THE METABOLIC PROBLEM POSED BY CANCER CACHEXIA

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ABSTRACT

Cachexia is often a vital consideration in assessing the survival prospects of cancer patients. Approximately two-thirds of all patients with malignant disease are cachectic at first diagnosis or become so at a later stage. The effect of the cachectic condition on the course and outcome of the cancer therapy can be very significant. At present, however, little is known about the mechanisms that result in this condition, its true frequency or heterogeneity, the extent of tumour involvement in causing metabolic alterations in the host or the secretion of "ectopic factor(s)" acting on host tissues. There appears no simple correlation between the degree of cachexia and the type, size, site or degree of metastatic spread of the tumour, but removal of the tumour has been associated with complete reversion of the cachexia syndrome.

An assessment of the degree of cachexia in certain cancer patients was undertaken. An epidemiological study involving anthropometric determination of the muscle and fat areas of 52 cancer patients and 258 control patients showed that cancer patients, other than those with cancer of the breast, had significantly lower muscle and fat areas when compared on the basis of matched standard values. Breast cancer patients as a group are not cachectic and have increased fat and muscle areas, irrespective of whether they are in remission or have progressive disease.

An *in vivo* study involving measurement of the arterial and arterio-venous concentration differences across the forearms of 12 cancer weight-losing patients; 8 noncancer weight-losing patients and 9 normal controls was undertaken. A significant increase in the number of amino acids in venous excess was found in the weight-losing cancer patient group as compared with both other groups, suggesting that enhanced muscle proteolysis occurred in the cancer patients or

alternately, that decreased utilization of amino acids by the muscle tissue i.e. decreased proteogenesis, was a factor in these patients.

Rates of fat and muscle protein breakdown were determined in vitro in tissue incubation systems; in the case of the latter, cell culture systems were also used, in order to try to determine a "direct" or "indirect" effect of tumours on host tissue, possibly contributing to overall weight loss. There were no differences in the effects of serum, either "whole" or desalted, obtained from weight-losing cancer patients and matched control patients, in respect of heterologous promotion of lipolysis or proteolysis, and thus no evidence was apparent for the presence of "ectopic factor(s)". However, this model is not definitive due to methodological limitations, and the true causes of cancer-associated cachexia remain unknown except in those patients whose appetite or gross intestinal function are being affected.

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Proverbs 3 : 5,6

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ABBREVIATIONS AND SYMBOLS

ATP	: adenosine 5'-triphosphate
$CaCl_2$	: calcium chloride
Ci	: curie (3.7×10^{10} dpm)
cm^2	: square centimeters
CO_2	: carbon dioxide
dpm	: disintegrations per minute
em	: emitter
ex	: exciter
g	: grams
g	: acceleration due to gravity
$[^3H]$	: tritium
hr(s)	: hour(s)
KCl	: potassium chloride
Kg	: kilogram
$KHCO_3$	: potassium bicarbonate
$KHPO_4$	: potassium dihydrogen phosphate
M	: molar concentration
mg	: milligram
$MgSO_4$	: magnesium sulphate
mins	: minutes
ml	: millitre
mM	: millimolar concentration
mm	: millimeter
µg	: microgram
NaCl	: sodium chloride
NAD	: nicotinamide adenine dinucleotide
NADH	: nicotinamide adenine dinucleotide, reduced
$NaHCO_3$	: sodium bicarbonate
n (prefix)	: nano ($\times 10^9$)
nm	: nanomoles
O_2	: oxygen
PEP	: phospho(-enol) pyruvate
pH	: negative logarithm of the hydrogen ion concentration
pg	: picogram

S.D. : standard deviation $= \frac{\{\Sigma x^2 - \frac{(\Sigma x)^2}{n}\}^{\frac{1}{2}}}{\{n - 1\}^{\frac{1}{2}}}$

S.E. : standard error of the mean

$$= \frac{\{\Sigma (x - \bar{x})^2 / (n - 1)\}^{\frac{1}{2}}}{n^{\frac{1}{2}}}$$

> : greater than

< : less than

% : percentage

CONTENTS

CHAPTER 1

THE FIELD OF CANCER CACHEXIA

Cachexia, or inanition, has long been recognised as a leading cause of death in cancer (1). As many as two-thirds of all patients with malignant disease are cachectic at the time of first diagnosis or become so, at a later stage (2). Indeed, many cancer patients initially present with significant weight loss as their first and only symptom, in the face of an apparently adequate diet (3). In such patients, a reduced intake of food may later contribute to cachexia. Clearly, a substantial number of cancer sufferers lose body mass as a result of gastrointestinal disease which depresses the appetite, impairs digestion or causes malabsorption. There may also be anxiety-caused anorexia, nausea produced by drug administration and post-operative catabolic states.

Cachexia is characterised primarily by weakness, anorexia, depletion (and occasionally redistribution) of body tissues and an inability to maintain normal regulatory functions (4,5). There is no simple relationship between the degree of cachexia and the type, size, site or degree of metastatic spread of the tumour (4).

The effects of cachexia on the course and outcome of cancer therapy is probably very significant. Median survival was significantly shorter in the case of patients with weight loss than in that of patients presenting without loss of weight and chemotherapy response rates were also lower in the patients with weight loss (6). The cachectic condition may even limit or preclude cancer therapy in certain instances (7).

Weight loss may arise as an indirect and mechanistically clear-cut consequence of malignancy or it may be an apparently inexplicable feature of the cancer-host relationship, as suggested by the lack of correlation that sometimes exists between

the degree of weight loss and various clinical parameters (8). Thus, Strain et al (9) have shown severe weight loss to occur (up to 25% body weight) in rats bearing a xenografted hypernephroma from a weight-losing human patient treated by surgical removal of his tumour. Dietary intakes were not decreased and malabsorption contributed little, if anything, to the loss of weight observed in these animals. This situation is very similar to that very commonly observed in spontaneous tumour malignancies.

Removal of tumours by surgical procedure or regression brought about by other treatment modalities have often been associated with complete reversion of the cachexia syndrome (10).

The mechanisms by which single tumours may cause the loss of body tissue and other metabolic alterations in their hosts is not known at this time. Various proposals have, however, been made based on diverse lines of research and clinical observation, and these will be reviewed in this chapter.

1.1. TUMOUR-HOST RELATIONSHIPS

The ability of tumour cells to establish themselves in host tissue is inherently necessary for their survival. The growing tumour relies on the host to supply all the requirements for cell replication and growth i.e. the precursors for protein and nucleic acid biosynthesis, as well as carbohydrate and lipid fuels to supply energy. The body fluids of the host provide a proximal source for these nutrients, the diet being a more distal source.

In principle, tumours may be able to exert diverse systemic effects to ensure their survival in the competition for important metabolites and trophic factors. The generalized, inter-organ reaction of the host to the effects of the tumour may constitute part or most of the basis for the cachectic phenomenon seen in many cancer patients.

1.1.1. Tumour Effects on Protein Metabolism.

Loss of total body protein, and in particular muscle protein, has been observed and quantified in both animal experimental models (11,12,13) and in cancer patients (14,15). Hypoalbuminaemia is a common sign encountered in malignant disease and may often provide evidence for marginal protein malnutrition in cancer patients (16,17).

1.1.1.1. Tumours acting as nitrogen "traps".

That tumours may act as nitrogen "traps" was postulated by Mider (18) and has since been confirmed by many workers (16,17). Tumours have the capacity to take up nitrogen derived from host tissue proteins. Once within the tumours, this nitrogen is not released again to the host tissues (16).

The nitrogen contents of various host tissues in rats bearing Walker 256 carcinomas were found to be decreased by Sherman *et al* (13). Similar results were obtained in rabbits carrying Brown-Pierce carcinomas (17). Costa *et al* (14), however, reported no changes in the nitrogen content of muscles from cancer patients compared with tissue obtained from controls.

There is no reason to believe that tumours cannot consume both exogenous (dietary) nitrogen and nitrogen from endogenous sources (17). An idea put forward by Sherman *et al* (13) is that dietary nitrogen is the major nitrogen source for tumours, during a short period of initial growth, but that thereafter the dietary intake may frequently be inadequate, so that dispensable stores of nitrogen from various host tissues are given up to meet the increasing needs of the tumour. This is clearly a somewhat simplistic notion in view of the integrated nature of whole body nitrogen metabolism.

Nitrogen balance studies in cancer patients have so far been equivocal, and a considerable variation in the picture from patient to patient must be anticipated (13,17).

1.1.1.2. Uptake of amino acids by tumours.

Tumour requirements for amino acids are influenced by the apparently slow rate at which non-essential amino acids are synthesized, by tumour tissues *in vivo* (16). Generally, amino acid requirements of tumour cells are beyond their own synthetic ability and there is therefore a demand on the host to provide essential and many non-essential amino acids. Glutamine, for example, is hardly detectable in tumour cells (16) and must be considered to be an absolute requirement. Tumour cell membranes appear to be readily permeated by this amino acid (19). Factors that may favour uptake of amino acids from the host are not known, but for those amino acids present at low concentration within tumour cells, a gradient may operate to promote efficient uptake of these essential nutrients.

The availability of the host amino acid pool is thus probably a factor of great importance for tumour nutrition. Hence systemic effects exerted by tumours may well influence the rates of proteolysis and proteogenesis in host tissue. Tumours have a unique capacity to grow in starving animals and to concentrate amino acids under extremely adverse conditions (16).

1.1.1.3. Increased protein degradation in the tumour-bearing host.

Increased degradation of muscle proteins has been observed by Lundholm *et al* (15) in cancer patients and in tumour-induced mice. A tumour-associated increase in some lysosomal enzyme activities was also found: cathepsin-D and β-glucuronidase activities were increased, while acid phosphatase activity remained unchanged.

The simultaneous increase in lysosomal enzyme activity *in situ* and in the fractional degradation rate of muscle proteins in cancer patients may thus point to a role of these host tissue

enzymes in the development of cachexia.

Clarke et al (20) failed to detect signs of excessive protein catabolism in the muscle tissue of five patients with progressive disease. The peripheral amino acid levels were measured and no evidence of an increased venous excess was found.

1.1.1.4. Decreased protein synthesis in the tumour-bearing host.

Isolated diaphragms derived from rats bearing Walker 256 carcinomas incorporated less [^{3}H]-4,5-lysine into myofibrillar proteins than did the muscle tissue of controls (12). A "toxohormone" preparation from the tumour itself, added to the incubation medium of control rat hemi-diaphragm pieces, decreased the uptake of the labelled amino acid. This also happened when the material was injected into live control rats, and theincorporation of thelabelled amino acid into hemi-diaphragm pieces prepared from these animals was measured (12).

Goodlad and Clarke (11) have found an inhibition of [^{3}H]-lysine incorporation in polyribosomal preparations from gastrocnemius, but not soleus, muscle of tumour-bearing animals. Their results indicated that the inhibition of protein synthesis occurred at the level of translation. Lundholm et al (21) have shown significantly decreased incorporation of radioactive leucine into skeletal muscle proteins in homogenates prepared from muscle material derived from cancer patients. Amino acid addition stimulated leucine incorporation, when the enrichment was effected at ten times the concentration of the individual amino acids in human plasma. They proposed, on the basis of this amino acid stimulation, that the decreased protein synthesis observed was not due to any defect in the mechanism for initiation, elongation or termination of protein biosynthesis.

The ability of added amino acids to restore the incorporation of label into muscle protein to control levels, suggests that amino acid unavailability may be the reason for decreased overall protein synthesis, in preparations not supplemented with added precursors. The ability of tumours to deplete the amino acid pools of their hosts may be brought about not only through sequestration of amino acids but also as a result of general stimulation of gluconeogenesis, in the host. A full amino acid complement for protein biosynthesis may consequently not be available to the host (12,20). This would decrease the overall rate of protein synthesis and increase the concentration of amino acids in the body available to the tumour, or an acceleration of host gluconeogenesis may be brought about. Specific requirements of tumours for particular amino acids (e.g. glutamine and asparagine) and their facilitated uptake into tumour cells (19), would support this concept, although no evidenceis available to show that preferential sequestration of amino acids by tumours occurs. The constant turnover of proteins, in the steady state, in the host would enable tumours to sequester the amino acids released from host tissues without any direct effect on the net protein content of host tissues.

1.1.2. Tumour Effects on Lipid Metabolism.

1.1.2.1. Lipid requirements of tumours.

The available evidence on the rate and sufficiency of synthesis of lipids by tumour cells is equivocal. Most experiments have dealt with synthesis of lipids *in vitro* and may thus not relate to the *in vivo* situation (16).

Tumours are thought to utilize host cholesterol and fatty acid stores and not to accumulate these lipids to any great extent (17). The exact role of lipids in tumour metabolism is not known, but it is certain that cholesterol plays a

structural role and is assimilated into tumour membranes. As an energy source, fatty acids appear to play a minor role. Experiments suggesting tumour oxidation of mobilized lipids have been performed mostly *in vitro* under aerobic conditions (22). *In vivo*, tumour cells are frequently in a relative and varying state of hypoxia and are generally, although not universally, capable of maintaining high rates of glycolysis even in the presence of oxygen (23). The primary energy source of tumour cells is thus glycolysis.

1.1.2.2. Loss of body fat in tumour-bearing hosts.

Swiss mice bearing Krebs-2 carcinomas have displayed profound and sustained fat loss during tumour growth (24). This depletion of body fat occurred in three phases: an initial rapid phase when 50% of body fat was lost even though the tumour was barely detectable; a second phase of steady-state fat loss when tumour growth was rapid; and a third terminal phase, characterized by substantial fat loss. The authors proposed that a lipolytic factor caused the initial phase of fat loss but provided no evidence for the factor.

Work done on compositional changes in human muscle during malignant growth have shown that the muscles of cancer patients have a fat content, per unit wet mass, that is 50% lower than that of muscles from control patients (14). Kralovic *et al* (25) using rats bearing Walker-256 carcinomas, showed a reduction of total body neutral lipid when tumours reached a mass equal to about 6-7% of total body mass, but no host lipid loss was observed when tumours were at about 4% of the host body mass.

1.1.2.3. Increased plasma fatty acid concentrations in tumour-bearing hosts.

Increased concentrations of unesterified plasma free fatty acids have been observed in cancer patients, as a group, when they were compared with non-cancer patients. Those

patients with active metastatic disease showed individually increased plasma free fatty acid levels (26).

This effect has also been seen in rats bearing induced tumours (27).

Mays (22), in a detailed study of serum lipids in 15 cases of human cancer, was unable to confirm the finding of increased fatty acid levels and concluded that, in the absence of liver dysfunction, plasma fatty acid levels of far-advanced cancer patients remained within the normal range even though marked loss of fat depots occurred. Holroyde et al (8), in a study of eight cancer patients having progressive weight loss compared to six cancer patients having no weight loss, found no difference in plasma fatty acid concentrations.

1.1.3. Tumour Effects on Carbohydrate Metabolism.

1.1.3.1. Tumour cell demand for glucose.

Aerobic tumour cells do not appear to use the Krebs cycle to the same extent as do normal cells. Warburg (23), writing authoritatively, at an early stage, on the metabolism of tumours, stated that an absence or defect in the oxidative Krebs cycle is inherent in tumour cell metabolism. It has become widely accepted that cancer cells oxidise glucose very actively even in the presence of oxygen. Tumour cells, even those derived from gluconeogenic tissues, are incapable of synthesizing glucose (16).

Lactate producing glycolysis does not produce as great an amount of energy as does complete oxidative catabolism of this carbohydrate; the needs of the tumours for a glucose source are, thus, considerable. Also, if intermediates of glycolysis such as glucose-6-phosphate and fructose-6-phosphate are channelled into the hexose-monophosphate shunt for the biosynthesis of purines, pyrimidines, DNA and RNA, a further demand for glucose is caused (28). The contribution to total

glucose metabolism of the hexose phosphate pathway in tumour cells in vivo has not yet been estimated, but it is known that the necessary enzymes are present (16).

Tumour cells contain undetectable amounts of free glucose (17) and appear to lack a storage capacity for glucose (16). Tumours, therefore, function as glucose "traps" in order to survive (17). Rapid uptake of glucose by tumour cells may be the result of a change in the permeability of the cell membrane. Alternatively, a large glucose gradient may drive the monosaccharide into tumour cells, since glucose is lacking in the cytoplasm. Many cancer patients show diabetic glucose tolerance (10,29,30,31).

Episodes of hypoglycaemia in cancer patients and in tumour-bearing animals have been reported (17). On the other hand, many tumour-bearing hosts do not become hypoglycaemic (32). For example, fasting blood glucose levels were normal in a group of weight-losing cancer patients seen in a study by Waterhouse and Kemperman (31). Maintenance of normoglycaemia in the face of increasing demands for glucose by a growing tumour could be accomplished by altering the metabolism pattern of the host, i.e. by increasing the gluconeogenic activity (32) or by using alternative fuels, e.g. free fatty acids, thus "sparing" glucose to be used by the tumour. An increased dietary intake of glucose would also balance the hypoglycaemic strain of the tumour on the host.

1.1.3.2. Increased glucose provision by the host.

Glycogen Depletion.

Depletion of glycogen stores in the muscles and liver of tumour-bearing hosts has been reported in the case of experimental animals bearing a variety of tumours (17,32). However, a hypoglycaemic condition developed in rabbits bearing Brown-Pierce carcinomas, showing substantial glycogen depletion (33). Thus the role of glycogen mobilization, in compensating

the hypoglycaemic pressure exerted by the tumour on the host, has been questioned. Shapot (17) provided evidence for a direct correlation between host liver glycogen depletion and the acceleration of liver gluconeogenesis.

Increased glucose synthesis.

The rate of gluconeogenesis is increased in many cancer patients (8,20) as well as in tumour-bearing animals (17). Singh et al (32) showed increased gluconeogenic rates in rats bearing transplantable sarcomas and treatment of the animals with an inhibitor of gluconeogenesis caused hypoglycaemia. The importance of an increased gluconeogenic rate in maintaining normal blood glucose levels was thus evident.

Gluconeogenesis is a process which occurs in liver, kidney and probably in white muscle tissue, under multihormonal control. Increased plasma levels of glucocorticoids increase the activity of key enzymes in the gluconeogenic pathway, as do increases in growth hormone. Singh et al (32) were unable to establish that changes in key hepatic enzymes caused the increased gluconeogenesis observed. An alteration in insulin release or binding, or insulin insensitivity, may lead to gluconeogenesis. A defect in insulin release in cancer-bearing patients was reported by Jasani et al (30), but normal though delayed release was shown by Schein et al (10). Schein et al also showed normal insulin receptors to be present on monocytes, although a resistance to administered insulin was seen in cancer patients showing glucose intolerance.

An effect of the tumour, therefore, on the various hormonal control points of gluconeogenesis can directly increase the glucose available to the tumour. The increased glucose demand of the tumour may deplete the glucose content of the plasma and of the tissue (glycogen) and physiological metabolic mechanisms in the host may then occur to increase gluconeogenesis.

Tumour effects on gluconeogenesis may also be indirect; thus an increasing substrate availability for gluconeogenesis (e.g. lactate or glucogenic amino acids) is known to increase the rate of glucose synthesis independently of other control mechanisms. Lactate does not accumulate in tumour cells, where it is also not metabolised (28). Transport of lactate into the plasma, to the liver of the host, results in gluconeogenesis and hence a cycling effect is set up between the tumour cells and the host liver cells. Increased glucose-lactate-glucose (Cori cycle) activity has been seen in many weight-losing cancer patients (10,34). Increased proteolysis and decreased proteogenesis in host muscles (as mentioned in preceding sections) increases the availability to the liver of glucogenic amino acids, such as alanine. The study by Clarke et al (20) on peripheral amino acid levels in cancer patients showed that alanine, glycine and threonine were decreased in arterial blood in the cancer patients, compared with controls. The preferential use of some amino acids either by the tumour itself e.g. glutamine or asparagine, or by increased host gluconeogenesis would serve further to decrease the synthesis of proteins, and thus to increase amino acid availability either for host gluconeogenesis or to the tumour.

1.1.3.3. Glucose intolerance in tumour-bearing hosts.

Normal fasting blood glucose levels were observed in weight-losing cancer patients (31), who subsequently, on the administration of glucose, showed a delayed clearance of glucose which was ascribed to defective metabolic adjustment. These cancer patients were also found to continue fatty acid oxidation after glucose administration i.e. they appeared to retain the metabolic patterns of the fasting state even in the fed state.

Glucose intolerance is a widely observed phenomenon in cancer patients (10,29,30). The role played by defective insulin

release or action has not been clarified, and this type of mechanism cannot generally be said to account for the delayed glucose clearance seen in cancer patients. Lundholm *et al* (15) showed decreased glycolytic and oxidative enzyme activities in tumour-bearing mice and men which supports previous suggestions from findings in man (21,31) that malignant tumours interfere with the ability of the host to utilize glucose. This may then result in the observed glucose intolerance.

The acceleration in the rates of gluconeogenesis may be so great that hyperglycaemia may occur and this may contribute to a diabetic glucose tolerance curve.

1.1.4. Tumour Effects on Nucleic Acid Metabolism.

A wide spectrum of tumours has been found to synthesize purines *de novo* from glycine, formate and other precursors.

Preformed purines in the diet can be used by the tumour, as shown by Henderson and Le Page (16); thus considerable amounts of host purines were transferred to, and utilized by, several transplantable mouse tumours.

Pyrimidine synthesis *de novo* has been less well studied in tumour cells, but the synthetic ability required for this is present. Shapot (17) reported that rapidly growing malignant tumours primarily used the "salvage" pathway for pyrimidine acquisition. Different tissues of the host may preferentially use either the *de novo* or "salvage" pathway and host tissues may be differentially affected by the tumour-caused drain on the necessary precursors. Successful competition of the tumour with the host for thymidine, as a precursor of DNA, has already been shown (17).

1.2. THEORIES AND PROPOSED MECHANISMS.

The tumour, by its active metabolism and proliferation within the host may cause alterations in the metabolism of the latter, such that wasting and a catabolic state prevails. Alternatively, the tumour may elicit a more direct response from the host by secreting an ectopic "factor" that would induce a catabolic state.

In accordance with the "indirect mechanisms", Gold (35) has proposed a mechanism for cancer cachexia dependent on an interplay of host gluconeogenesis and tumour glycolysis. The production of lactate by the tumour may have a two-fold significance. The energy produced by the formation of lactate would constitute an energy source for the tumour, but since lactate formation produces significantly less energy per molecule of glucose than the corresponding oxidative pathway, Gold has proposed an additional reason for the apparent obligatory production of lactate: lactate production enhances gluconeogenesis of the host. However, since gluconeogenesis occurs in host tissue and is an energy consuming process, an increased energy drain on the host is brought about and the result is the wasting of host tissues.

This proposal is supported, in theory, by much of the evidence reviewed in the preceding sections i.e. evidence that gluconeogenesis is generally increased in tumour-bearing organisms. Hyperlactataemia has not been observed, however. Normoglycaemia found in weight-losing cancer patients suggests that the rate of glucose utilization may accommodate the increased supply.

