
      XC=X(5)+X(6)+X(7)+X(8)
      TUMS=(XT-XC)**2/(8*GP)
      XP=X(1)+X(2)+X(5)+X(6)
      XM=X(3)+X(4)+X(7)+X(8)
      TNS=(XP-XM)**2/(8*GP)
      U=X(1)+X(3)+X(5)+X(7)
      XS=X(2)+X(4)+X(6)+X(8)
      TMS=(U-XS)**2/(8*GP)
      XX=X(8)+X(2)+X(7)+X(1)
      XY=X(3)+X(4)+X(5)+X(6)
      TXI=(XX-XY)**2/(8*GP)
      YX=X(8)+X(4)+X(1)+X(5)
      YY=X(2)+X(3)+X(6)+X(7)
      XMI=(YX-YY)**2/(8*GP)
      Y1=X(8)+X(3)+X(6)+X(1)
      Y2=X(2)+X(4)+X(5)+X(7)
      TXM=(Y1-Y2)**2/(8*GP)
      TMI=TRS-TUMS-TNS-TMS-TXI-XMI-TXM
      SRS=SRS/(GP-1)
      SER=SER/(7*(GP-1))
      DF1=GP-1
```

```
      DF2=1
      DF3=7*(GP-1)
      DF4=8*GP-1
      ZF1=SRS/SER
      ZF2=TUMS/SER
      ZF3=TNS/SER
      ZF4=TMS/SER
      ZF5=TXI/SER
      ZF6=XMI/SER
      ZF7=TXM/SER
      ZF8=TMI/SER
      WRITE (6,301) BGD
      WRITE (6,260)
      WRITE (6,270)
      DO 100 I=1,GP
      WRITE (6,300)  (CPM(I,J),J=1,8)
  100 WRITE (6,302)  (W(I,J),J=1,8)
      WRITE (6,275)
      WRITE (6,270)
      WRITE (6,305)  (D(1,J),J=1,8)
      DO 110 I=2,GP
  110 WRITE (6,310)  (D(I,J),J=1,8)
      WRITE (6,350) (AM(J),J=1,8)
      WRITE (6,351) (SE(J),J=1,8)
      WRITE (6,390)
      WRITE (6,391)
      WRITE (6,400)  DF1,ZF1
      WRITE (6,403)  DF2,ZF2
      WRITE (6,404)  DF2,ZF3
      WRITE (6,405)  DF2,ZF4
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      WRITE (6,406)   DF2,ZF5
      WRITE (6,407)   DF2,ZF6
      WRITE (6,408)   DF2,ZF7
      WRITE (6,409)   DF2,ZF8
      WRITE (6,402)   DF3
  200 FORMAT (I3,I5)
  210 FORMAT (I5,F10.2)
  260 FORMAT (14H0ORIGINAL DATA)
  270 FORMAT (1H0,4X,4H TMI,5X,4H TSI,5X,4H TMS,5X,4H TSS,5X,4H CMI,5X,
     14H CSI,5X,4H CMS,5X,4H CSS)
  275 FORMAT (7H1CPM-MG)
  300 FORMAT (1H0,3X,8(I5,4X))
  301 FORMAT (13H1BACKGROUND :,I5)
  302 FORMAT (1H ,3X,8(F6.2,3X))
  305 FORMAT (1H0,3X,8(I5,4X))
  310 FORMAT (1H ,3X,8(I5,4X))
  350 FORMAT (1H0,3X,8(I5,4X),5X,6H MEANS)
  351 FORMAT (1H0,8(F9.2),5X,11H STD.ERRORS)
  390 FORMAT (9H1F VALUES)
  391 FORMAT (1H0,15X,5H D.F.,6X,3H F.)
  400 FORMAT (1H ,15X,I3,6X,F6.2,5X,11H REPLICATES)
  403 FORMAT (1H ,15X,I3,6X,F6.2,5X,15H TUMOUR-CONTROL)
  404 FORMAT (1H ,15X,I3,6X,F6.2,5X,15H INSULIN-SALINE)
  405 FORMAT (1H ,15X,I3,6X,F6.2,5X,16H MUSCLE FRACTION)
  406 FORMAT (1H ,15X,I3,6X,F6.2,5X,20H XION TUMOUR-INSULIN)
  407 FORMAT (1H ,15X,I3,6X,F6.2,5X,20H XION INSULIN-MUSCLE)
  408 FORMAT (1H ,15X,I3,6X,F6.2,5X,19H XION TUMOUR-MUSCLE)
  409 FORMAT (1H ,15X,I3,6X,F6.2,5X,14H 3 FACTOR XION)
  402 FORMAT (1H ,15X,I3,17X,9H RESIDUAL)
      END
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