Waterhouse and Kemperman (31) found a limited adaptation of cancer patients to glucose administration, but the patients tended to show increased fatty acid oxidation even after glucose infusion. The "hosts", therefore, were apparently in a continual state of energy "deficiency" i.e. mimicking

fasting state conditions. This would be the case if the tumour was utilizing much of the carbohydrate produced in the host.

Gold's proposal of a systemic energy-losing cycle is attractive and theoretically likely. Grubbs et al (36) have tested this proposal and shown in rats bearing the Morris hepatoma 7777, that inhibition of gluconeogenesis with concomitant maintenance of high blood glucose levels resulted in maintenance of host body weight and increased nitrogen retention.

Cancer patients have an increased daily energy expenditure and resting metabolic rate, when compared with controls (8, 37), although this was not universally observed (38). A greater energy demand on the host may produce an increased resting metabolic rate if this were to incorporate an inherently increased rate of gluconeogenesis. The overall failure of a tumour-bearing host to adapt to semi-starvation may also be limited to a gluconeogenic increase in the host (5,10,31,37). Bland et al (39) proposed that inefficient substrate utilization, of the host, in the presence of an adequate dietary intake, may contribute to a cachectic condition in cancer patients.

Young (38) has challenged the proposal that increased Cori cycle activity could impose an energy "drain" on the host. He calculates that if only 15% of the lactate produced is oxidised, the energy produced would balance the energy loss if the remaining 85% of the lactate were recycled to glucose. This argument, however, begs the question of the net energy cost of these processes.

The postulation of chemical mediators produced by various tumours cannot be ignored. Effects of tumours, independent of anatomical alterations may occur at sites remote from the site of tumour involvement. Costa (4) has proposed that the destruction of normal cells by neoplastic cells may be

due to the production, by the former, of chemically definable toxic mediators, active in the immediate vicinity of the tumour or at distant sites.

Greenstein (1943)(40), in work on the liver catalase activity of tumour-bearing animals, found decreased enzyme activity in rats and mice bearing rapidly growing tumours. This effect was reversed by tumour removal. He proposed that the tumours liberated toxic substances into the blood stream or alternately that they removed components from the blood stream of the host, to cause the inhibition of liver catalase.

Toporek in 1973 (41) investigated the effects of whole blood and also the albumin fraction from tumour-bearing rats, on liver protein synthesis. The whole blood significantly decreased serum protein synthesis, as did the broad albumin fraction. The albumin fraction in addition caused a decrease in liver protein synthesis. The whole blood and albumin fractions from these Walker 256-induced rats may thus have contained some kind of "signal factor(s)".

As mentioned briefly in the section on protein synthesis in the tumour-bearing host, "toxohormone" produced from purified extracts of Walker 256 tumours (12) caused marked inhibition of the *in vitro* incorporation of [^{3}H]-lysine into protein in isolated rat diaphragms, both when injected into the animal prior to the assay and when it was added to the incubation medium of control diaphragms. A fraction was isolated comprising two components of the same molecular size as the active "toxohormone" preparation of other workers.

Ectopic hormone secretion by various histologic types of lung carcinoma has been investigated by Gropp *et al* (42). The fact that some tumours are known to secrete various small peptides which themselves cause or induce the host to elicit inappropriate secretions of hormones, e.g. ACTH, HCG, calcitonin, etc., points to the possibility that

various humoral factors may be released by tumours of various kinds.

The proposal of a lipolytic factor in serum from tumour-bearing hosts has not been substantiated (25). Costa and Holland (24) proposed that a lipolytic factor caused increased fat loss in Swiss-mice bearing Krebs-2 carcinoma, but provide no conclusive evidence, and their attempts to fractionate their tumour suspensions were also inconclusive.

Mays (22) investigated the lipid-mobilizing activity of serum from cancer patients but found no evidence for any fat-mobilizing substance. Carter et al (29) in 1975, using serum from breast cancer patients added to incubations of control rat epididymal fat pads, failed to show an increased lipolytic rate, nor was there any evidence for a lipolytic factor.

If the increased fatty acid oxidation in tumour-bearing hosts were merely a normal host response to an energy-deficient system (as proposed by an increased Cori cycle activity), then the likelihood of a lipolytic factor being produced by the tumour would be slight.

Theologides (43) has proposed a mechanism for cachexia in cancer patients which depends on the induction of a chaotic metabolic state in the host. He proposed that tumours could produce (unspecified) allosterically-active metabolites, as a result of the derepression of various genomes, which would then be capable of modifying and controlling various host enzyme activities. The alleged allosteric metabolites may induce a chaotic metabolic state by senseless activation and inactivation of enzymes. This would disturb metabolic patterns in the host and subvent metabolic equilibrium; tumours would be able to trap any nutrients released by these reactions. The primitive nature of the tumour cell would render it less vulnerable to the effects of the peptides it may produce.

This idea is attractive in view of the specific patterns of enzyme regulation observed in cancer patients (15). Schein et al (10) have proposed that release of a metabolic toxin by the tumour may be responsible for the failure of the host to adapt to "fed" conditions, i.e. to regulate fatty acid oxidation accordingly. It is possible, however, that removal of tumours does not remove the source of a "toxin" but that it removes the glycolytic "machine" proposed by Gold, reducing Cori cycle activity and restoring glucose homeostasis to the overall pattern found in normal adaptation to starvation and refeeding.

Cachexia of cancer patients probably arises through a metabolic interplay of tumour "ectopic factor" production and energy wasting metabolism and may even vary mechanistically in the case of different tumours. The most striking example of cachexia induced by a tumour has been provided by Strain et al (9), who found significant weight loss in mice bearing a xenografted hypernephroma from a weight-losing cancer patient. The tumour, therefore, not only caused weight loss in the human host (up to 30 kg in the two months prior to surgery) but also caused weight loss (>25%) in immunosuppressed mice, when the tumour comprised only 5% of the host body weight.

Tumours may thus produce "ectopic factors" that can act across species barriers or they may, by their metabolism, cause metabolic "chaos" in any host type, by the preferential sequestration of fuel nutrients and by inefficient energy-producing systems.

1.3. OUTLINE OF RESEARCH UNDERTAKEN.

The work that will be presented in the next chapters was undertaken to investigate various important areas that appear lacking so far in studies conducted on the cachexia of cancer patients.

Initially work was done to evaluate the frequency of weight loss in a spectrum of tumour types. No reports of any epidemiologic studies of mass loss in cancer patients could be located. Using anthropometry, data on arm muscle areas and arm fat areas of control non-cancer patients (n = 258) were obtained in order to define tissue losses in a series of fifty-two cancer patients bearing a variety of tumour types.

In vivo studies on arm muscle proteolysis in human subjects were then undertaken to investigate the status of muscle protein metabolism in situ. Arterio-venous amino acid differences were accordingly measured across the forearms of a group of weight-losing cancer patients; a group of non-cancer weight-losing patients and a group of non-weight-losing patients served as controls.

The rates of net proteolysis and lipolysis in rat muscle and adiposetissue respectively were measured to determine whether serum from weight-losing cancer patients would elicit a catabolic state in vitro; a positive result would enable the demonstration that the serum of cancer patients contained a proteolytic or lipolytic factor that metabolically contributes to the cachectic state developing in those patients.

CHAPTER 2

AN ANTHROPOMETRIC STUDY OF MUSCLE AND FAT WASTING IN PATIENTS SUFFERING FROM CANCER

2.1. INTRODUCTION

As reviewed in Chapter 1, cachexia of cancer patients is a vital criterion in the ultimate survival of the patient, and appears to have no correlation with calorie intake, tumour burden, tumour cell type or anatomical site of involvement (4,6,7,44). Breast cancer patients, however, seem to have a particularly low occurrence of weight loss.

An anthropometric study was thus undertaken, to determine muscle and fat areas in 52 cancer patients and 258 control patients suffering from minor, non-cancer, conditions, in order to assess the degree of cachexia in patients with cancer. Anthropometry is an accepted means of showing protein-calorie undernutrition and is easily applied to clinical use (45,46,47,48,49,50,51,52,53,54). The cancer patients studied formed predominantly two groups: patients having breast cancer and patients having other types of cancer. The series of other patients suffering from minor conditions was studied to derive standard data for both sexes and for white and black individuals, respectively. Fat area and muscle area values for all the patients were then calculated and the cancer patients compared with controls on the basis of mean standard values appropriate for their sex and racial group.

2.2. MATERIALS AND METHODS

2.2.1. Selection of Cancer Patients.

Twenty breast cancer patients (10 patients in remission, 10 with progressive disease), 17 lung cancer patients, 5 cervical cancer patients and a mixed group of eight patients having other tumour types, including hepatoma, lymphoma and

carcinoma of the bladder, anus and post-nasal space, were studied (total 52). Only twelve of the patients were on treatment, none were receiving hormones, none were diabetic, and patients with tumours of the oro-pharynx or gastro-intestinal tract were excluded. All patients were ambulatory and were graded 1-10 according to performance status (55).

2.2.2. Anthropometric Measurements.

All anthropometric measurements were taken by one person to eliminate inter-observation error (47,50,56). The measurements taken were the upper mid-arm circumference and triceps skinfold thickness, according to the method described by Jelliffe (45) A measurement of the triceps skinfold thickness (TST) assesses the patient's available fat reserve, while the upper midarm muscle circumference (MAC) is an indicator of lean body mass or muscle tissue (45,48,57). In each case the non-dominant arm was measured (47,56). Women with cancer of the left breast, who were right-handed, were excluded as lymphatic obstruction resulting from tumour spread or treatment could have affected the M.A.C. and T.S.T. measurements in these patients.

Triceps Skinfold Thickness (T.S.T.)

The triceps skinfold thickness was measured at the midpoint on the upper arm, between the acromial process of the scapula and the olecranon process of the elbow. In each case, the upper arm was held parallel to the body in a relaxed manner, while the forearm was supported at the abdomen by the arm not being measured. The measurement was taken at the back of the arm, by pinching the skin at the midpoint and applying a Ponderax Mark II skinfold caliper, this being an accepted caliper for use in such studies (47,58).

Upper Mid-arm Circumference (M.A.C.)

The upper mid-arm circumference was measured at the midpoint described above, using a non-stretchable tape measure.

Calculation of fat area and muscle area from the measurements of M.A.C. and T.S.T.

Arm muscle area (M) and arm fat area (F), which are more precise indices of body muscle and fat than M.A.C. and T.S.T. (46,50,56), were derived using the following formulas:

$$M = \frac{(C - \pi T)^2}{4\pi} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (a)$$

$$F = \frac{C^2}{4\pi} - M \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (b)$$

where C = M.A.C.
T = T.S.T.

2.2.3. Mean Standard Values of Fat Area (F) and Muscle Area (M)

(a) Normal white patients:

Accepted standard values for adult upper mid-arm circumference and triceps skinfold thickness (45) were modified using the equations for (M) and (F), set out above, to yield standard (M) and (F) values. Although these standard values were those of an American population, there was no difference between these values and those obtained for twenty normal white South Africans.

(b) Normal black patients (mixed race or "coloured"):

The standard values referred to above (45) were found to differ from those of black South African persons, and therefore mean standard (M) and (F) values for adult black males and females were determined separately. Accordingly, two hundred and thirty-eight black men and women who attended a general out-patient clinic at

Groote Schuur Hospital for minor, non-endocrionological illnesses were assessed for mid-arm circumference and triceps skinfold thickness. There were 118 females (mean age 49,7 ± 1,4 years (mean ± S.E.) years, and 120 males (mean age 49,7 ± 1,3 years). The (M) and (F) values obtained from cancer patients were then expressed as a percentage of the relevant standard mean.

2.3. RESULTS

There was no difference in tumour load when comparing breast cancer patients with patients with other types of tumour (see Table 2.1). The muscle and fat area measurements of patients suffering from cancers, other than those of the breast, showed the presence of wasting when they were compared to matched standard values and expressed as a percentage of the mean standard value. The patients with breast cancer were significantly different in these respects (Fig. 2.1). They yielded (M) and (F) values that were greater by 10-20% than those of a matched standard group. The increase in fat area was particularly noticeable. Thus the breast cancer patients had 30% more muscle area and 70% more fat area when compared with the other cancer patients. It is also interesting that there was no significant difference between (M) and (F) values in breast cancer patients with progressive disease when compared with patients in remission, although patients in the former group had slightly increased (M) and (F) values compared with those in the latter (Fig. 2.2).

TABLE 2.1.

TO COMPARE TUMOUR LOAD IN BREAST CANCER PATIENTS AND PATIENTS WITH OTHER TYPES OF CANCER

Grade	1	2	3
Breast Cancer	0	5	5
Other Cancer	4	19	7

The tumour load was arbitrarily assessed in the following way: Grade 1 = local disease < 3 cm; Grade 2 = local disease > 3 cm; Grade 3 = metastatic disease.

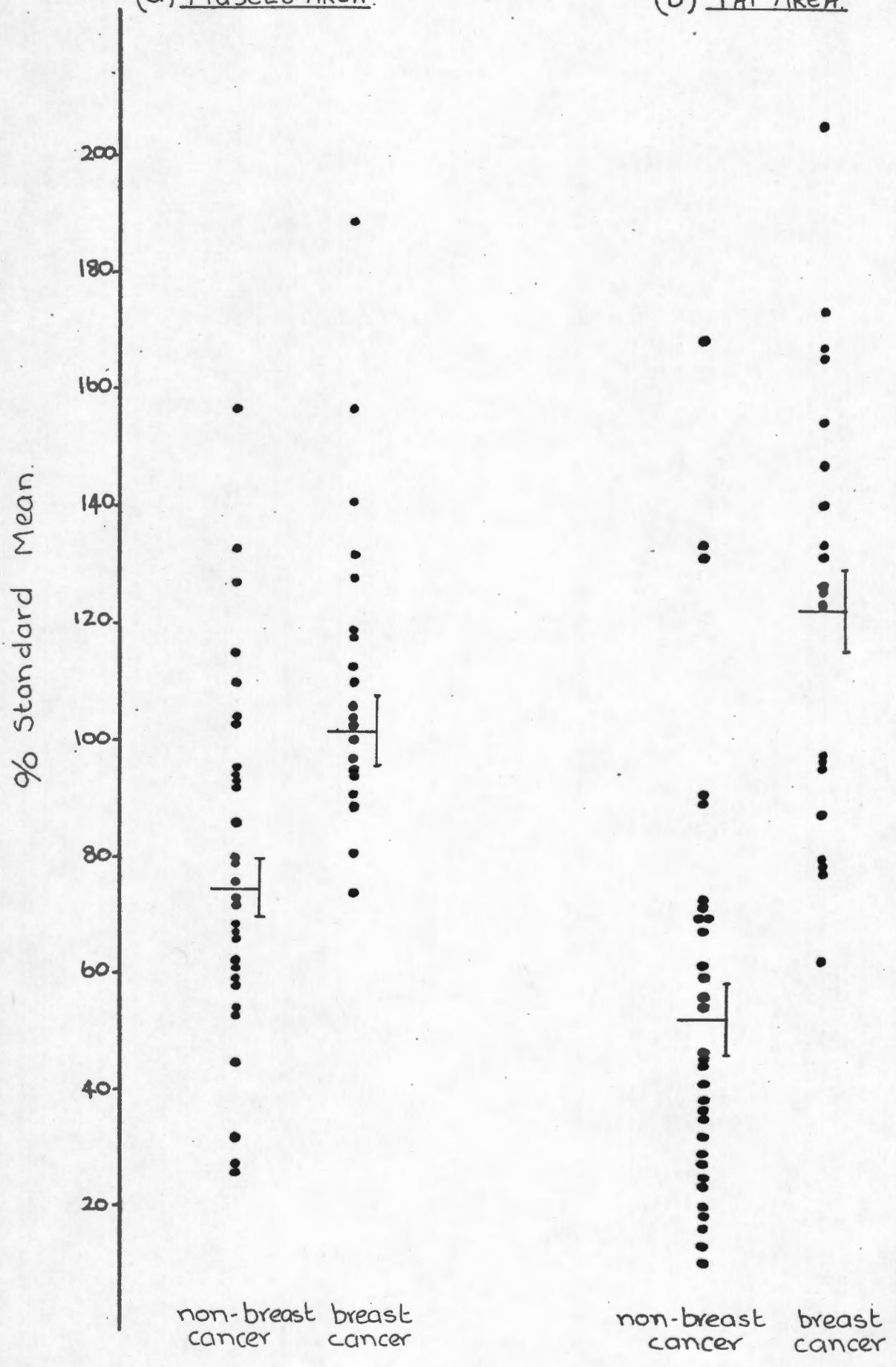
(a) MUSCLE AREA.
(b) FAT AREA.
% Standard Mean.
200
180
160
140
120
100
80
60
40
20
non-breast cancer
breast cancer
non-breast cancer
breast cancer

Figure 2.1.

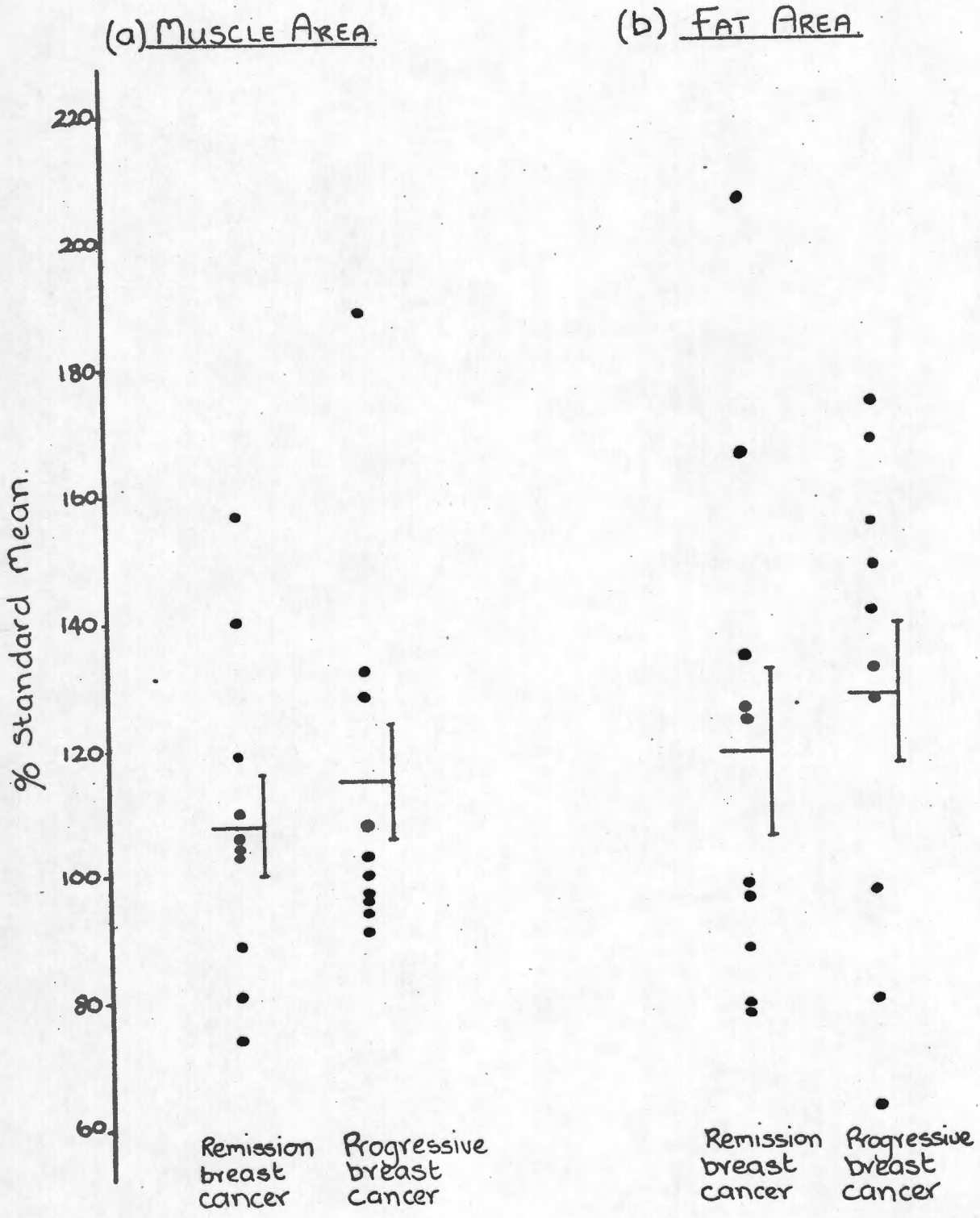

Figure 2.2. Muscle and fat areas of patients with breast cancer; in remission or with progressive disease.

Results are expressed as the percentage of a standard control mean value; the mean ±S.E. for each group is given.

2.4. DISCUSSION

The use of anthropometric measurements as an indicator of protein-calorie undernutrition is considered valid by many workers, when the measurements are conducted as described in this article. The measurement of muscle and fat areas, as opposed to mid-arm circumference and triceps skinfold thickness, is advocated by many (46,48,49,50,56), and expressing the overall result as a percentage of a standard value is considered to be more appropriate (44,45,47,48,49, 51).

The formulae for determining (F) and (M) assume that the upper arm is cylindrical, an assumption which may be inaccurate. In determining (M) there is also no provision made for variations in humoral diameter, while in determining (F) there is no adjustment made for variations in skin compressibility. However, the validity of the above assumptions was examined by Heymsfield *et al* (49), using computerised axial tomography. It was found that by expressing M.A.C. and T.S.T. as muscle areas and fat areas, and also by expressing these values as the percentage of a standard value, there was reasonable agreement between the two methods, so that anthropometry is acceptable for clinical use. Furthermore, T.S.T. correlates well with body fat, as measured by body density (58,59). The choice of an appropriate standard group is important and appears to be lacking in many anthropometric studies. Expressing each value as a percentage of the relevant standard mean allowed us to make a direct comparison between cancer patients of different sex and race.

This epidemiological study showed anthropometrically that patients without breast cancer had profound loss of fat area, and a less-pronounced loss of muscle area. The fact that fat areas were reduced by a greater percentage than muscle areas in the non-breast cancer patients is interesting, as fat is a greater source of energy, during starvation,

than muscle. This greater decrease in fat areas compared with muscle areas, in weight-losing cancer patients, has also been documented by other workers (50,59). The breast cancer patients, however, did not show reduced muscle or fat areas but tended, rather, to show increased values when compared with those of a matched standard population. The finding that there was no apparent difference between (M) and (F) values in breast cancer patients with progressive disease when compared with breast cancer patients in remission runs counter to current notions, as well as the findings of De Wys *et al* (6), which pointed to an increase in weight loss with an increasing number of anatomic sites involved in the tumour progression. However, these observations do not exclude the possibility that the patients with progressive disease had excessively raised (F) and (M) values at the onset of disease and that these values had subsequently decreased. Indeed, the greater increase in (F) values over (M) values in breast cancer is interesting in view of the strong association between dietary fat intake and breast cancer incidence (57,60,61,62,63,64). Women who are obese are thought to have an increased risk of breast cancer, especially in post-menopausal years (64). De Waard *et al* (62,63) have shown this fact to correlate well with their proposal of a bimodal age distribution in breast cancer incidence. The ability of adipose tissue to function as a slow-release depot for lipid-soluble carcinogens has also been proposed as a possible cause of the association between obesity and breast cancer (65). This study has shown that muscle and fat depletion occurs in many cancers; this is generally independent of the anatomical sites of involvement or the extent of the disease, but appears to be very much reduced in breast cancer patients. The increased fat area seen in those breast cancer patients studied, correlates well with present ideas on nutrition and breast cancer incidence.

CHAPTER 3

SERUM AMINO ACIDS IN WEIGHT-LOSING PATIENTS WITH CANCER, AS WELL AS WEIGHT-LOSING PATIENTS NOT SUFFERING FROM CANCER AND NORMAL CONTROLS

3.1. INTRODUCTION

There are numerous potential abnormalities in protein metabolism in weight-losing cancer patients, as reviewed in Chapter 1, and which could predictably produce a severe imbalance in protein metabolic homeostasis.

A study was undertaken to investigate serum amino acid levels *in vivo*, in 12 weight-losing cancer (CWL) patients, 8 weight-losing noncancer patients, with active pulmonary tuberculosis (TWL) and 9 normal controls, in order to ascertain which changes are unique to cancer patients.

The arterial and venous amino acids across the forearm of each patient were measured and the arterio-venous amino acid concentration differences determined. Increased muscle proteolysis is characterized by a venous excess of amino aicds and elevated arterial levels of essential branched chain amino acids (66). Conversely, decreased proteogenesis, as may occur in protein-calorie malnutrition, is characterized by decreased arterio-venous amino acid differences and reduced arterial plasma levels of essential branched chain amino acids (66, 67, 68).

3.2. MATERIALS AND METHODS

3.2.1. Patient Selection.

Approval for this study was obtained from the Hospital Ethical Committee, and informed consent was obtained from all patients studied.

Patients with various solid tumours (excluding those of

the gastrointestinal tract), who had lost at least 10% body weight during the preceding 6 weeks, had received no radiotherapy, surgery or chemotherapy during this time and had no evidence of intestinal obstruction or dysphagia, were selected for study. All patients had locally advanced disease and there was no clinical evidence of metastatic disease, with the exception of one patient with adenocarcinoma of the lung who had a cerebral metastasis. Seven patients had lung cancer (three adenocarcinoma, two oat-cell, one squamous-cell and one large-cell carcinoma), three patients had stage III carcinoma of the cervix and one patient had stage III carcinoma of the ovary.

In addition, patients with active pulmonary tuberculosis who had lost at least 10% body weight during the previous 6 weeks were selected, as well as "normal" patients who had been admitted to hospital for minor surgical procedures, such as hernia repair, haemorrhoidectomy and varicose vein stripping. All control patients were studied pre-operatively and none gave a history of recent weight loss.

None of the patients studied had diabetes or other endocrine abnormalities, nor was there biochemical evidence of hepatic or renal dysfunction in any of them.

In all, 12 CWL patients (8 males, 4 females; average age 53 $\pm$ 12 years), 8 TWL patients (5 males, 3 females; average age 43 $\pm$ 12 years) and 9 normal controls (6 males, 3 females; average age 46 $\pm$ 14 years) were studied.

3.2.2. Sample Collection and Preparation.

Blood samples were collected after a 12 hour overnight fast. Patients in all three groups were ambulatory in a hospital environment and blood specimens were obtained early in the morning before any physical exertion. Venous blood was collected from a large antecubital vein without stasis and arterial blood was collected by radial artery puncture from

the same arm. The clotted blood was centrifuged immediately and the serum was deproteinized by treatment with sulphosalicylic acid and centrifugation before storage at -20°C until analysis. Amino acids were measured by ion-exchange chromatography, using lithium buffers on a Model 121-M Beckman Amino Acid Analyser. The experimental error was found to be less than 5%.

3.2.3. Statistical Evaluation of Data.

Individual amino acids were compared in the three groups by applying a two-tailed Student's T test, while individual arterio-venous amino acid differences were compared by applying the Mann-Whitney U-test (69) to take into account the wide scatter of results in each group. The numbers of amino acids in venous excess in each group were compared by applying a simple Chi-square test.

3.3. RESULTS

3.3.1. Arterial Amino Acids.

Mean values for arterial amino acid levels are shown in Table 3.1.

Branched-chain amino acids: All branched-chain amino acids were present at significantly lower concentrations in the arterial blood of CWL patients when compared with TWL patients. Although the mean values for branched-chain amino acids were lower in the CWL patients when compared with normal controls, these differences were not statistically significant.

Gluconeogenic amino acids: The gluconeogenic amino acids, serine and alanine, were also present at significantly lower concentrations in the CWL patients when compared with TWL patients. However, in the case of alanine (which is thought to be one of the most important gluconeogenic amino

TABLE 3.1.

ARTERIAL AMINO ACID LEVELS

ESSENTIAL AMINO ACIDS	CANCER µmol/ℓ	TUBERCULOSIS µmol/ℓ	CONTROL µmol/ℓ
Valine	135.6±13.4 † p<0.01	181±11.4	165±12.1
Leucine	84.3±8.7 † p<0.05	115.4±7.5	97.5±8.6
Isoleucine	39.4±3.1 † p<0.01	53.4±3.1 † p<0.06	43.8±3.3
Total Branched Chain Amino Acids	263±90 † p<0.05	349±52	305±73
Threonine	77.9±7.0 † p<0.06	98±5.9	93.3±13.2
Methionine	14.3±1.5	16.2±0.9	16.3±1.2
Lysine	122.1±10.7	129.6±7.0	126.5±8.9
Phenylalanine	60.9±5.3	72.0±6.5	50.3±6.2
NON-ESSENTIAL AMINO ACIDS			
Serine	107.7±9.1 † p<0.01	158.1±14.9	131.4±12.5
Alanine	228.4±19.5 † p<0.05	294.2±19.5 * p<0.05	227.9±23.4
Proline	132.6±13.5	157.1±5.6	141.4±16.2
Citrulline	14.7±1.4 † p<0.01 * p<0.01	24.1±4.3	28.3±3.8
Glycine	184.8±14.2	240.1±12.6	219.2±13.5
½-Cystine	71.8±5.9	71.1±4.4	69.3±4.1
Tyrosine	39.3±3.2 † p<0.05	49.0±3.3	37.9±2.8
Ornithine	106.1±16.1 † p<0.01	167.1±8.6 * p<0.001	72.5±13.6
Histidine	47.3±3.0	43.7±1.9	55.4±3.9
Arginine	82.3±7.0	100.6±6.7	85.1±8.0
Glutamine and Glutamic Acid	382.8±49.3	432.6±40.3	405.0±30.3
Asparagine and Aspartic Acid	105.5±12.5 † p<0.06	149±19 * p<0.06	105.0±6.2

Results are the mean ± S.E.
† Significant difference compared with TB group (TWL).
* Significant difference compared with control group.

acids) this difference was brought about by the significantly raised alanine levels in TWL patients when compared with normal controls (294 ± 19.5 vs 227.9 ± 23.4, $p < 0.05$). There was no decrease in gluconeogenic amino acids in CWL patients compared with normal controls.

Glycine: One of the hallmarks of chronic malnutrition is an elevation of plasma glycine. Neither the CWL nor TWL patients had glycine concentrations which differed significantly from those of the normal control group. However, glycine values were higher in the TWL group compared with CWL patients, the difference being marginally significant ($p < 0.06$).

Citrulline and ornithine: Citrulline concentrations were significantly decreased in the CWL patients when compared with TWL patients (14.7 ± 1.4 vs 24.1 ± 4.3, $p < 0.01$) and control patients (14.7 ± 1.4 vs 28.3 ± 3.8, $p < 0.01$), whereas there were no differences in this respect between TWL patients and controls.

Arterial ornithine concentrations were very high in TWL patients when compared with cancer patients (167.1 ± 8.6 vs 106.1 ± 16.1, $p < 0.01$) and controls (167.1 ± 8.6 vs 72.5 ± 13.6, $p < 0.0001$), while there was no difference in ornithine values comparing CWL patients with normal controls.

3.3.2. Arterio-Venous Differences of Amino Acids.

The means of arterio-venous differences for individual amino acids in the three groups of patients are shown in Table 3.2. Qualitative assessment of the data showed a significantly increased release of the branched-chain amino acids leucine and isoleucine from forearm muscles in CWL patients, compared with normal controls. Although there appeared to be other amino acids with increased venous excess in CWL patients,

TABLE 3.2

AMINO ACID ARTERIO-VENOUS DIFFERENCES

ESSENTIAL AMINO ACIDS	CANCER µmol/ℓ	TUBERCULOSIS µmol/ℓ	CONTROL µmol/ℓ
Valine	-1.6±8.6	+16±4.4	+8.6±12
Leucine	-9.0±5.3 * p<0.02	+0.57±5.8	+13.8±5.8
Isoleucine	-2.0±3.1 * p<0.02	+2.2±2.9	+1.7±2.9
Total Branched-Chain Amino Acids	-12.6	+7.1	+24.1
Threonine	-3.5±8.8	-6.8±8.1	-1.9±8.3
Methionine	-1.6±1.2	-0.4±1.4	-2.0±1.2
Lysine	-8.4±10.2	-3.8±11.2	-4.9±8.0
Phenylalanine	-4.7±5.6	-2.5±3.3	+0.8±3.4
NON-ESSENTIAL AMINO ACIDS			
Serine	-2.1±10.3	+3.4±11.7	+5.7±10.9
Alanine	-26.4±85.7	-23±31.5	-18±16.5
Proline	-9.5±13.3	-0.14±8.8	+4.7±9.3
Citrulline	+0.38±0.9	+3.3±1.6	+1.8±1.9
Glycine	-11.6±14.4	+4.8±10.9	+10.8±18.2
½-Cystine	-0.5±6.1	-4.5±5.3	-0.6±6.0
Tyrosine	-4.8±2.5	+1.0±2.5	-1.0±3.1
Ornithine	-8.3±11.1	+1.4±11.9	+1.2±5.1
Histidine	-2.1±3.9	-0.3±2.2	-1.6±3.9
Arginine	+2.3±5.0	-7.4±10.2	-0.7±5.9
Glutamine & Glutamic Acid	-23.3±44.2	-29.0±27.0	+38.7±31.9
Asparagine & Aspartic Acid	-3.2±8.2	-10.7±14.3	+3.8 ±7.5

Results are the Mean ± S.E.

* Significant difference compared with Control Group.

The wide scatter of data accounts for the large standard errors of the means. Nevertheless, there is a significant venous excess for the 2 essential branched chain amino acids leucine and isoleucine in cancer patients compared with normal controls.

Minus sign denotes venous excess; plus sign denotes arterial excess.

the small numbers of patients studied may have affected significance levels. The data were also assessed quantitatively by counting the number of amino acids in venous excess in each group (Table 3.3). The groups were compared in a 2 x 2 contingency table and a simple Chi-square test applied.

It can be seen from Table 3.3 that there were significantly more amino acids in venous excess in the CWL patients when compared with TWL patients or normal controls ($p < 0.01$).

3.4. DISCUSSION

Ideally, muscle blood flow should be determined in this type of study, as differences in flow may theoretically affect the uptake and efflux of amino acids in a muscle group. We were unable to make such measurements for a variety of practical and ethical reasons and simply endeavoured to ensure that patient conditions and ambient surroundings were as constant as possible for each sampling episode.

The venous excess of a greater number of amino acids in the CWL group implies either

(i) that there was enhanced proteolysis in the muscles of these patients;
(ii) that there was reduced proteogenesis without any increase in proteolysis;
(iii) that blood flow was generally slower in the cancer patients; or
(iv) that the differences arose by chance.

The finding that venous excesses for leucine and isoleucine were significantly increased in the CWL patients when compared with normal controls does not really help to distinguish between these possibilities. During enhanced muscle proteolysis resulting from starvation, there is known to be an increase in the intracellular pool size of branched-

TABLE 3.3

NUMBER OF AMINO ACIDS IN VENOUS EXCESS

	AMINO ACIDS IN VENOUS EXCESS	
	0-10	11-20
Cachectic Cancer (CWL)	4	8 (67%)
Cachectic T.B. (TWL)	6	2 (25%)
Normal Controls	7	2 (22%)

The numbers of patients with 0-10 amino acids in venous excess appear in the left-hand column, while those with 11-20 amino acids in venous excess appear in the right-hand column.

There are significantly more amino acids in venous excess in the CWL group compared with TWL and controls ($p < 0.01$).

chain amino acids which is associated with release of these amino acids, leading to elevated arterial levels (70). Likewise, the under-utilization of these amino acids by skeletal muscle might also lead to an increased venous excess. If there is an under-utilization of amino acids in CWL patients, it is unlikely to be wholly on the basis of an underlying protein-calorie malnutrition, as in the latter situation there is a decreased venous excess of amino acids, probably as a result of decreased proteogenesis to facilitate protein sparing (71).

Under-utilization of amino acids might result from decreased proteogenesis, which has been demonstrated in skeletal muscle of cancer patients (11, 15). Alternatively, preferential sequestration of certain amino acids by the tumour may result in an incomplete complement of amino acids presented to skeletal muscle, thereby reducing its capacity for proteogenesis.

Although one would expect an increase in arterial branched-chain amino acids to occur in patients with an increased release of amino acids from muscle, the normal plasma concentrations of these substances in the CWL patients may have arisen from their enhanced sequestration by the tumour itself, or from altered hepatic amino acid metabolism.

Normal arterial glycine concentrations in CWL and TWL patients are consistent with our impression that these patients did not suffer from chronic malnutrition. The finding of normal glycine values in CWL patients is in accordance with the findings of Clarke et al (20).

The arterial branched-chain amino acid levels were significantly lower in CWL compared with TWL patients. It is not possible from these data to assess whether these differences were due to a decrease in the concentration of branched-chain amino acids in CWL patients relative to

controls which, however, was not statistically significant. Conversely, the differences may have been brought about by the increase in these amino acids in TWL patients relative to controls, which was also not statistically significant. Whatever the case, the data define a distinct difference in arterial branched-chain amino acids in weight-losing cancer patients compared with weight-losing patients with a chronic infectious illness, and this has not been reported before.

Clarke et al (20) showed normal plasma concentrations of branched-chain amino acids in weight-losing cancer patients, whereas malnourished patients had low levels of these amino acids. However, their non-cancer malnourished group had anorexia nervosa or previous gastrectomies, which would not make them strictly comparable with weight-losing cancer patients, although the latter did have anorexia as well.

The comparison made between CWL and TWL patients is more meaningful, as neither group was biased regarding excessive hypophagia or intestinal malfunction secondary to gastro-intestinal surgery, both of which could produce distinctive metabolic changes in their own right.

The lower arterial concentrations of serine and alanine in the CWL patients when compared with TWL patients was the result of an increase of these amino acids in TWL patients, alanine being significantly elevated in the TWL group when compared with both the CWL patients and with controls. It is generally assumed that high arterial concentrations of gluconeogenic amino acids reflect decreased hepatic uptake of these amino acids and therefore implies decreased hepatic priming for gluconeogenesis. Conversely, low arterial alanine levels imply increased hepatic gluconeo-genesis. This did not, therefore, confirm the findings of Clarke et al (20), who found a significant decrease in glucoenogenic amino acids in their CWL patients.

The only clear-cut difference in arterial amino acid concentrations between CWL and all other patients was the significant hypocitrullinaemia. Citrulline is an intermediate in the urea cycle, and hypocitrullinaemia may reflect a block in synthesis, since ornithine levels were normal.

These data therefore suggest enhanced muscle proteolysis or decreased utilization of amino acids by muscle in CWL patients. Lower arterial amino acid levels in CWL patients may be accounted for by tumour sequestration of these amino acids or by altered hepatic metabolism of amino acids.

3.4.1. Acknowledgement.

This work was done while the candidate was an assistant to Dr L. Levin (Dept. of Medicine, UCT Medical School), and is presented here with Dr Levin's permission.

CHAPTER 4

HAS SERUM FROM CACHECTIC CANCER PATIENTS A PROTEOLYTIC EFFECT ON MUSCLE? USE OF HETEROLOGOUS IN VITRO ASSAY SYSTEMS

4.1. INTRODUCTION

The cachectic condition of some cancer patients has led to the proposal of a tumour-produced ectopic factor(s) which could stimulate muscle proteolysis and thus cause increased loss of lean body mass (ref. Chapter 1). Alternately, the tumour may provoke a host response which might give rise to the cachectic condition (ref. Chapter 1). This idea was investigated in the work being reported in this chapter. Heterologous *in vitro* assays of skeletal muscle proteolysis were established in order to determine what influence, if any, serum from cachectic cancer patients might have on proteolysis in muscle and if this influence could contribute to the cachectic condition.

Investigations of muscle protein degradation have been limited by methodological difficulties. Intact animal systems are prone to a variety of difficulties and possible artefacts (72), monitoring degradation by measuring the disappearance of radioactively labelled proteins. In contrast, isolated mammalian muscles incubated *in vitro* permit controlled investigations of muscle protein metabolism.

Isolated rat diaphragms have many advantages that are attributed to their rapid dissection and preparation; the thin (often translucent) nature of the sheet of muscle fibres (73, 74), and because quarter diaphragms isolated from one animal (allowing direct comparisons to be made) provide obvious advantages for statistical analysis of complex biological variables (73, 75). The measurement of trichloroacetic acid (TCA)-soluble tyrosine, released into the medium from the isolated diaphragms, provides a simple and reproducible determination of net protein degradation in the system (73).

In this work, two proteolysis systems were used: the well-established rat diaphragm system (73,76,77,78,79) and a cell culture system using an established myoblast cell line, L8, originally isolated from rat skeletal muscle.

Day-to-day variations between experimental animals (73) present a very significant problem in the isolated tissue system. To permit a valid comparison of results obtained using different rats, results were expressed as a series of percentage differences in respect of tissue fragments from single animals. Because of the short incubation periods for which the diaphragm assay is suited, and the necessity to desalt the serum samples to remove high tyrosine blanks, the second *in vitro* system was tested in a similar manner to the tissue system. The L8 skeletal muscle cell line has been well-characterized (80).

4.2. TISSUE INCUBATIONS

4.2.1. Experimental Procedures.

(Note: See Appendix for the suppliers of important materials used).

4.2.1.1. Preparation and incubation of rat quarter diaphragms.

Male rats of between 60 - 90 g were sacrificed by cervical dislocation and the diaphragms removed and rinsed in ice-cold Krebs Ringer Bicarbonate (KRB) buffer (0.12 M NaCl; 0.005 M KCl; 0.001 M $CaCl_2$; 0.001 M $MgSO_4 \times 7H_2O$; 0.001 M KH_2PO_4; 0.025 M $NaHCO_3$; pH 7.4 equilibrated with 95% O_2 - 5% CO_2).

The central tendon and ribs were carefully dissected away and each hemidiaphragm bisected parallel to the muscle fibres to yield four diaphragm pieces. The pieces were weighed and placed into 25 ml Erhlenmayer flasks containing 2 ml KRB-buffer, equilibrated with 95% O_2 - 5% CO_2, and preincubated for 30 mins at 37°C, with shaking. A 30-minute preincubation

is recommended (75) to allow resealing of damaged muscle fibres.

The muscle pieces were then transferred to incubation flasks containing 2 ml incubation medium, equilibrated with 95% O_2 - 5% CO_2 and incubated for 3 hours (unless otherwise stated), at 37°C with shaking. The incubation medium contained 60 µg/ml penicillin G and 100 µg/ml streptomycin sulphate during every incubation period lasting 3 hours. After incubation, the muscle tissue was removed and usually discarded, except for the determination of the intracellular muscle pools of tyrosine, when the muscle was rinsed, blotted and homogenized in 1 ml cold 10 mM potassium phosphate buffer, pH 7.4, using an Ultraturrax homogenizer (Janke & Kunkel, FRG). The protein was precipitated by adding an equal value of 20% TCA, keeping the mixture on ice for 30 mins and then centrifuging for 30 mins at 2 000 *g*, at 0°C in a Beckman TJ-6R centrifuge.

The incubation medium was routinely collected and the TCA-soluble supernatant prepared (as described above). The tyrosine content of the TCA-soluble fraction of the medium (or muscle homogenate) was determined fluorometrically by the method of Waalkes and Udenfriend (74) which is sensitive and specific for tyrosine, either in the free form or in oligopeptides. In incubations where an "antiproteolytic" mixture of agents (insulin, 0.1 units/ml; glucose, 10 mM; branched chain amino acids (BCA), 5 x normal rat plasma concentrations (80), or hormones such as hydrocortisone (in amounts varying from 50 mg to 5.0 mg) and glucagon (varying from 50 pg to 500 µg) was added, these were added to both the preincubation and incubation media. Incubations performed in 50% serum or cell culture medium were preincubated in KRB-buffer alone.

4.2.1.2. Tyrosine determination

To 2 ml of acid supernatant were added 1 ml 1-nitroso-2-naphthal reagent and 1 ml nitric acid reagent; the tubes were stoppered, shaken well and incubated at 55°C for thirty minutes. After cooling, 9 ml 1,2-dichloroethane was added, the tubes again shaken and spun at 700 *g*, briefly, to separate the organic phases. The unchanged nitrosonaphthal reagent was extracted into the organic phase, and discarded. The upper aqueous phase, containing the nitrosonaphthal tyrosine derivative, was removed and assayed in a Perkin-Elmer fluorescence spectrophotometer (λex: 460 nm; λem: 570 nm). The tyrosine content of samples was determined by reference to a standard curve obtained for free L-tyrosine, and the rate of tissue proteolysis was finally expressed as the net release of tyrosine per mg wet muscle weight per hour.

4.2.1.3. Selection, preparation and incubation of patient serum samples.

Cancer patients who were not undergoing treatment, who had normal liver functions and were not diabetic nor suffering from other obvious endocrinological disorders, were selected for this study. Five such patients having substantial weight loss within the six months preceding the blood sampling and bearing a variety of malignant tumours (including lung and cervical carcinomas, but excluding any oro-pharyngeal or gastro-intestinal obstructions) were chosen as the weight-losing cancer patient group. As a non-weight losing cancer patient group, 5 patients with progressive cancer of the breast were chosen, prior to mastectomy or any other form of treatment.

For each cancer patient selected, there was a sex-, age- and race-matched control patient (not suffering from cancer), selected. Paired internal comparisons of effects, on muscle proteolysis *in vitro*, of serum from cancer and matched control patients were performed in duplicate within single experimental animals.

Blood samples were obtained from patients in the fed state; all had given their informed consent. The blood samples were centrifuged at 1000 *g* in the cold, for 20 mins , and the serum was then desalted on a Sephadex G-25 column, equilibrated with 0.9% NaCl. The desalting procedure was necessary in order to reduce the high background levels of tyrosine present in the serum. The incubation medium was comprised of equal volumes of serum and double-strength KRB-buffer. The tyrosine content of the incubation flask, without muscle, was determined for each serum sample. This tyrosine "blank" was then subtracted from each respective experimental tyrosine value to give a "corrected" tyrosine release value. The tyrosine "blank" of desalted serum was less than 5% of that released by the tissues.

4.2.1.4. Treatment of rats.

Adult male rats of approximately 400 g body weight were subjected to various treatments in order to impose physiological modulations *in vivo*; the serum of such rats was tested as a "model" for the experiments conducted with human sera, i.e. the response of the target diaphragm pieces to serum of varying "proteolysis promoting" potential could be assessed.

Two rats were subjected to each of the following treatments: Fasting (rats maintained on water alone for 90 hrs or 130 hrs); induced diabetes mellitus (rats treated with a dose of 8 mg Alloxan/100 g body weight (83) and sacrificed on Day 5 after injection); Hypercortisolism (rats treated with hydrocortisone sodium succinate at 5 mg/100 g body weight/day for 5 days, and were either fed or fasted for 130 hr, being sacrificed 24 hrs after the last injection), and Hyperthyroidism (rats injected intraperitoneally with 25 µg, L-Triiodothyronine (T_3) per day per 100 g body weight, for a period of 17 days, being sacrificed on Day 18 (78,82)).

The carotid artery of each serum donor rat was carefully, but rapidly, severed after the animals were anaesthetized

with Sagatex. Blood was drained into collecting tubes (about 10 mls), allowed to clot and centrifuged to obtain the serum. The serum for each treatment was pooled and then desalted and incubated as described (4.2.1.3). The effect of serum from the treated rats was directly compared in internal paired comparisons with serum from a fed control rat.

4.2.1.5. Medium from transformed cell cultures.

$R_9Cl_{10}T$ cells are spontaneously transformed cells which have arisen from an R_9Cl_{10} cell line, derived from neonatal rat hearts in the 16th passage. The transformed cells were characterized as such by the following criteria: they showed anchorage independence on soft agar plates; they had lost the capacity to lay down elastin and collagen matrices; they secreted very high rates of plasminogen activator; their rates of cell division were at least twice those of the R_9Cl_{10} cells; their morphology was altered from that of longitudinal rod-like cells, to small spherical cells; and cells from $R_9Cl_{10}T$ cultures, when injected subcutaneously into immunosuppressed rats, developed into local fibrosarcomas, on two occasions of testing.

Medium (lacking serum) from these cell cultures was supplied by Dr T. Scott-Burden, after it had been exposed to the cells for three days. Desalting, and subsequent incubation, procedures were as described previously (4.2.1.3.).

4.2.2. Results.

4.2.2.1. Optimalization of the assay.

Rat diaphragm quarters incubated in KRB-buffer, preceded by a thirty-minute preincubation period, released tyrosine into the medium at a linear rate for three hours, both when quarter diaphragms from one experimental animal were incubated for different time periods (Figure 4.1) and when the average of

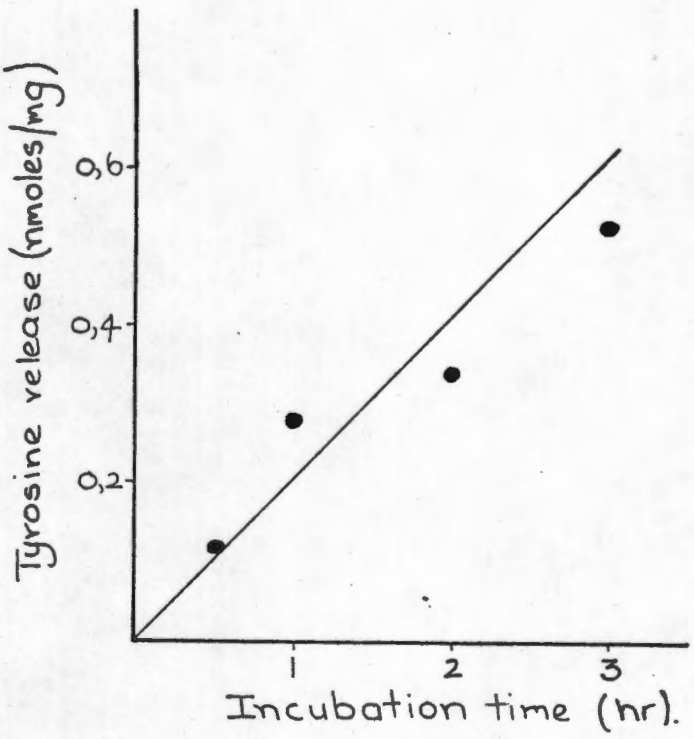

Figure 4.1. Release of tyrosine from quarter diaphragm pieces of one experimental animal, with time of incubation.

Quarter diaphragm pieces incubated in KRB-buffer for 0.5 hr, 1 hr, 2 hrs, 3 hrs. Each point represents the tyrosine released by a quarter diaphragm taken from a single experimental animal.

tyrosine release was plotted as the (mean ± S.E.) of 3-5 rats for each time point (Figure 4.2). Determining the mean values for each time point incorporates day-to-day variation between experimental animals; however, the mean rate of proteolysis, 0.22 ± 0.04 (mean ± S.D.) nmoles of tyrosine released per mg wet tissue weight per hour, is comparable with literature values (73). The release of tyrosine into the medium was not the result of a non-specific leakage of material from the intracellular pools (Figure 4.3). The intracellular pool of tyrosine remained constant during three hours of incubation; the overall slower rate of proteolysis was due to the presence of inhibiting factors (see below). The isolated diaphragm may leak intracellular components during the first half hour after the ribs are trimmed away, but subsequently appears stable by several criteria (75) (and hence a 30 min preincubation was routinely performed).

The net production of tyrosine was taken to indicate net protein breakdown since tyrosine is neither synthesized nor degraded in muscle tissue (73); the tissue tyrosine content is unchanged over 3 hr of incubation (suggesting a specific release of tyrosine from the intracellular pools) (73), and since tyrosine rapidly equilibrates between intracellular pools and the medium.

Reutilization of amino acids from the intracellular pools occurring concurrently with their release into the medium is an important consideration in protein degradation studies. Inhibiting protein synthesis allows absolute rates of proteolysis to be obtained. Cycloheximide, an inhibitor of protein synthesis, did not substantially affect the proteolytic rate in this investigation (Table 4.1), suggesting that reutilization of tyrosine during 3 hour incubation is minimal. Cycloheximide has been known to reduce absolute rates of protein degradation concomitantly with inhibition of protein synthesis (73). For purposes of this study, where absolute rates of proteolysis were not required, net tyrosine release was

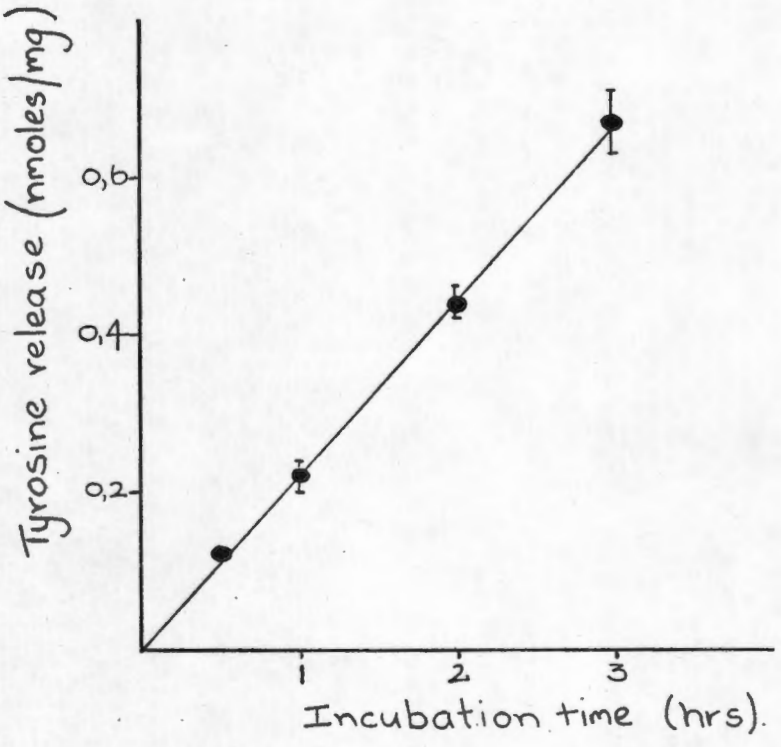

Figure 4.2. Mean release of tyrosine with time of incubation of 3-5 experimental animals.

Release of trichloracetic acid (TCA) soluble tyrosine from muscle protein at different times of incubation, where each point excluding 30 mins incubation is the mean ±S.E. for muscle pieces from between 3-5 rats.

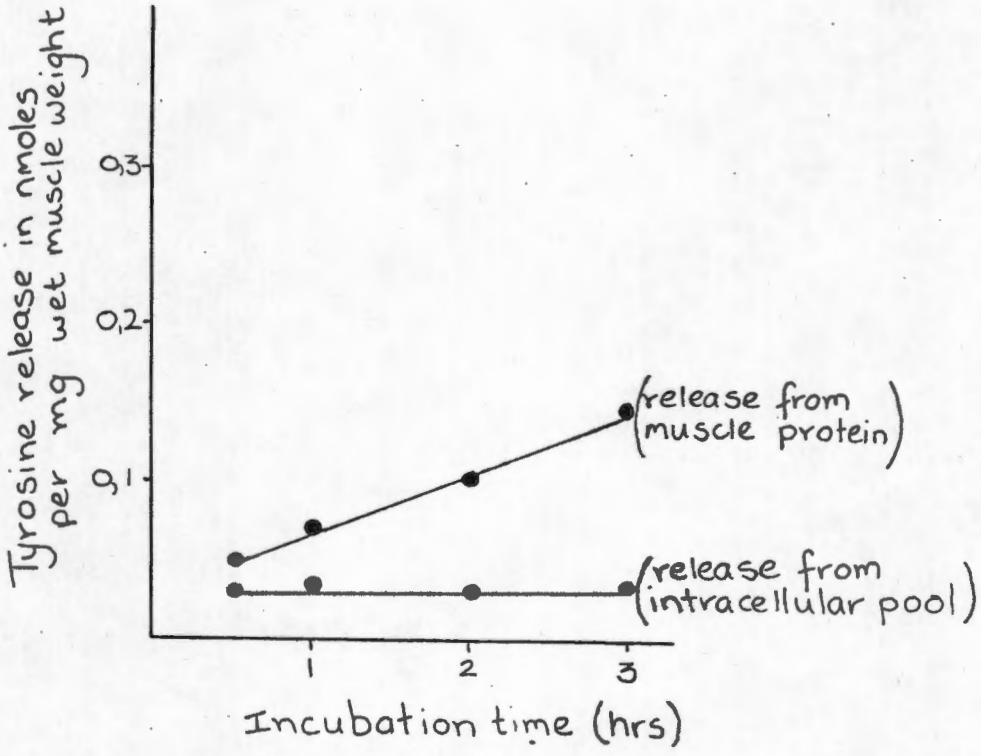

Figure 4.3. The intracellular pool of tyrosine over three hours in incubation.

Release of TCA-soluble tyrosine from muscle protein over 3 hours of incubation, following a 30 min pre-incubation period, in KRB-buffer + insulin (0,1 units/ml) glucose (10 mM) branched chain amino acids (x5 plasma concentration).
Tyrosine in the intracellular pool was measured after varying incubation times in the case of individual diaphragm quarters.

TABLE 4.1

ADDITION OF CYCLOHEXIMIDE(0.5 mM) TO THE KRB-BUFFER INCUBATION MEDIUM

Incubation medium KRB-buffer	Net Tyrosine Release (nmoles/mg/hr)
No cycloheximide	0.31 ± 0.01
+ 0.5 mM cycloheximide	0.32 ± 0.02

(Mean ± S.D.)

Results are the (mean ± S.D.) for paired duplicate samples within one experimental animal. The percentage variation is 3%.

measured and overall rate of proteolysis determined, in the face of ongoing protein synthesis.

Tyrosine release determined fluorimetrically was exactly reproducible (Figure 4.4) and gave experimental values that were 30-40% higher than those for the same experimental samples given by amino acid analysis (Table 4.2); the fluorimetric procedure also measures small tyrosyl-peptides in the TCA-soluble fraction of the precipitated medium. In the course of this report, all tyrosine release data include these small acid-soluble peptides.

The system responded to various physiological stimuli. Fasted rat diaphragms released more tyrosine than diaphragms from fed rats (Figure 4.5). It was hoped that diaphragms from fasted rats may have released sufficient tyrosine to accommodate the tyrosine blank of serum and allow addition of "whole" serum to the incubation flasks. Unfortunately, this was not the case.

Addition of factors known to inhibit tyrosine release from isolated diaphragms gave a decrease of 31 $\pm$ 0.9% (mean $\pm$ S.E.) in the proteolytic rates of isolated diaphragm quarters from 5 different rats (Figure 4.6). This effect was higher than that reported for the influence of each separate agent, i.e. insulin 0.1 units/ml; glucose, 10 mM; branched chain amino acids (BCA), 5 times normal rat plasma concentrations) (73) and may have represented additive effects.

Fed rat diaphragms were routinely used in the investigations on serum from cancer patients, since diaphragms from fasted rats, in a proteolytic mode, would require any proteolytic factor present in the serum to enhance already "activated" processes.

Known promotors of catabolism present in serum in varying amounts, which could be responsible for altered proteolytic

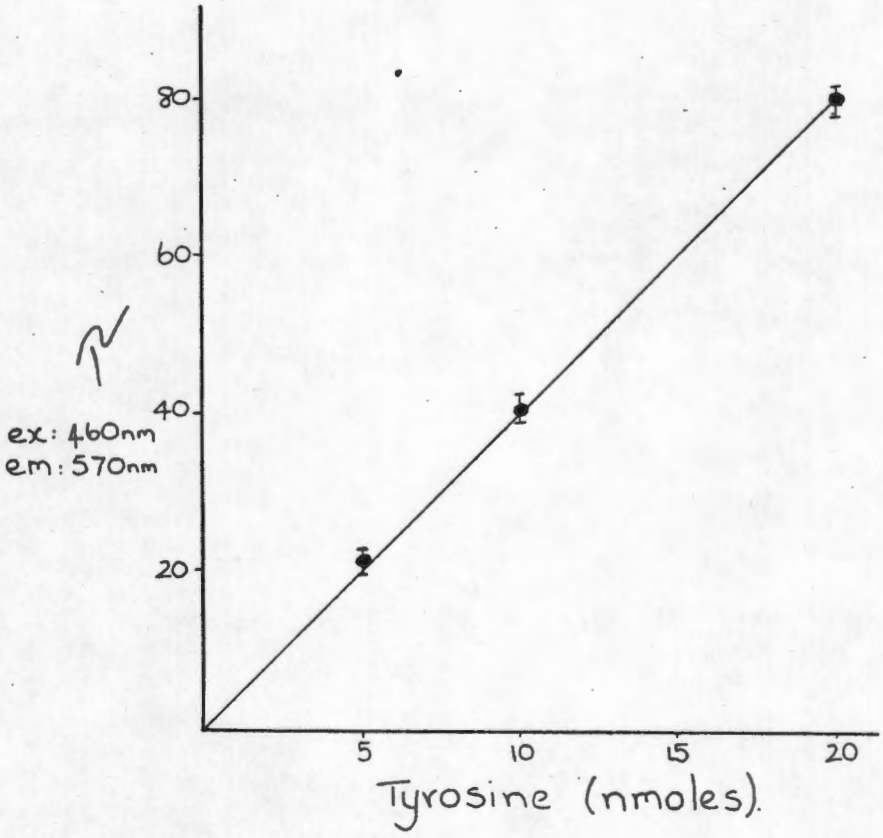

Figure 4.4. Standard curve for the determination of tyrosine.

The range of 0-20 nmoles tyrosine was measured fluorimetrically (λex: 460 nm; λ em: 570 nm). Each point is the mean ±S.D. of 20 determinations.

TABLE 4.2

TYROSINE RELEASE MEASURED BY FLUORIMETRY OR BY AMINO ACID ANALYSIS

Incubation conditions	nmoles Tyrosine determined fluorometrically	nmoles Tyrosine using amino acid analysis	% diff.
KRB-buffer	12.0	7.0	40
KRB-buffer + insulin + glucose + BCA	6.0	4.0	33

TCA-soluble tyrosine released into KRB-buffer or KRB-buffer + insulin (0.1 units/ml); glucose 10 mM; branched chain amino acids, 5 x normal rat plasma concentrations, during 3 hr incubation periods.

Amino acid analysis measured using lithium buffers, and a Model 121-M Beckman amino acid analyser.

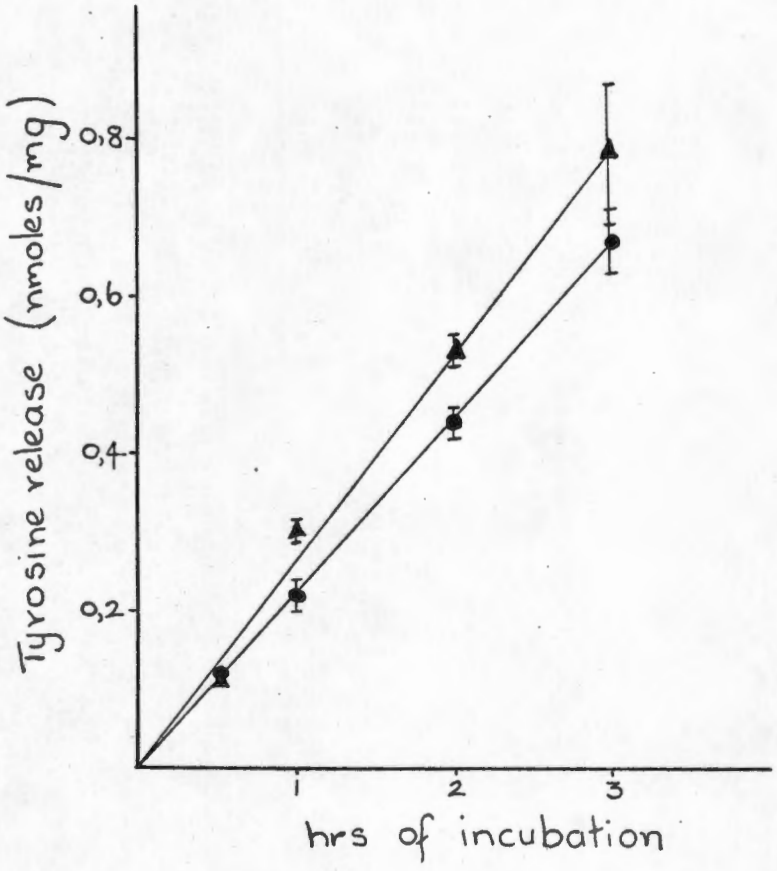

Figure 4.5. Tyrosine release from diaphragms of fed or fasted rats.

TCA-soluble tyrosine released from muscle protein, into KRB-buffer, using diaphragms from fasted (▲) and fed (●), during various periods of incubation. Each point, excluding 30 mins incubation, is the mean ±S.E. of 3-5 rats.

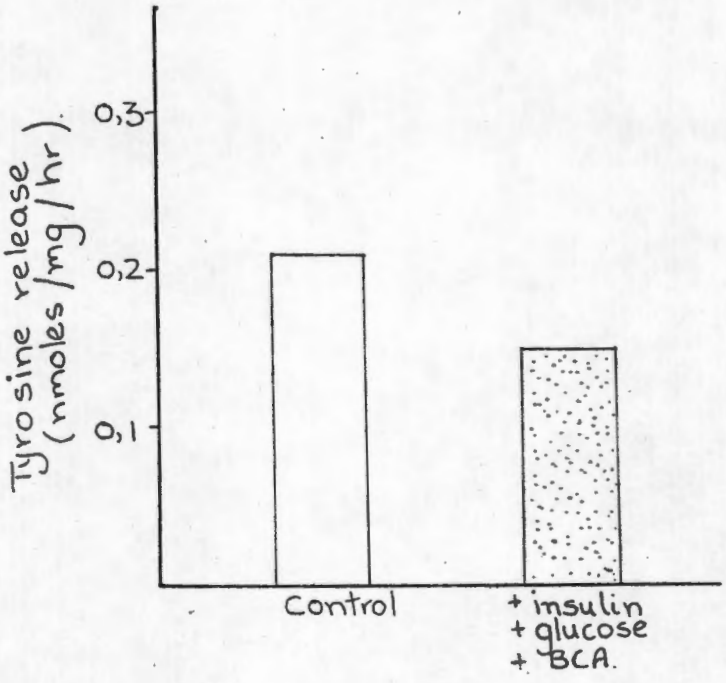

Figure 4.6. Release of tyrosine into KRB-buffer and supplemented KRB-buffer.

Proteolytic rates of quarter diaphragms from fed rats in KRB-buffer alone or in medium supplemented with insulin. 0,1 units/ml; glucose, 10 mM; branched chain amino acids (BCA) at 5x normal rat plasma concentrations. Each value represents the mean ±S.E. for 6 rats.

rates independently of cancer-specific serum factors, were tested in the system to assess the significance of their possible contributions. Glucagon and hydrocortisone added at concentrations in the human physiological range exerted no significant effects on proteolytic rates (Figures 4.7a; 4.8a); the concentration of glucagon to 10^6 times the physiological level, caused an increase in proteolysis of about 90%; this effect was dose dependent (Figure 4.7b). However, the likelihood of such glucagon levels being present in serum samples from patients selected by the criteria set out (4.2.1.3), is very small and was ignored.

Supra-physiological amounts of hydrocortisone did not elicit any response from the muscle tissue (Figure 4.8b). This may not be surprising in view of a finding by Tomas *et al* (76) which indicates that the active hormone in the rat is corticosterone. The influence of varying physiological levels of either glucagon or hydrocortisone in the serum samples, and hence added to the incubation medium, was ignored.

4.2.2.2. Effects of serum from cancer patients.

Serum from either fed or fasted patients contained, on average, 50-60 nmoles of tyrosine per ml of serum. This serum tyrosine blank was reduced to an acceptable level for experimental purposes by desalting on a Sephadex G-25 column, namely about 2 nmoles/ml tyrosine, which was less than one-tenth the experimental values.

Serum from human control subjects, incubated in a 1:1 ratio with KRB-buffer caused a slight decrease in the release of tyrosine from muscle (Table 4.3). The mean percentage decrease was 11 $\pm$ 4.4% (mean $\pm$ S.E.) for paired internal comparisons and was hardly above the 5-7% error inherent in the system. Serum addition to supplemented medium (i.e. medium containing insulin; glucose; BCA) also caused a decrease of 20 $\pm$ 6.3% (mean $\pm$ S.E.) for paired internal comparisons.

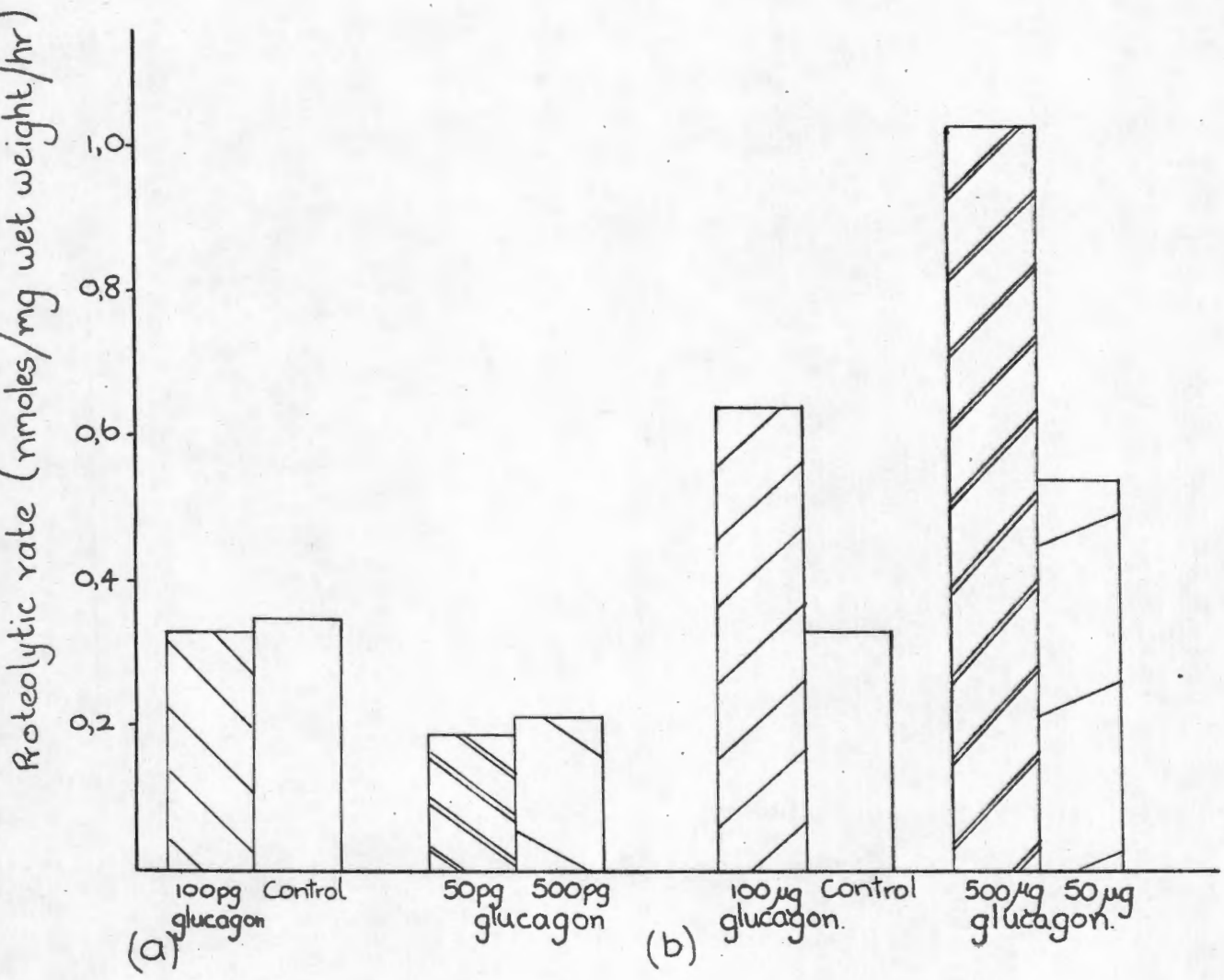

Figure 4.7(a)&(b). Effect of glucagon in (a) physiological and (b) supraphysiological amounts on the proteolytic rate of isolated rat diaphragms.

Glucagon added in (a) physiological amounts (50 pg; 100 pg; 500 pg); (b) supraphysiological amounts [x10^6] (50 µg; 100 µg; 500 µg).
Histogram shows duplicate paired comparisons within one experimental animal.

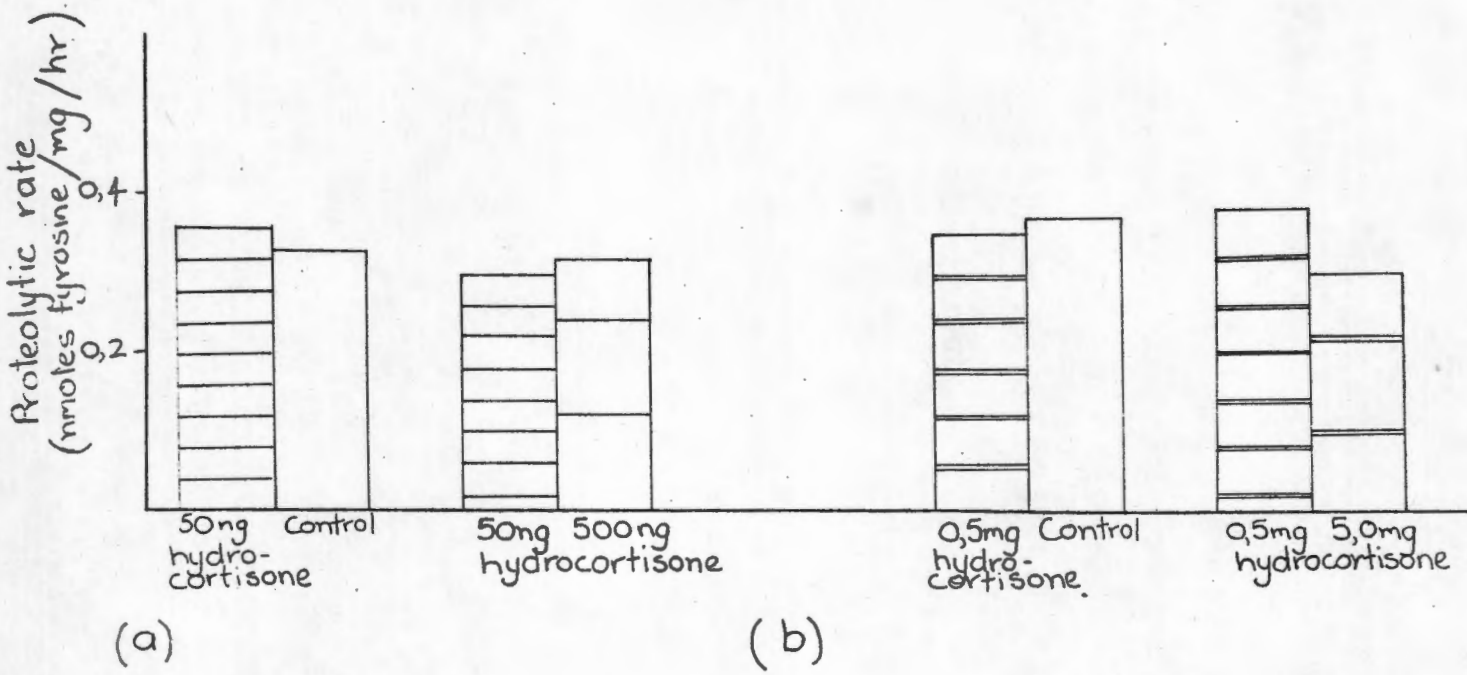

Figure 4.8(a) & (b). Effect of hydrocortisone in (a) physiological and (b) supraphysiological amounts on the rate of proteolysis in isolated rat diaphragms.

Histogram shows duplicate paired comparisons within tissue samples from individual experimental animals. Hydrocortisone added in (a) physiological amounts (50 ng; 500 ng) and (b) supraphysiological amounts (0,5 mg; 5,0 mg).

TABLE 4.3.

EFFECT OF CONTROL HUMAN SERUM ON THE RATE OF TYROSINE RELEASE FROM TISSUE

Incubation condition		nmoles tyrosine release per mg per hour
No additions	KRB-buffer...	0.21 ± 0.04
	50% serum....	0.18 ± 0.01
+ insulin + glucose + BCA	KRB-buffer...	0.14 ± 0.01
	50% serum....	0.11 ± 0.03

Diaphragm pieces incubated in medium containing 50% desalted serum from a human control patient, or KRB-buffer alone and in the presence or absence of insulin, 0.1 units/ml; glucose, 10 mM; branched chain amino acids (BCA) at 5 x normal concentration. Results are the mean ± S.D. of 3 rats and serum from 3 control patients.

This suggests that human serum contains one or more inhibitory factor(s). Incubations investigating serum from cancer patients were routinely performed in unsupplemented medium.

Comparisons of serum from a control patient whether in a fed or fasted state, showed no significant differences in tyrosine release (Table 4.4), the slight inhibitory effect of serum addition on the overall rate of proteolysis (normal rate, 0.22 nmoles/mg/hr) is apparent in both cases. There appeared no advantage therefore in using serum from fasted patients, hence serum samples were routinely obtained from patients who were fed, which in any case could be more closely monitored.

Serum samples from 5 weight-losing cancer patients were compared, in internal paired experiments with serum samples from 5 matched control patients. No difference in proteolytic rate was observed (Table 4.5). Likewise, serum samples from 5 non-weight losing cancer patients compared with 5 matched control patients also showed no difference in proteolytic rate (Table 4.5). Expressing the results as percentage differences of paired internal comparisons gave a mean 9% increase in proteolytic rate attributable to serum from weight-losing cancer patients, and no increase for serum from non-weight-losing cancer patients (Table 4.6). The 9% increase seen was hardly above the 5-7% error inherent in the system.

4.2.2.3. Effects of transformed cell culture medium.

A continued investigation of any cancer-mediated effect on *in vitro* muscle proteolysis was carried out using medium from the $R_9Cl_{10}T$ cell cultures. It was hoped that any factors produced by the transformed rat cells and secreted into the medium would elicit a response in the incubation system, and because a rat cell line was used, species differences would be eliminated.

Medium obtained from these cell cultures failed to show any

TABLE 4.4.

TYROSINE RELEASE IN THE PRESENCE OF SERUM FROM ONE CONTROL PATIENT IN A FED OR FASTED STATE

Serum source	nmoles Tyrosine release from protein/mg/hr
Fed control patient	0.14 ± 0.01
Fasted control patient	0.15 ± 0.01

Serum addition was 50% of the incubation medium. The "fasted" serum sample was collected after a 20 hr fast, the "fed" serum sample was collected 2½ hours post-eating, and both samples came from one control patient and were used fresh, following desalting.
The results are the mean ± S.D. of an internal paired duplicate experiment.

TABLE 4.5.

PROTEOLYTIC RATE OF ISOLATED DIAPHRAGM PIECES IN THE PRESENCE OF SERUM FROM WEIGHT-LOSING AND NON-WEIGHT LOSING CANCER PATIENTS

Source of serum	Proteolytic rate (nmoles/mg/hr)
Weight-losing cancer patients	0.18 ± 0.02
Control (non cancer) patients	0.17 ± 0.02
Non-weight losing cancer patients	0.19 ± 0.03
Control (noncancer) patients	0.19 ± 0.03

Incubation in 50% desalted serum. Internal paired comparisons between serum from cancer and matched control patients performed using 5 weight-losing and 5 nonweight-losing cancer patients. Each determination performed in duplicate.
Results are the mean ± S.D. of 10 animals in each group.

TABLE 4.6.

THE PERCENTAGE INCREASE IN PROTEOLYTIC RATE OF INCUBATIONS IN THE PRESENCE OF "CANCER" SERUM COMPARED WITH "CONTROL" SERUM

Cancer Patient Group	% Increase in Proteolytic rate
Weight-losing.....	9 ± 11.1
Non-weight losing....	0 ± 5.8

Expressing the change in proteolytic rates as a percentage increase for paired duplicate determinations on single experimental animals. Results are the mean ± S.D. Percentage increase in proteolytic rates determined individually for cancer and control patient serum samples; and for both cancer patient groups. The % increase in proteolytic rate =

$$\frac{\text{Proteolytic rate in "cancer" serum - proteolytic rate in "control" serum}}{\text{Proteolytic rate in "control" serum}}$$

effect, either when comparing this medium with KRB-buffer (Table 4.7) or when comapring it to the effect obtained using medium from untransformed R_9Cl_{10} cell cultures (Table 4.8). The percentage difference for paired internal comparisons was 7% in both cases.

4.2.2.4. Effects of serum from rats.

The response of the *in vitro* rat muscle proteolysis system was further investigated using various rat serum samples obtained from rats subjected to a variety of hormonal and physiological treatments. The response of rat muscle to factors present in rat serum, was thus investigated.

Serum from control fed rats had no significant effect on the KRB-buffer system (Table 4.9), the percent difference for paired internal comparisons being $3 \pm 2.8\%$ (mean $\pm$ S.E.). Addition of serum from rats fasted for 90 hr or 130 hr compared with serum from fed rats, produced no difference in the mean proteolytic rates (Table 4.10).

A paired internal comparison of the proteolytic rates of rat muscle pieces incubated in serum from fed rats with serum from diabetes-induced rats and from hydrocortisone-treated rats, either in a fed or 130 hr fasted state, showed no differences either (Table 4.11). Serum from fed rats compared with serum from T_3-treated rats showed no significant difference (Table 4.12).

In all these cases the percentage difference of the paired internal comparisons showed no variation above the error for the system. All the treatments, however, caused increased proteolysis of isolated muscle of the animals *in vitro* (78,79,83). Serum from these animals may be expected to contain a variety of hormones and other substances, so that incubated diaphragm pieces should reflect their activity, in so far as they survive the desalting procedure required to prepare the serum. Animals may, however, vary in their

TABLE 4.7.

TYROSINE RELEASE IN THE PRESENCE OF MEDIUM FROM TRANSFORMED CELL CULTURES

Incubation condition	Proteolytic rate (nmoles/mg/hr)
KRB-buffer.....	0.21 ± 0.06
1:1 ratio KRB-buffer and medium from $R_9Cl_{10}T$ cell cultures.....	0.19 ± 0.05

The release of TCA-soluble tyrosine in the presence of 50% medium from transformed cells i.e. $R_9Cl_{10}T$ cell cultures compared with the release into KRB-buffer. Results are the mean ± S.D. of paired duplicate experiments using 4 rats.

TABLE 4.8

TYROSINE RELEASE IN THE PRESENCE OF CELL CULTURE MEDIUM FROM TRANSFORMED AND UNTRANSFORMED CELL CULTURES

Source of cell culture medium	Proteolytic rate (nmoles/mg/hr)
R_9Cl_{10} cell cultures.....	0.19 ± 0.09
$R_9Cl_{10}T$ cell cultures.....	0.21 ± 0.09

(Mean ± S.D.)

Quarter diaphragms incubated in the presence of 50% medium from transformed cell cultures ($R_9Cl_{10}T$) or medium from untransformed R_9Cl_{10} cell cultures (control). The rates are the means ± S.D. of paired duplicate determinations.

TABLE 4.9.

TYROSINE RELEASE IN THE PRESENCE OF SERUM FROM FED RATS

Incubation condition	Proteolytic rate nmoles tyrosine/mg/hr
KRB-buffer.....	0.27 ± 0.04
1:1 ratio of serum from a fed rat and KRB-buffer	0.25 ± 0.05

Quarter diaphragms incubated in the presence of 50% serum from a fed control rat and compared with incubation in KRB-buffer alone. Results are the mean ± S.D. of five rats.

TABLE 4.10.

TYROSINE RELEASE IN THE PRESENCE OF SERUM FROM FED RATS AND FASTED RATS

Nutritional state of serum donor rat	Proteolytic rate (nmoles/mg/hr)
Fed.....	0.25 ± 0.05
90-hour fasted.....	0.25 ± 0.03
130-hour fasted.....	0.25 ± 0.03

Release of tyrosine in the presence of serum from fed rats compared with the release in the presence of serum from 90-hour and 130-hour fasted rats. Results are the mean ± S.D. using 4 separately pooled sets of sera and performing each set in duplicate.

TABLE 4.11.

TYROSINE RELEASE IN THE PRESENCE OF SERUM FROM RATS SUBJECTED TO VARIOUS PHYSIOLOGICAL STRESSES

Physiological status of serum donor rat	Proteolytic rate (nmoles/mg/hr)
Fed	0.23 ± 0.007
Diabetic ..... (Alloxan treated)	0.24 ± 0.02
Hydrocortisone-treated	0.22 ± 0.02
Hydrocortisone-treated + 130-hour starvation.....	0.24 ± 0.04

Quarter diaphragms incubated in the presence of 50% serum from rats subjected to various treatments. Each serum sample was pooled from 2 rats. Results are the mean ± S.D. of duplicate paired determinations.

TABLE 4.12.

TYROSINE RELEASE IN THE PRESENCE OF SERUM FROM TRIIODOTHYRONINE (T_3) - TREATED RATS

Treatment of donor rat prior to serum collection	Proteolytic rate (nmoles/mg/hr)
Control	0.26 ± 0.02
T_3-treated	0.25 ± 0.05

Quarter diaphragms incubated in the presence of 50% serum from T_3-treated rats and compared with incubation in serum from control rats. Results are the mean ± S.D. of duplicate matched internal comparisons.

response to the treatment, or alternately the factors produced may be present in different amounts and may cancel out the effects such that overall no variation is seen. No prediction can confidently be made of what one would expect to find in this type of experiment.

The overall lack of response was disturbing since it suggested a lack of sensitivity in the system. Alternately, the desalting process or insufficient length of incubation could have contributed or invoked this loss of sensitivity. An alternate proteolysis system was therefore investigated, which deviated the problem of incubation time, and desalting of the serum: in order to confirm the absence of proteolysis promoting factor(s) in serum as tested by the diaphragm assay.

4.3. CELL CULTURE ASSAYS

4.3.1. Experimental Procedures.

4.3.1.1. Culture of cells.

The established myoblast cell line L8, originally isolated from rat skeletal muscle (80) was used.

Cells were thawed from storage in liquid nitrogen and grown to confluence in 75 cm^2 stock flasks in a 4:1 mixture of Dulbecco's Modified Eagle's Medium and Medium 199 containing 10% horse serum (see Appendix), 60 µg/ml penicillin G and 100 µg/ml streptomycin sulphate. Cells were incubated at 37°C in a humidified incubator with 5% CO_2 in air. After myoblast proliferation to confluence in the stock flask, the cells were dissociated with Trypsin-EDTA solution and seeded at 10^5 cells per 35 mm petri dish containing 2 ml of growth medium. The medium was changed every three days. Once confluent, the cells progressively fused to form large multinucleate syncytia (myotubes) which synthesized muscle-specific proteins and contracted spontaneously after 7-8 days growth.

All experiments were performed on cells between sixth and tenth passage and were initiated on dishes showing at least 80% of nuclei in fused myotubes.

4.3.1.2. Radioactive labelling of cell proteins.

Cell cultures were labelled for 16 hours in growth medium containing 1 μCi/ml of [^{3}H]-phenylalanine; this resulted in the preferential labelling of proteins having relatively long half-lives i.e. "long-lived" proteins (80). The radioactive medium was then removed and the cells were washed rapidly in ice-cold sterile phosphate-buffered saline (PBS), in order to remove all extracellular [^{3}H]-phenylalanine (4 such washes were sufficient)(80). A series of four consecutive 30-minute incubations at 37°C, adding 1 ml fresh growth medium after each one, was carried out to "wash out" the intracellular free [^{3}H]-phenylalanine. This resulted in a stepwise extraction of the intracellular [^{3}H]-phenylalanine since it has been shown that the intracellular concentration of [^{3}H]-phenylalanine is directly proportional to the extracellular concentration after 30 mins at 37°C (80). A minimum level of intracellular [^{3}H]-phenylalanine was reached which remained constant throughout any subsequent incubations. The release of [^{3}H]-phenylalanine was measured as the net increase in acid-soluble radioactivity during incubations with test media. Following the washes at 37°C, 1 ml of fresh "chase" medium containing 1 mM excess non-labelled phenylalanine was added to the cell layers. The excess "cold" phenylalanine decreased isotope reutilization. The release of labelled amino acid into this medium for various time periods, was measured to determine the rate of intracellular proteolysis: Cells were incubated at 37°C, after which the medium was removed and assayed for TCA-soluble radioactivity by precipitation with equal volumes of 20% TCA for 30 mins at 0°C. The cell layers were washed with PBS and 1 ml 1% sodium dodecyl sulphate (SDS) was then added to solubilize the cells, which were removed from the culture

dish after 10 minutes, using a "rubber policeman". This solubilization was repeated. The SDS lysate (2 ml) was then briefly sonicated, a 1 ml aliquot counted to determine the total radioactivity within the cells and the remaining 1 ml was supplemented with 1% bovine serum albumin (acting as a carrier), precipitated with equal volumes of 20% TCA at 0°C for 30 mins and then spun at 1200 *g* for 15 mins using a Beckman TJ-6R centrifuge. The supernatant from the cell lysate was counted to obtain the residual amount of intracellular TCA-soluble radioactivity.

All samples were counted in Instagel, in a Beckman scintillation counter. The samples were checked for chemiluminescence after 24 hours, but due to the acidic nature of the samples, this was never a significant problem (80).

Intracellular protein degradation in each case was expressed as the percentage of initial total protein radioactivity that was released into the "chase" medium as free amino acid:

$$\% \text{ Proteolysis} = \frac{\text{dpm } [^3\text{H}]\text{-Phe in TCA-sol. medium}}{\text{dpm in Total Protein at time 0}}$$

, where dpm in Total Protein at time 0 = (Total intracellular dpm - intracellular TCA-soluble dpm).

4.3.1.3. Intracellular proteolysis in the presence of human serum.

Cells were grown as described and labelled for 16 hours under the conditions described for basal proteolysis measurements. During the two hour, step-wise extraction, washing procedure, and during the following chase period, the cell layers were incubated in medium containing 10% human serum (from either control, non-cancer bearing patients or from weight-losing cancer patients) added in the place of the 10% horse serum.

Where the percentage human serum substitution was >10% (i.e. 15% or 40%) the medium was present only during the chase period. The proteolysis rate was measured and the results

expressed, as described for basal proteolysis measurements.

Control human serum samples were obtained from a healthy, noncancer-bearing patient, in the fed state (see 4.2.1.3). Serum samples were obtained from cancer patients satisfying the criteria set out (see 4.2.1.3), and having substantial weight loss. No non weight-losing cancer patients were used in this study (i.e. patients having cancer of the breast).

The serum obtained from centrifugation of the blood samples was added to the medium following sterile filtration, and did not undergo prior desalting as described previously (see section 4.2).

All serum samples were used on the day they were obtained from the patients.

4.3.1.4. Accelerated intracellular proteolysis.

Cells were grown as described and labelled for 16 hours as for basal proteolysis measurements. As described above, during the 2 hour step-wise extraction washing procedures, and during the subsequent chase period, the cell layers were incubated in a medium which contained no horse serum (or human serum substitute) and no chick embryo extract (i.e. minimal medium causing nutritional step-down conditions). The "accelerated" proteolysis rate was measured as described above.

4.3.1.5. Intracellular proteolysis in the presence of inhibiting factors.

Cells were grown and labelled, as described, and the washing procedures were performed as for basal proteolysis. The chase medium contained insulin (0.1 units/ml); glucose (10 mM) and BCA at five times the plasma concentrations.

The proteolysis rate in the presence of these factors (known

to decrease proteolysis in muscle incubations in vitro (73) was then measured.

4.3.2. Results

4.3.2.1. Validation of methods.

[^{3}H]-phenylalanine was chosen as a marker for proteolysis since this amino acid is largely unmetabolized in muscle (80) due to the absence of the enzyme phenylalanine hydroxylase. Cultures of L8 skeletal muscle cells were used since they were a line already in use, and characterized by Dr T. Scott-Burden and co-workers in this laboratory (unpublished experiments).

Release of [^{3}H]-phenylalanine was linear up to 4 hours, the release of radioactive label being expressed as a percentage of the initial intracellular protein radioactivity (Figure 4.9). The proteolytic rate of 1.5 - 1.7% per hour was similar to that reported by other workers (80).

4.3.2.2. Intracellular proteolysis in the presence of human serum.

The substitution of horse serum by 10% human control serum, present on the cell layers following the labelling period, did not significantly alter the proteolysis rates of the cells (Figure 4.9). Serum from 3 weight-losing cancer patients at 10% final concentration did not show any difference in percent proteolysis when compared with the effect of serum derived from patients without cancer (Figure 4.10). The proteolytic rate was 1.5% per hour.

Increasing the serum substitution to 15% and 40% final concentration also caused no significant differences to appear in comparisons with 10% horse serum, both in the case of serum from cancer and "noncancer" patients (Figure 4.11). Adding 40% "human control" serum did cause a slight decrease in the overall rate of proteolysis, as was seen with 40%

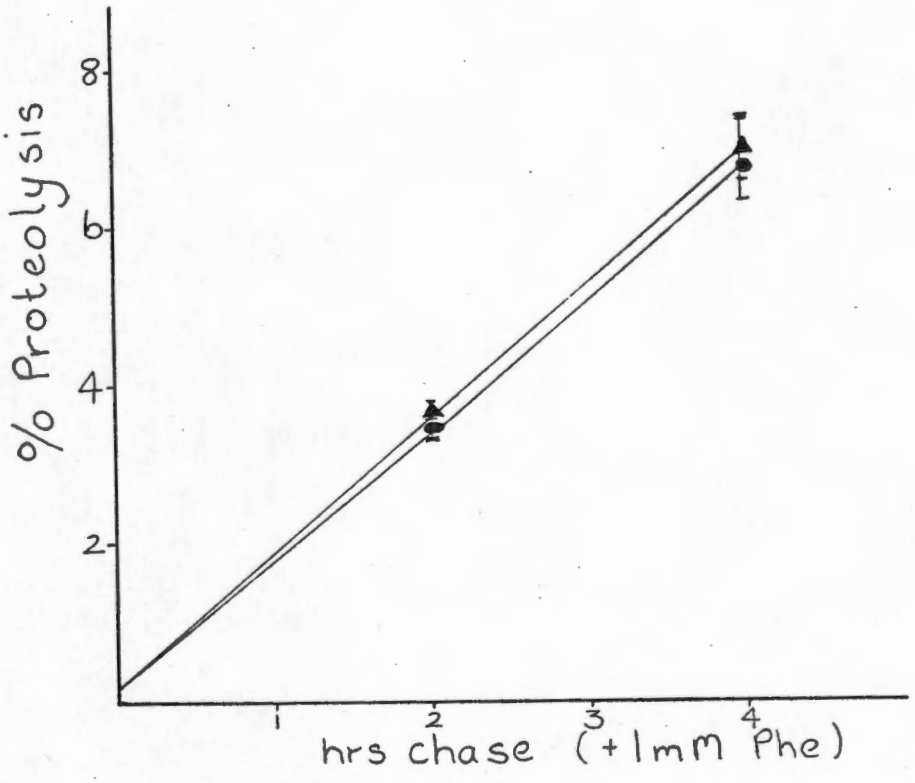

Figure 4.9. Intracellular basal proteolysis and proteolysis of cell layers exposed to serum from a control patient, with increasing time.

% Intracellular proteolysis of cell layers exposed to (▲) 10% human control serum, (●) 10% horse serum (where the serum is present for the period post-labelling of the cell layers). Each point is the mean ±S.E. of 4 dishes.

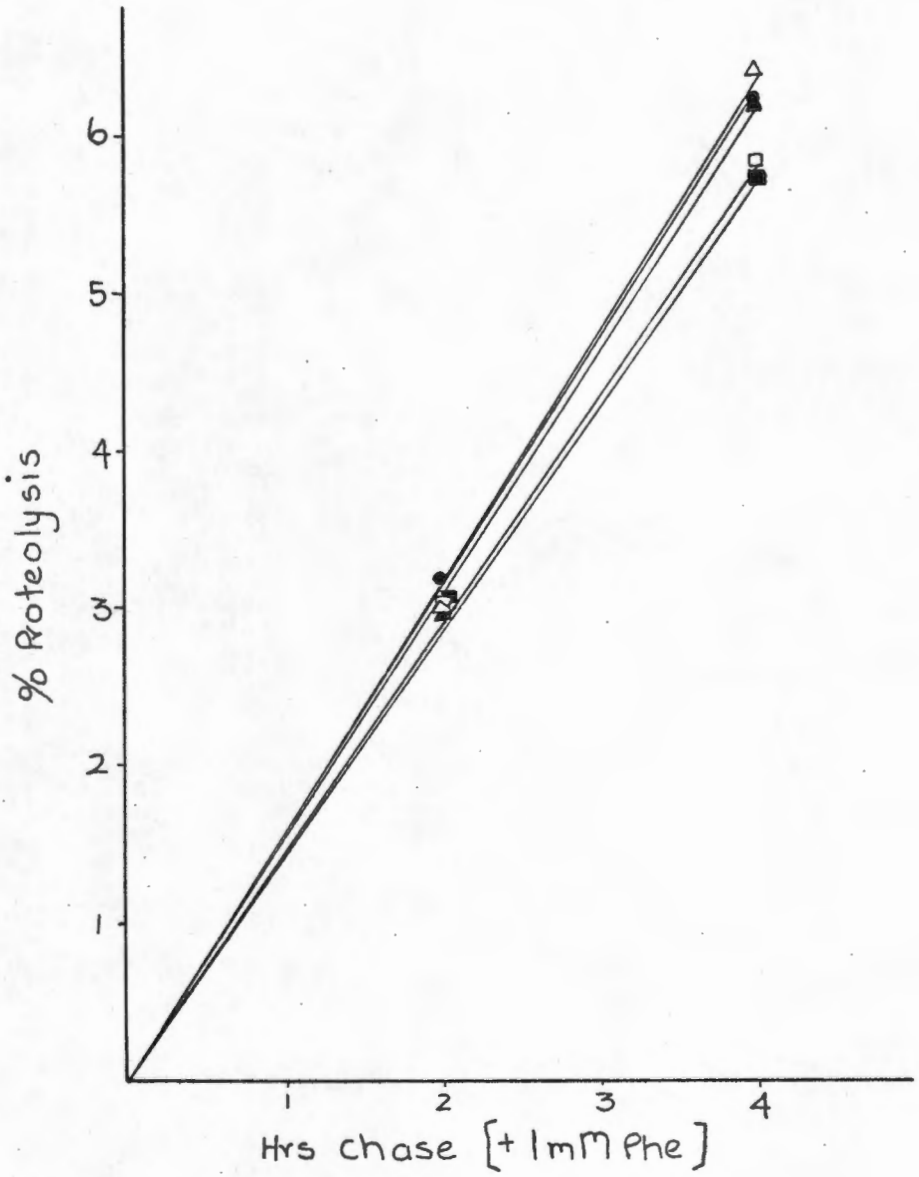

Figure 4.10. Intracellular proteolysis of cell layers in the presence of serum from weight-losing cancer patients.

Intracellular proteolysis of cell layers in the presence of 10% serum from "non-cancer" patient (●) and serum from 4 weight-losing cancer patients:

(Δ-Δ) cancer patient 1
(▲-▲) " " 2
(□-□) " " 3
(■-■) " " 4

Each point is the mean of 2 dishes and results are expressed as the percentage proteolysis occurring.

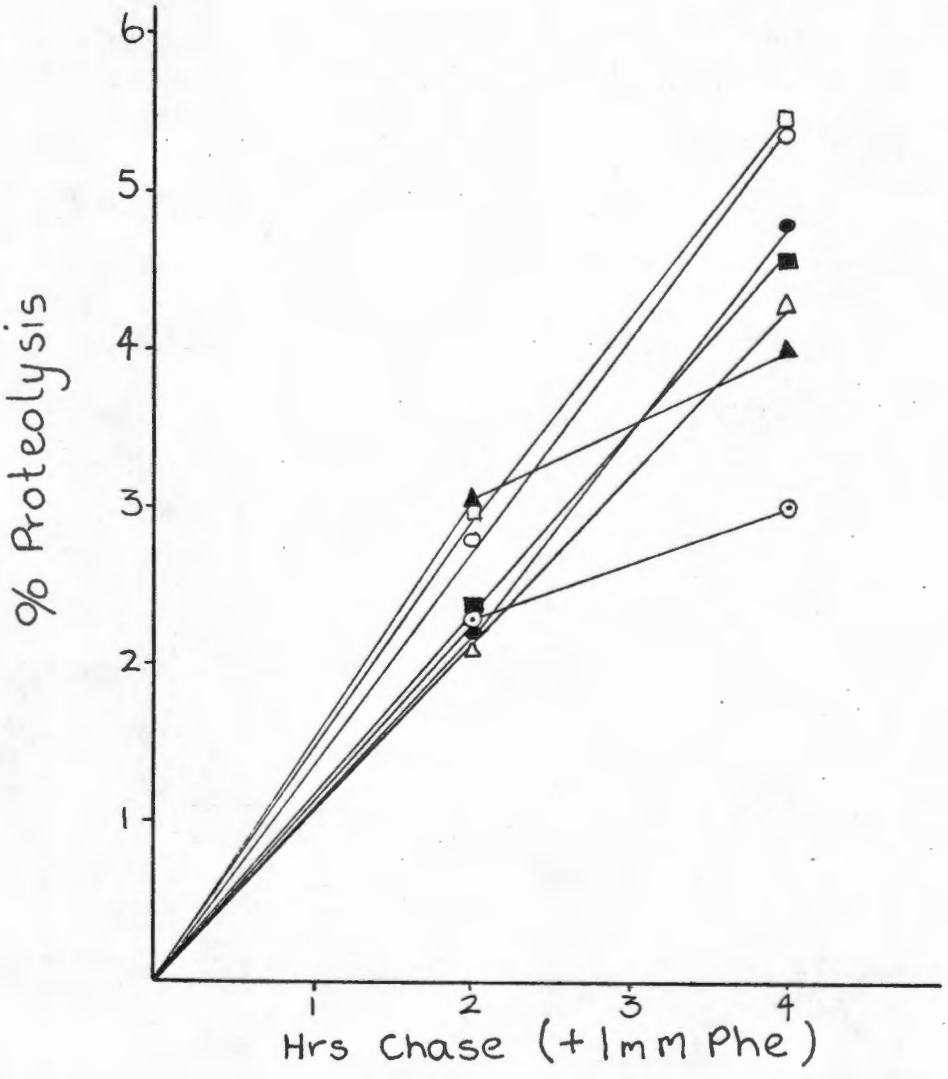

Figure 4.11. Intracellular proteolysis of cell layers exposed to increasing concentrations of serum from both cancer and non-cancer patients.

Concentrations of human serum increased to 15% and 40%; non-cancer patient (Δ-Δ , 15%); (▲-▲ , 40%), and weight-losing cancer patient (1) (□-□ : 15%; ■-■ : 40%), and patient (2) (0-0 : 15%; ⊙-⊙ : 40%). Intracellular proteolysis under basal conditions; (●-●), 10% horse serum Each point is the mean of 2 dishes and results expressed as a percentage of the proteolysis occurring.

substitution of one cancer patient serum sample. This may indicate some sort of effect arising due to the increased concentration of the serum.

Placing serum from a weight-losing cancer patient on the cell layers for a longer time period caused a more marked effect (Figure 4.12). Cell layers were exposed to serum from the same cancer patient at 10% final concentration for the 24 hr pre-label period; the post-label period or both pre- and post-label period. The longer exposure to serum from the cancer patient showed an increase of 20% in the percent proteolysis of the cell layers, when compared with those cell layers exposed for shorter periods of time. The presence of serum from cancer patients during the labelling period did not alter the total label incorporation into protein in 16 hr.

4.3.2.3. Accelerated intracellular proteolysis.

Under conditions of "starvation" i.e. in minimal medium, an increase of almost 50% (48%) in the percentage proteolysis was seen (Figure 4.13), when compared with the percentage proteolysis under standard conditions. This represents a normal proteolytic response to a physiological stress, and has been found by other workers (80).

4.3.2.4. Intracellular proteolysis in the presence of inhibiting factors.

The addition of exogenous factors known to inhibit proteolysis unexpectedly caused a stimulatory effect of 20% in percentage proteolysis compared with the percentage proteolysis in unsupplemented medium. The similarity between the increase effected by these factors and by the serum exposed to the cell layers for a longer time period may indicate that increases observed with serum were not true increases but artefacts arising from these factors in the serum.

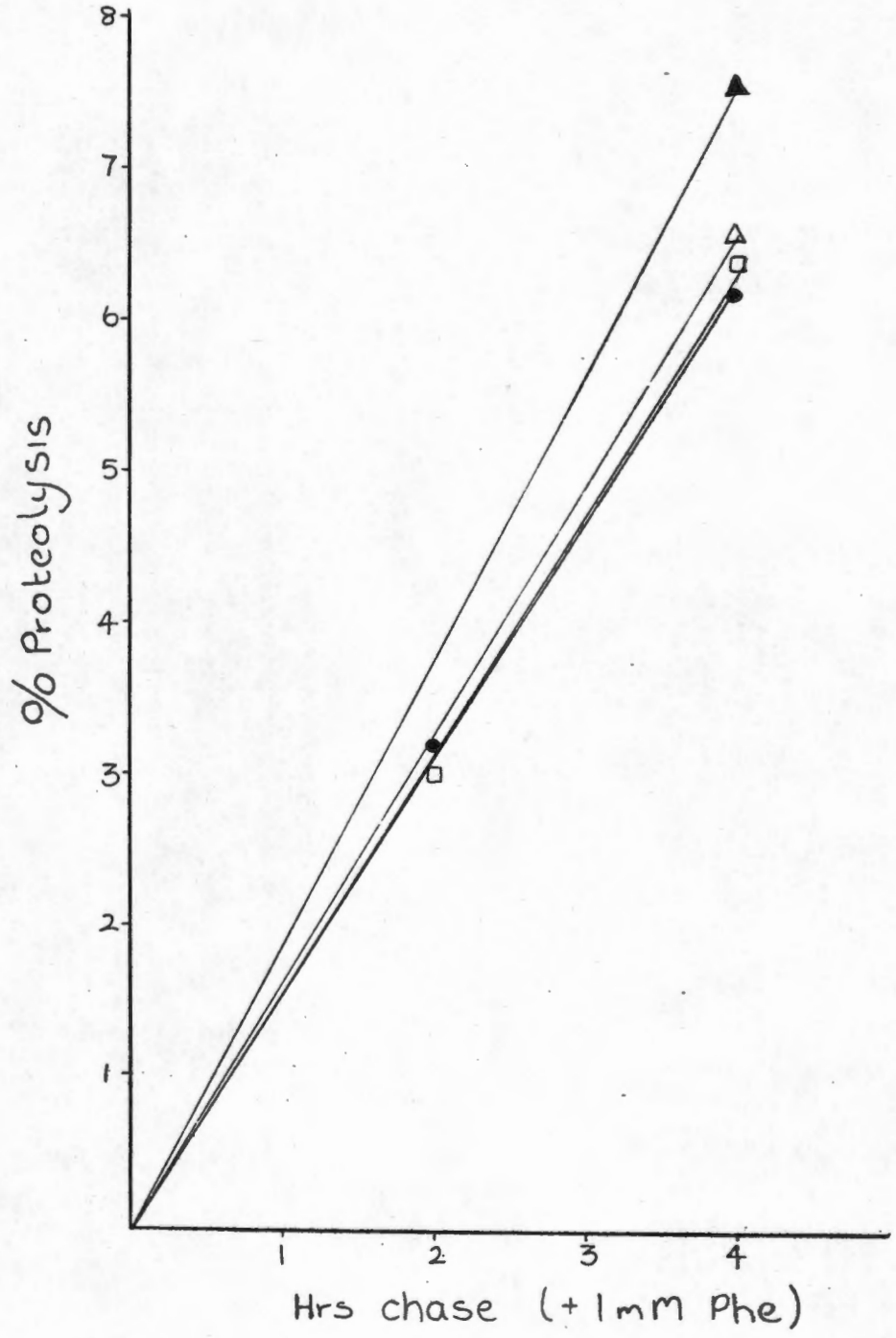

Figure 4.12.

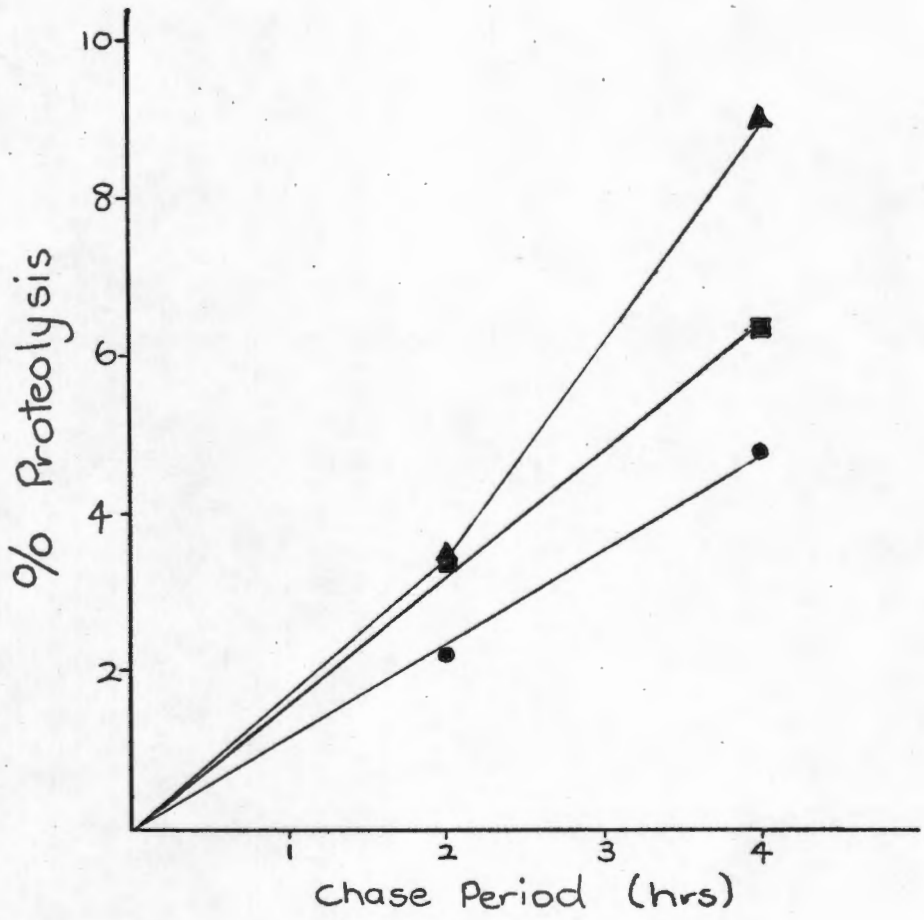

Figure 4.13. Response of system to starvation and addition of proteolytic inhibiting factor.

Intracellular proteolysis of cell layers under basal conditions (●-●); under "starvation" conditions, i.e. minimal medium (▲-▲) and under basal conditions + insulin (0,1 units/ml), glucose (10 mM) and branched chain amino acids at x5 plasma concentration (■-■). Each point is the mean of 2 dishes and results are expressed as the percentage proteolysis occurring.

4.4. DISCUSSION

The two independent heterologous *in vitro* proteolysis assay systems failed to unequivocally demonstrate the absence or presence of proteolytic factor(s) in the serum of cachectic cancer patients.

The rat diaphragm assay is well-characterized (73,75,76,77, 79) and in my hands satisfied the functional criteria for proteolysis determinations established by other workers. The system appears, however, to be far from ideal for the sophisticated proteolysis determinations required in order to detect any tumour-produced ectopic factor(s). The system failed to respond to physiological levels of exogenous hormones or to endogenous hormones present in the serum of physiologically manipulated rats.

The hormones may not act at the muscle cell level or alternately the diaphragm is not sufficiently sensitive to respond to the effects. Different skeletal muscles differ in the relative proportions of varying fibre types and therefore can differ appreciably in their metabolic, endocrinological and pharmacological responses (75). The diaphragm, comprised predominantly of red fibres, may therefore not respond to a variety of stimuli that other muscle types might do. The failure to demonstrate any tumour-associated proteolytic effect, both with human serum and with a homologous system using medium from transformed cell cultures (a rat cell line) not only suggests insensitivity of the diaphragm muscle but may have been an artefact of the desalting procedure. The cell culture proteolysis system was optimally functional and satisfied some of these unsuitable aspects of the diaphragm assay system. However, this heterologous system also failed to indicate any proteolytic tendency of "cancer" serum, while proving responsive to other proteolytic stimuli, such as "starvation".

This suggests the lack of a suitable system with which to

test these effects and which will need to be established and characterized before it can be said that no ectopic factor(s) exists in the serum of cachectic cancer patients.

A homologous human system suing human muscle biopsies in a similar in vitro assay to the rat diaphragm, is technically difficult, but has been reported (21). Teasing of the muscle fibres and sampling of the muscle pieces from various patients, to obtain similar and reproducible effects is extremely difficult and not without its pitfalls. The cachectic tumour model of Strain et al (9) appears thus far the most satisfactory system to work with. Providing cachexia-producing tumours can be successfully xenografted into experimental animals, extensive and comprehensive investigations into tumour effects on proteolysis could be conducted with this system.

CHAPTER 5

HAS SERUM FROM CACHECTIC CANCER PATIENTS A LIPOLYTIC EFFECT ON ADIPOSE TISSUE? USE OF A HETEROLOGOUS IN VITRO ASSAY SYSTEM

5.1. INTRODUCTION

Loss of body fat is commonly associated with experimental (24,25,27) and human cancer (7,14,26), as has been reviewed in Chapter 1.

The study of lipid loss in cancer has frequently raised the question of a tumour-induced humoral factor(s) which may stimulate adipose tissue lipolysis. Alternately, tumours have been considered possibly to cause the host to produce lipolytic hormones, to provide perhaps, a fuel source.

In this chapter is reported an investigation on the ability of serum from cancer patients (both weight-losing and non-weight-losing cancer patient groups) to stimulate adipose tissue lipolysis, in a heterologous assay.

Patient selection and the preparation of serum samples were conducted as described in 4.2.1.3.; experiments on *in vitro* proteolysis and lipolysis were run concurrently, using the same fresh serum samples. This parallel approach applied to all conditions studied.

Rat epididymal fat pads provided a means for directly comparing the effects of serum from cancer patients with serum from control patients, on lipolysis of fragments of adipose tissue derived from single experimental animals. By expressing the results as a percentage effect, mean values of paired experiments done with different animals could be obtained. This approach was applied to all comparisons made in this chapter.

5.2. METHODS

5.2.1. Experimental Procedure.

(Note: See Appendix for suppliers of important materials).

Long-Evans male rats of between 200-250 g were routinely used as adipose tissue donors. The rats were killed by cervical dislocation. The epididymal fat pads were excised and sections of the fat pad (around 150-200 mg each) were cut directly into preincubation flasks containing 4 mls KRB-buffer (see 4.2.1.1.), gassed with 95% O_2 - 5% CO_2, and pre-incubated for 15 mins, at 37°C, with shaking. The fat pieces were then transferred to incubation flasks containing 2 mls of incubation medium, pH 7.4, with 0.2% bovine serum albumin (BSA) and 12 mM glucose present under all incubation conditions.

Additions of various hormones were made in the following concentration ranges: insulin: 0.2 - 0.4 units/ml; adrenalin: 10^{-8}, 5×10^{-7}, 10^{-7}, 5×10^{-7}, 10^{-6} Molar.

The incubation flasks, after gassing, were incubated with shaking at 37°C, for 90 mins (unless otherwise stated), after which time the fat pads were removed, blotted and weighed. The medium was deproteinized with 0.4 mls 20% perchloric acid and the supernatant neutralized with 1 ml 3 M $KHCO_3$, on ice. After centrifugation at 1200 *g* for 15 mins, on a Beckman TJ-6R centrifuge at 0°C, an aliquot of the supernatant was assayed for glycerol content by an enzymatic procedure.

5.2.2. Assay for Glycerol.

Scheme:

(i) Glycerol + ATP $\xrightarrow{\text{glycerokinase}}$ L-glycerol-3-phosphate + ADP

(ii) PEP + ADP $\xrightarrow{\text{pyruvate kinase}}$ Pyruvate $\xrightarrow[H + NADH \; NAD^+]{\text{lactate dehydrogenase}}$ Lactate

The disappearance of NADH at 340 nm was recorded on a Beckman MSL spectrophotometer, and the micromoles of glycerol derived from the molar extinction coefficient of NADH at 340 nm in a 1 cm light path and having 1 ml volume in the cuvette.

The procedure was as follows:- a mixture containing triethanolamine (TEA) 0.2 M; Mg_2SO_4, 0.1 M; KCl, 1 M; ATP, 4 mM; PEP, 10 mM; NADH, 2 mM (final concentrations) was placed in disposable Kartell cuvettes with pyruvate kinase (3.5 units); lactate dehydrogenase (260 units) and an aliquot of deproteinized medium to a final volume of 1 ml. After mixing, the absorbance at 340 nm, after 10 mins, was recorded to give (E_1). Glycerokinase (1,0 unit) was then added, with mixing, to each cuvette; after 45 mins at room temperature, the absorbance at 340 nm was read to give (E_2). The change in absorbance ΔE i.e. (E_1-E_2) was determined, corrected for the blank (ΔE obtained in a cuvette containing the enzyme system as described, with buffer but no added glycerol), and the micromoles of glycerol in each sample were then determined from the corrected ΔE using the molar extinction coefficient of NADH at 340 nm. The rate of lipolysis was expressed as the total amount of glycerol released into the medium (micromoles) per gram wet tissue weight per hour. A standard glycerol curve (50; 100; 150 nmoles) was run concurrently with each glycerol determination, as a check on the sensitivity and reproducibility of the assay system.

5.2.3. Serum Samples (human and rat) and Transformed Cell Culture Medium Samples.

The selection, collection and preparation of the above were exactly as described in (4.2.1.3; 4.2.1.4; 4.2.1.5), the only, and most important difference being that "whole" and not desalted serum (or cell culture medium) was used.

A glycerol blank (representing the content of the added

serum cell culture or medium) was determined for each incubation condition (i.e. the glycerol content of the incubation flask, minus tissue) over 90 mins in incubation and was subtracted from the experimental glycerol value to provide a corrected amount of glycerol release from the tissue.

5.3. RESULTS

5.3.1. Optimalization of the Assay.

Kralovic et al (25), performing experiments similar to the ones being reported, detected appreciable lipase enzyme activity in serum at pH 7.4. This complicated the interpretation of their results since they measured free fatty acid release and found the apparent increase in this release to arise from lipase activity of the added serum.

In this work, the rate of lipolysis was assessed by measuring the amount of glycerol released, from adipose tissue, into the incubation medium. Measurement of glycerol offered additional advantages: it is not insoluble and does not require a carrier, such as albumin; it is not reutilized by adipose tissue since glycerokinase is absent in this tissue (84). The accumulation of glycerol is a valid reflection of the net rate of lipolysis (85), although the rate of accumulation in the medium does not necessarily reflect the true total rate of production because of dysequilibrium which may arise between tissue glycerol and medium (86). The glycerol content of freshly excised tissue is negligible, however, in relation to that found in the medium in incubation experiments (85), and thus for the purpose of this study, the glycerol content in the medium was taken to represent net lipolysis occurring in the system, and tissue glycerol measurements were not routinely performed (85).

Incubation in the presence of glucose and albumin did not affect glycerol release from the medium (85,86). Glucose was added for the following reasons: the amount of this

metabolite was expected to vary in the serum samples added; glucose also provides a source of α-glycerophosphate for the reesterification of tissue free fatty acids such that these do not become toxic to the adipocytes of the tissue.

The presence of albumin also served to prevent toxic accumulation of tissue free fatty acids, with time in incubation (absence of albumin from the medium leads to free fatty acid accumulation in the tissue (84)).

The enzymatic method for glycerol determination provided a simple, reproducible means of measuring glycerol. The glycerol standard curve (Figure 5.1) taken as the mean of 8 separate determinations, was always linear.

The error involved in the entire system i.e. tissue excision, incubation, medium deproteinization and enzymatic glycerol determination, was around 10%, variations being caused mainly by harsh handling of the fragile epididymal fat pads.

In order to determine any changes in the rate of glycerol release during 2 hours of incubation, pairs of tissue fragments from single experimental animals were incubated for periods of 1 and 2 hr (Figure 5.2). Release of glycerol (μmoles glycerol released/gram wet tissue weight) was linear for up to 2 hr; 90 min incubation periods were accordingly chosen for all further assays.

Glycerol release in μmoles per hour was linear with increasing wet tissue weight of adipose tissue (Figure 5.3).

The use of adipose tissue from fasted rats was considered as a possible means of increasing the rate of glycerol release from tissue, such that background serum glycerol levels would be negligible in comparison, and "whole" serum could be used. Adipose tissue from fasted rats (90-hour fast) released glycerol at a higher rate than did adipose tissue from fed rats (Table 5.1.), but this higher lipolytic

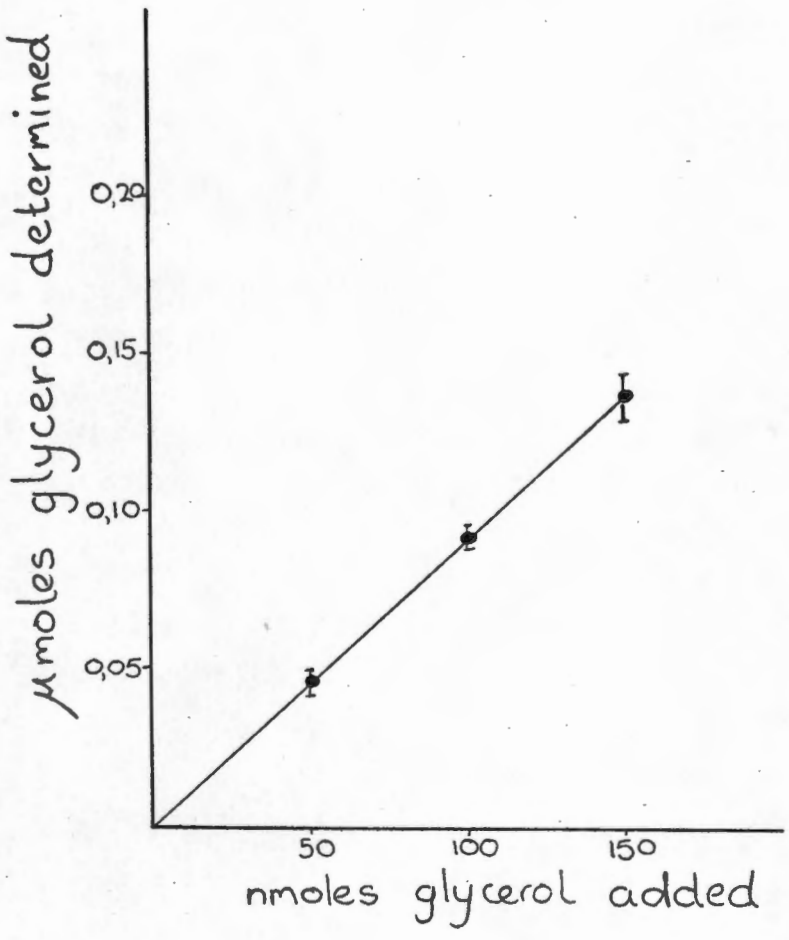

Figure 5.1. Standard curve of glycerol determined by enzymatic assay.

Each point is the mean ±S.E. of 8 determinations. Glycerol was determined using the molar extinction coefficient of NADH at 340 nm, in a 1 ml volume for a 1 cm light path.

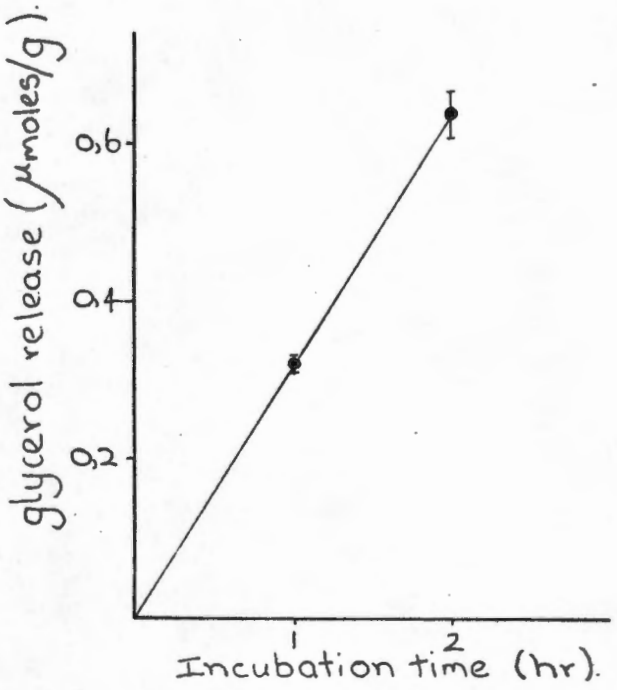

Figure 5.2. Glycerol release from adipose tissue with increasing time in incubation.

Each point is the mean ±S.E. of 2 separate determinations (using tissue from one animal) in KRB-buffer incubation medium during 2 hours.

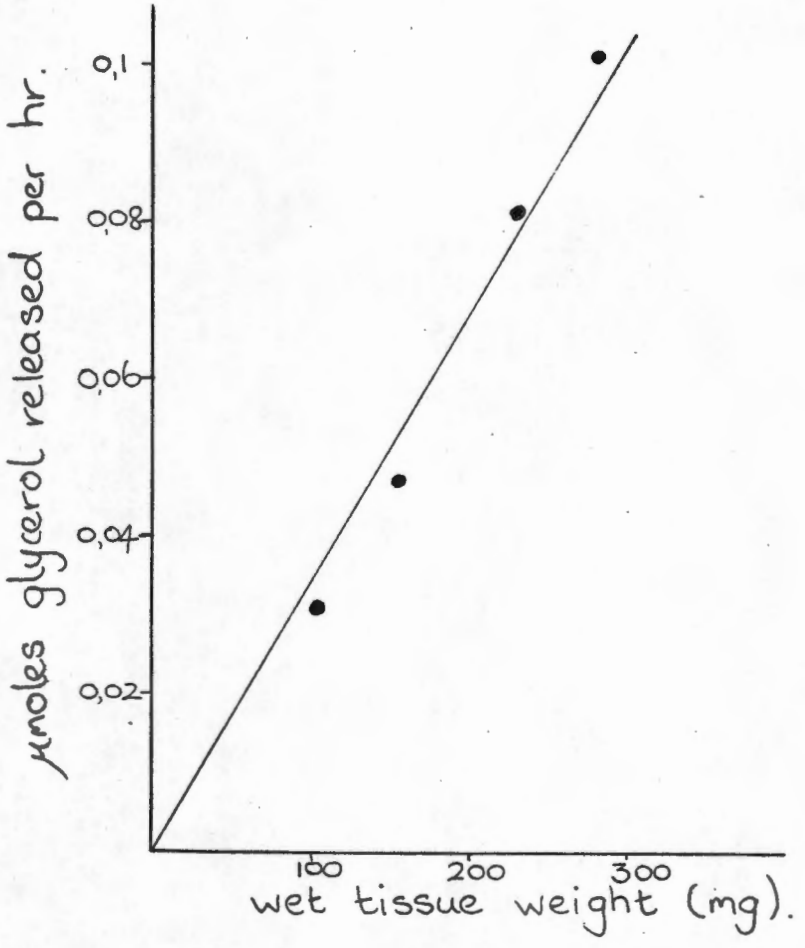

Figure 5.3. Glycerol release from adipose tissue as a function of increasing wet tissue weight.

Each point represents the glycerol release (μmoles/hr) of a separate tissue piece from a single animal.

TABLE 5.1.

GLYCEROL RELEASE FROM EPIDIDYMAL FAT PADS FROM FED AND FASTED (90-HR) RATS

Epididymal fat pad donor	Lipolytic rate as μmoles glycerol/g/hr
Fed rat	0.64 ± 0.02
90-hr fasted rat	1.60 ± 0.05

Lipolytic rate as the mean ± S.E. for 7 animals in each physiological state; a duplicate sample from each animal.

rate was not linear over 2 hr of incubation (Figure 5.4).

Accordingly, epididymal fat pads from fed rats were used, pieces of 150-200 mg providing sufficient glycerol release during 90 min incubation to accommodate the background serum glycerol level of "whole" serum. Fed rats provided a control physiological state on which to test the effects of the serum samples (as discussed in 4.2.2.1).

5.3.2. Response of the System to Exogenous Hormones.

The incubation system responded to addition of exogenous hormones. Adrenalin stimulated lipolysis in a dose-dependent manner (Figure 5.5.). The greatest stimulation occured between concentrations of 10^{-7} M - 10^{-6} M, giving the curve a sigmoid shape. Other workers (87) have noted a bimodal dose response curve with increasing adrenalin concentrations, around 10^{-6} M - 10^{-3} M, a sharp increase in glycerol release occurring just below 10^{-6} M.

Insulin, at concentrations between 0.2 - 0.4 units/ml, gave a slight inhibition of about 8%, in internally paired comparisons with tissue from 10 animals; this was not outside the error of the system. Some variability may have been due to inactivation of the hormone prior to its addition to the incubation flask; commercial preparations of insulin contain glycerol to stabilize the hormone and it was necessary to remove this high background glycerol by gel filtration. Insulin, in medium containing glucose, has been found to cause mild inhibition of glycerol release, but a greater inhibition of free fatty acid release (84). Insulin inhibition of adrenalin-stimulated lipolysis is well reported and substantiated (84,86,87).

5.3.3. Response of the System to Human Serum (Cachectic cancer, cancer, control patient serum).

Background glycerol levels in the incubation flasks (i.e.

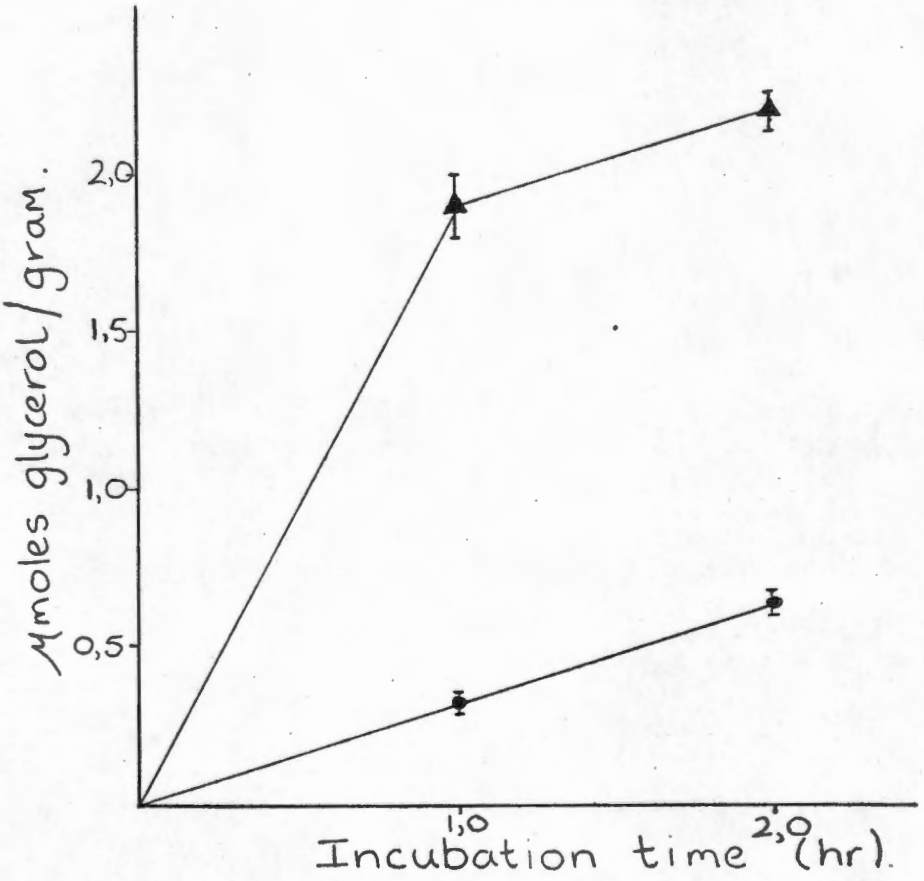

Figure 5.4. Release of glycerol from adipose tissue of a fasted (▲) and fed (●) rat.

Each point is the mean ±S.E. for 2 rats in each physiological state, where each time course describes data for tissue from a single animal.

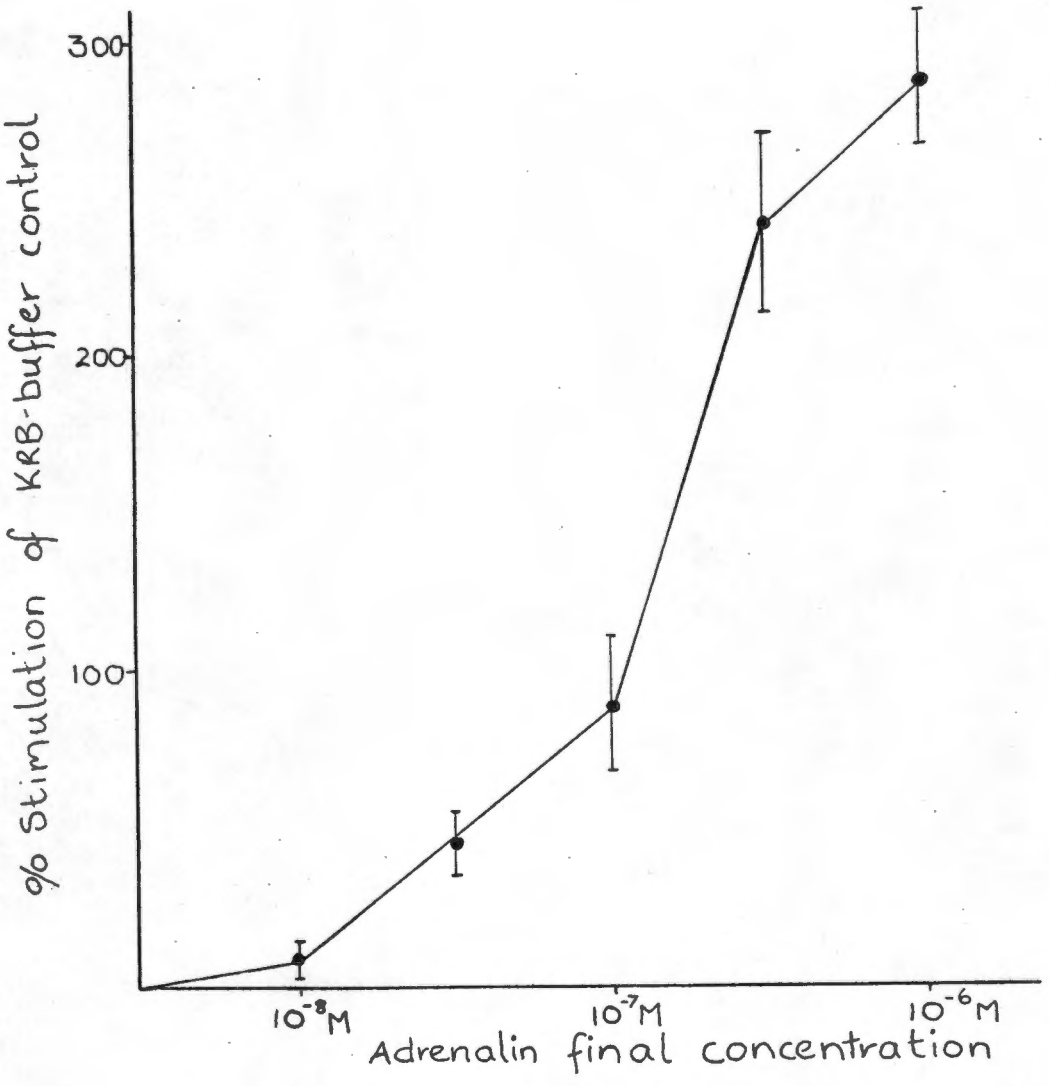

Figure 5.5. Effect of adrenalin on glycerol release from adipose tissue <u>in vitro</u>.

Each point is the mean ±S.E. of 2 animals, where the % stimulation was determined for tissue from the same animal, and the mean then deterined. The % stimulation =

$$\frac{\text{lipolytic rate in adrenalin - lipolytic rate in KRB-buffer}}{\text{lipolytic rate in KRB-buffer}}$$

1:1 KRB-buffer : serum) ranged from 10-80 nmoles, and comprised one-quarter to one-third of the total medium glycerol after 90 min incubation. The serum did not generate glycerol over 2 hours in incubation.

Serum from a fed control patient caused 24 ± 7.0% (mean ± S.E.) inhibition of glycerol release (Table 5.2), for paired internal comparisons with tissue from 3 animals.

In comparison, serum from a fasted control patient gave a higher lipolytic rate than did serum from the same control patient in a fed state (Table 5.3) i.e. serum from the fed state was more inhibitory than serum from the fasted state. The difference was 10%, for paired internal comparisons, and may have reflected differences in serum insulin levels - this hormone being present at lower concentrations during fasting. Serum samples from fasted patients had higher background glycerol contents. Serum samples from fed patients were accordingly, routinely used (see 4.2.2.2.).

Serum from cachectic cancer patients and from cancer patients having no evidence of weight loss, showed an inhibition of glycerol release compared with matched control patient serum samples (Table 5.4). The mean percentage inhibition determined for internally paired comparisons between cancer and control sera was 14 ± 3.4% (mean ± S.E.) for weight-losing cancer patients, and 17 ± 1.8% (mean ± S.E.) for non-weight-losing cancer patients (Table 5.5). The variation between cancer groups (i.e. 17% and 14% inhibition) was slight and may have reflected varying hormone levels in the serum samples. The difference between addition of cancer-derived and control serum was evidently not adequate to indicate a cancer-specific effect (regardless of weight loss). If the effect was in fact real, it was uniformly slight and not related specifically to the cachectic condition of cancer patients. The effect, in any case, was one of inhibition, and not stimulation, of lipolysis.

TABLE 5.2.

GLYCEROL RELEASE IN KRB-BUFFER AND KRB-BUFFER CONTAINING HUMAN SERUM (NON-CANCER CONTROLS)

Incubation condition	Lipolytic rate (μmoles/g/hr)
KRB-buffer	0.54 ± 0.04
KRB-buffer: serum ... (1:1)	0.40 ± 0.04

Lipolytic rate as the mean ± S.E. for 3 animals. The comparison was performed in "internal, paired" experiments to determine the percentage effect of serum presence in the medium.

TABLE 5.3.

GLYCEROL RELEASE FROM TISSUE INCUBATED IN SERUM FROM A FED OR FASTED CONTROL PATIENT

Serum source	Lipolytic rate (μ moles/g/hr)
Fed control patient	0.58 ± 0.04
Fasted control patient..	0.61 + 0.04

The rates are expressed as the mean ± S.E. of paired duplicate internal comparisons.

TABLE 5.4.

GLYCEROL RELEASE FROM ADIPOSE TISSUE INCUBATED IN SERUM FROM A WEIGHT-LOSING AND A NON-WEIGHT-LOSING CANCER PATIENT, COMPARED WITH SERUM FROM A MATCHED CONTROL PATIENT

Serum source	Lipolytic rate (μmoles/g/hr)
Weight-losing cancer patient	0.42 ± 0.01
Cancer patient	0.53 ± 0.02
Non-weight-losing cancer patient.....	0.70 ± 0.03
Control patient	0.77 ± 0.03

Results are the mean ± S.E. for 5 paired sets of weight-losing cancer : control, patient sera and non-weight-losing cancer:control patient sera where each set of cancer:control serum samples was performed in duplicate.

TABLE 5.5.

PERCENTAGE STIMULATION OF LIPOLYTIC RATE CAUSED BY CANCER-DERIVED COMPARED WITH CONTROL SERUM

Serum source	% Stimulation in lipolytic rate
Weight-losing cancer patient	-14 ± 3.4
Non-weight-losing cancer patient ...	-17 ± 1.8

As the mean ± S.E. where the % stimulation =

$$\frac{\text{Lipolytic rate in cancer serum - lipolytic rate in control serum}}{\text{Lipolytic rate in control serum}}$$

determined for paired internal comparisons and the mean was taken of 10 determinations in each cancer serum type.

In Chapter 2 of this thesis, evidence was given to show that breast cancer patients have increased fat areas compared with control patients, and the present ideas relating diet and breast cancer (see Chapters 1 and 2) may be reflected in the fat-conserving effect seen with serum from breast cancer patients i.e. the non-weight-losing cancer patient group. The fat-conserving effect seen in the cachectic cancer patient group was contrary to what was expected in the light of present ideas reviewed in Chapter 1, and the evidence presented in Chapter 2. It is possible that cachectic cancer patients, losing increased body fat, mount a host response involving the production of hormones which conserve lipid stores.

5.3.4. Response of the System to Medium from Transformed Cell Cultures.

The possibility of a lipolytic factor produced by a malignant cell line, which would cause increased lipolysis in this *in vitro* system, was investigated using medium from a transformed rat cell line ($R_9Cl_{10}T$ cell cultures) (refer 4.2.1.5.) in which the factor of a species difference was eliminated (i.e. a malignant rat cell line producing factors effective against rat tissue).

Medium from transformed and control cell cultures caused substantial inhibition of glycerol release (Table 5.6) which was most marked using medium from the transformed cell layers. It did not appear to be a medium effect but rather an effect specific to the transformed cell cultures. This effect was not investigated further since it was a diversion from the primary investigation, and thus the exact reason for this inhibition is not known. It may be that as the transformed cells had a greater rate of division, there would be more cellsper dish which would be better able to "condition" the medium (e.g. with metabolites, by-products and waste products) such that glycerol release from adipose tissue in the presence of this medium *in vitro* was reduced by 68%. The modulation

TABLE 5.6.

GLYCEROL RELEASE FROM ADIPOSE TISSUE INCUBATED WITH MEDIUM FROM TRANSFORMED CELL CULTURES

Incubation condition	Lipolytic rate (μmoles/g/hr)	% Inhibition
KRB-buffer	0.62 ± 0.03	
		68 ± 1.2
Medium T : KRB-buffer	0.18 ± 0.07	
Medium T : KRB-buffer	0.20 ± 0.02	
		54 ± 5.0
Medium : KRB-buffer (1 : 1)	0.47 ± 0.07	

Where Medium T = medium from transformed cell cultures ($R_9Cl_{10}T$)

Medium = medium from untransformed cell cultures (R_9Cl_{10}).

The lipolytic rate was the mean ± S.E. for 2 animals in duplicate internal comparisons.

The % inhibition =

$$\frac{\text{Lipolytic rate in KRB-buffer - lipolytic rate in Medium T}}{\text{Lipolytic rate in KRB-buffer}}$$

or alternately

$$\frac{\text{Lipolytic rate in Medium - lipolytic rate in Medium T}}{\text{Lipolytic rate in Medium}}$$

and is the mean of separately determined internal comparisons.

of these cells may have caused metabolic changes, as yet not apparent, that would cause the inhibitory effect of the medium.

5.3.5. Response of the System to Rat Serum.

As described for the proteolysis system in Chapter 4, the response of this in vitro lipolysis system to rat serum (both control rat serum and physiologically modulated rat serum) was investigated. Control rat serum had a negligible inhibitory effect on the KRB-buffer system (4% as determined in paired internal comparisons with 4 animals) (Table 5.7). Comparing serum from fed and fasted rats, no differences were observed with serum from fed rats and from rats fasted for 90 hours. Serum from 130 hr fasted rats increased the lipolytic rate by 40 ± 7.1% (mean ± S.E.) compared with the rate using serum from fed animals (Figure 5.6). This effect was greater than that seen with serum from fasted humans, and may indicate a response of rat tissue to rat serum i.e. a species barrier to adipose tissue response in vitro.

The other hormone treatments i.e. Alloxan-induced diabetes; hydrocortisone treatment, either alone or coupled with 130-hr starvation, yielded serum that reduced the lipolytic rate of adipose tissue, when compared internally, with control fed rat serum (Figure 5.7). The 16 ± 7.4% (mean ± S.E.) inhibition in diabetic rat serum is not likely to be an insulin effect. The insulin in the serum would be negligible and the serum would be expected to stimulate lipolysis. The increased glucose in the serum would not have affected the glycerol release directly.

Hydrocortisone treatment yielded serum that gave the greatest inhibitory effect, which was diminished, but not eliminated, by concomitant starvation of the serum donor rat. The hydrocortisone influence appeared greater than the starvation influence (refer to Figure 5.6). Thyrotoxic rats gave serum that caused over 100% stimulation of lipolysis, in paired

TABLE 5.7.

GLYCEROL RELEASE FROM ADIPOSE TISSUE INCUBATED WITH CONTROL RAT SERUM

Incubation condition	Lipolytic rate (μmoles/g/hr)
KRB-buffer	0.51 ± 0.03
KRB-buffer : rat serum (1:1)	0.46 ± 0.07

Lipolytic rate is the mean ± S.E. for 4 controls. Each determination was performed by paired internal comparison to allow direct comparison of the % difference between animals.

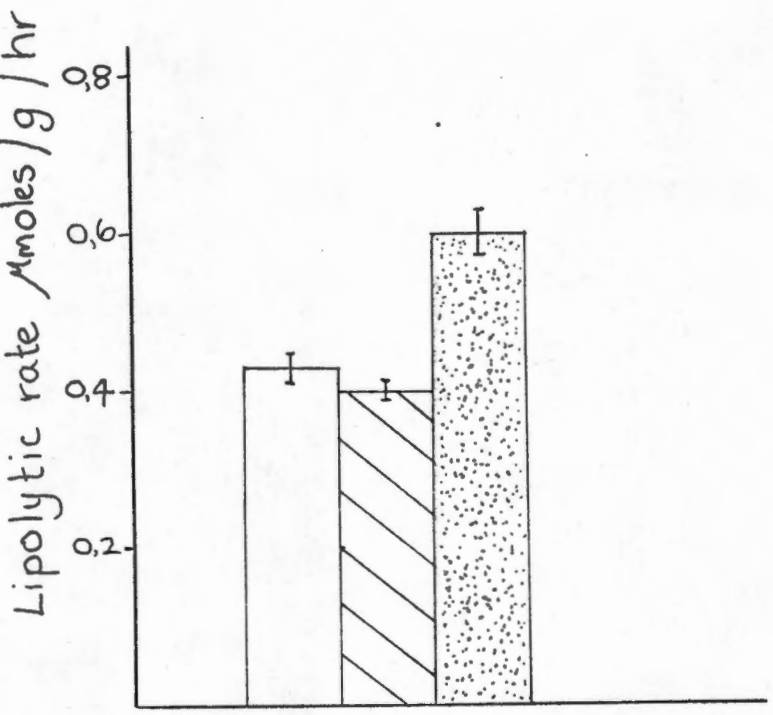

Figure 5.6. Glycerol release in the presence of serum from rats in a fed state (□) in a 90 hr fasted state (▨) and a 130 hr fasted state (▩).

Each value is the mean ±S.E. for 7 animals, where each of the serum types was compared with tissue from a single animal.

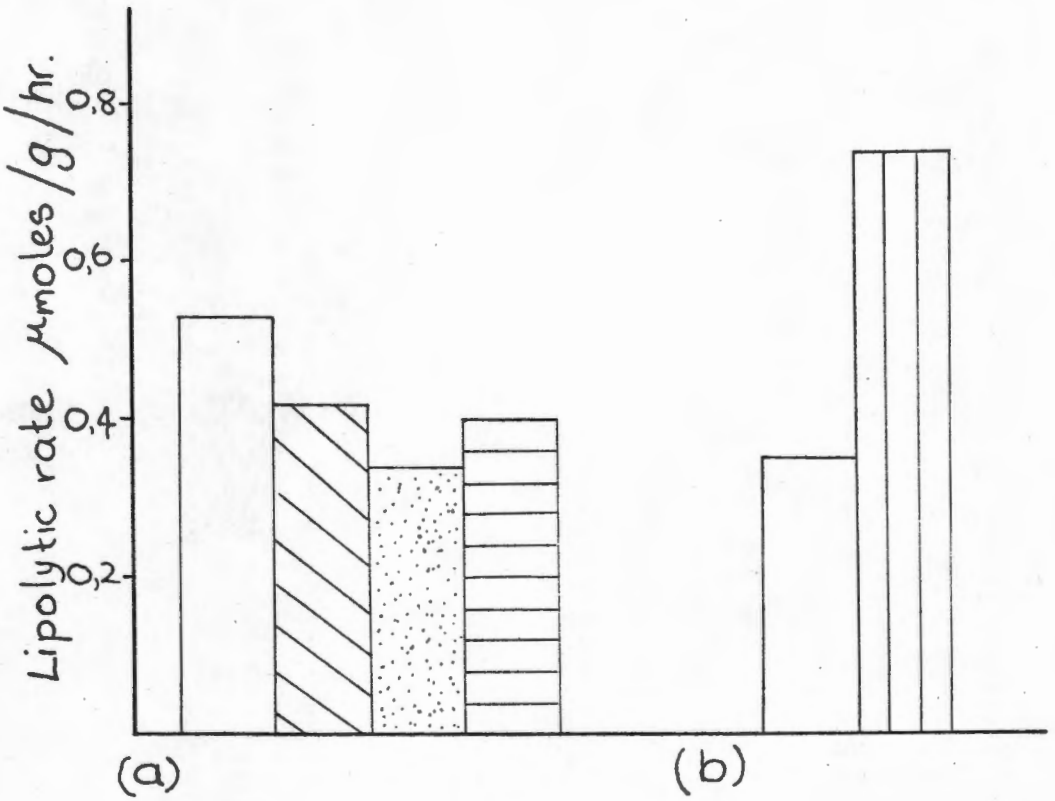

Figure 5.7. Release of glycerol from adipose tissue incubated in the presence of serum from rats under various hormonal treatments.

Each value is the mean rate of 2 separate determinations, where (a) and (b) were each performed within one animal. The rat serum source was as follows:

(□) - fed control rats
(▨) - alloxan-treated rats
(▩) - hydrocortisone-treated rats
(▩) - hydrocortisone-treated + 130 hr fasted rats
(▩) - triiodothyronine-treated rats.

internal comparisons with control rat serum. This is in accord with what one would expect, knowing the stimulatory effect of thyroxine on lipolysis. Experiments of this nature rely on the assumption that animals react to hormone treatments to produce serum which contains some factor (hormone or metabolite) in sufficient quantity, absent or present (within physiological limits) to produce responses *in vitro* in incubation systems.

One would expect increased lipolysis rates *in vivo* to arise from the treatments described. However, different animals do not always respond as expected (78,82) and if they did respond, the substances produced by the response may be in amounts which manifest overall as zero net change in lipolysis rate, *in vitro*. One can therefore not predict confidently any effect with this type of experiment.

The system did, however, show a response to rat serum as evidence of its functional capacity and sensitivity.

5.4. DISCUSSION

The question of whether a lipolytic factor is present in the serum of cancer patients remains unanswered. Previous workers using similar *in vitro* systems, were unable to reach any conclusion regarding the lipolytic tendencies of serum, both from tumour-induced rats (25) and human cancer patients (22,29). This indicates the lack of a suitable system with which comprehensively to investigate this problem in order to reach a decision.

In my hands, epididymal fat pads released glycerol in a linear manner, *in vitro*, for 2 hours and were responsive to exogenous hormones.

The responses to added serum, both from humans and from rats, were unfortunately difficult to interpret. This may indicate

the need to use a homologous assay system.

A human mammary adipose cell line may provide a suitable human system for human cancer serum investigations. Human adipose tissue biopsies would not be feasible to isolate or incubate *in vitro*.

Qualitative *in vivo* lipolysis studies on cancer patients are somewhat difficult to perform and very complex in character.

The tumour model of Strain *et al* (9) remains the most suitable system yet reported for cachectic cancer studies, provided the cachexia causing tumours can be excised and xenografted successfully in animals. Adipose tissue can be isolated from these weight-losing mice hosts, and lipolytic rates established *in vitro* and compared with controls. *In vivo* mouse host studies could also be done.

The serum of mouse hosts could be fractionated to investigate tumour factor(s) or alternately to qualitate host hormones and thus establish any increase in these compared to normal, or to assess if normal responses to stress situations were applicable.

This may comprehensively answer the question of ectopic hormone production or alternately provide evidence for the indirect hypothesis of Gold (35).

CHAPTER 6

CONCLUDING DISCUSSION

This study has failed to substantiate the proposal of a tumour-produced ectopic factor(s), which could cause cachexia of cancer patients. Nevertheless, we have not conclusively ruled out the possibility that such a factor(s) exists. Other workers investigating this question and using minor variations of the same *in vitro* assay systems, have also not provided equivocal evidence for ectopic factor production (see Chapter 1).

It is my opinion that present *in vitro* methods used to tackle this question are inadequate, and that the field of cancer cachexia lacks a clear-cut intense investigation into the frequency, tumour type and extent of disease associated with this condition. A clearer understanding epidemiologically into cancer cachexia, together with further investigations, using the *in vivo* human model, into changes in serum amino acids, including 3-Methylhistidine, metabolites, minerals and waste products such as creatinine, would be invaluable in aiding biochemical investigations, as revealed by Chapters 2 and 3.

Present *in vitro* assay systems yield equivocal data and rely on a vast number of assumptions that may not be justified. One such important consideration is the relevance of animal models: induction of cachexia in experimental animals by animal carcinomas e.g. Krebs-2, Walker 256, etc., may be far removed from the human system. Unfortunately, because of their convenience, these methods continue to be widely used. The use of serum from cachectic cancer patients on experimental animal models, *in vivo* provides a more direct approach but is plagued by the question of possible inactivation of a factor(s), species barriers, and the general sensitivity of the system with respect to the concentration of factor(s) and length of time required to observe an effect. The model

of Strain *et al*, as discussed in sections 4.4, 5.4, is thus far the most feasible and advanced system, which remains convenient for a comprehensive investigation into this area. Until a system is available to provide exact, reproducible data, and which could become widely used, the proposal of ectopic factor production remains equivocal, and our understanding of cachexia of cancer patients is ill-defined.

APPENDIX

The following chemical suppliers were used:

BDH Chemicals (Pty) Ltd Poole, England.	:	Alloxan
Boehringer, Mannheim GrubH, Germany	:	Glycerokinase; lactate dehydrogenase; pyruvate kinase.
Boots Company (SA) Pty Ltd Isando, Transvaal.	:	Insulin.
Eli Lilly & Co., Indianapolis, USA	:	Glucagon
Gibco Laboratories, New York, USA	:	Dulbeccos Modified Eagles Medium, Medium 199.
Merck, Darmstadt, Germany	:	D(-1) Glucose.
Miles Laboratories (Pty) Ltd Epping, S.A.	:	Bovine serum albumin.
New England Nuclear, Cape Town, S.A.	:	L-$[H^3]$-phenylalanine.
Pharmacia Fine Chemicals Uppsala, Sweden	:	Sephadex G-25 (medium)
Sigma Chemicals Co. St Louis, USA	:	Arterenal HCl; adenosine 5' triphosphate; phospho(enol) pyruvate; β-nicotinamide: adenine dinucleotide; L-tri-iodothyronine; cycloheximide; L-leucine; L-isoleucine; L-valine; L-tyrosine; L-phenylalanine.

Upjohn (Pty) Ltd
Isando, RSA : Hydrocortisone sodium succinate.

Animals: Male Long Evans rats used and supplied by Physiology Animal House, UCT Medical School.

Chick Embryo Extract: Prepared from 11-day chick embryos, under sterile conditions, filtered and frozen. Before it was added to the media, the thawed extract was centrifuged at 400 g to remove insoluble material.

Horse serum: Prepared from freshly collected blood obtained from the State Vaccine Institute, Cape Town.

REFERENCES

1. Warren, S. Am. J. Med. Sci. 184, 610-615 (1932).
2. Theologides, A. Curr. Concepts Nutr. 6, 75-94 (1977).
3. Gold, J. Ann. N.Y. Acad. Sci. 230, 103-110 (1974).
4. Costa, G. Cancer Res. 37: 2327-2335 (1977).
5. Waterhouse, C. Ann. N.Y. Acad. Sci. 230: 86-93 (1974).
6. Dewys, W.D. et al. Am. J. Med. 69: 491-497 (1980).
7. Watson, W.S. and Samman, A.M. Cancer 46: 2041-2046 (1980).
8. Holroyde, C.P., Gabuzda, T.G., Putnam, R.C., Paul, P. and Reichard, G.A. Cancer Res. 35: 3710-3714 (1975).
9. Strain, A.J., Easty, G.C. and Munro Neville, A. J. Nat. Cancer Inst. 64: 217-221 (1980).
10. Schein, P.S., Kisner, D., Haller, D., Blecher, M. and Hamosh, M. Cancer 43: 2070-2076 (1979).
11. Goodlad, G.A. and Clarke, C.M. Eur. J. Cancer 8: 647-651 (1972).
12. Goodlad, G.A. and Raymond, M.J. Eur. J. Cancer 9: 139-145 (1973).
13. Sherman, C.D., Morton, J.J., and Mider, G.B. Cancer Res. 10: 374-378 (1950).
14. Costa, G., Samal, B.A., Brennan, J. and Pickren, J.W. Proc. Am. Assoc. Cancer Res. 6: 12 (1965).
15. Lundholm, K., Edström, S., Ekman, L., Karlberg, I., Bylund, A.C. and Schersten, T. Cancer 42: 453-461 (1978).
16. Henderson, J.F. and Le Page, G.A. Cancer Res. 19: 887-902 (1959).
17. Shapot, J.S. Adv. Cancer Res. 30: 89-150 (1979).
18. Mider, G. Cancer Res. 11: 821-829 (1951).
19. Levin, L. and Gevers, W. S. Afr. Med. J. 59: 553-556 (1981).
20. Clarke, E.F., Lewis, A.M. and Waterhouse, C. Cancer 42: 2909-2913 (1978).
21. Lundholm, K., Bylund, A-C., Holm, J. and Scherstén, T. Eur. J. Cancer 12: 465-473 (1976).
22. Mays, E.T. J. Surg. Res. 9: 273-277 (1969).
23. Warburg, O. Metabolism of Tumours. Arnold Constable, London (1930).
24. Costa, G. and Holland, J.F. Cancer Res. 22: 1081-1083 (1962).

25. Kralovic, R.C., Zepp , E.A. and Cenedella, R.K. Eur. J. Cancer 13: 1071-1079 (1977).
26. Mueller, P.S. and Watkin, D.M. J. Lab. Clin. Med. 57: 95-108 (1961).
27. Mider, G.B., Sherman, C.D. and Morton, J.J. Cancer Res. 9: 222-224 (1949).
28. Levin, L. and Gevers, W. S. Afr. Med. J. 59:518-521 (1981).
29. Carter, A.C., Lefkon, B.W., Farlin, M. and Feldman, E.B. J. Clin. Endo. Metab. 40: 260-264 (1975).
30. Jasani, B., Donaldson, L.J., Ratcliffe, J.G. and Sokhi, G.S. Br. J. Cancer 38: 287-292 (1978).
31. Waterhouse, C. and Kemperman, J.H. Cancer Res. 31: 1273-1278 (1971).
32. Singh, J., Grigor, M.R. and Thompson, M.P. Cancer Res. 40: 1699-1706 (1980).
33. Shapot, V.S. and Blinov, V.A. Cancer Res. 34: 1827-1832 (1974).
34. Waterhouse, C. Cancer Res. 33: 66-71 (1974).
35. Gold, J. Ann. N.Y. Acad. Sci. 230: 103-110 (1974).
36. Grubbs, B., Rogers, W. and Cameron, I. Oncology 36: 216-223 (1979).
37. Warnold, I., Lundholm, K. and Schérsten, T. Cancer Res. 38: 1801-1807 (1978).
38. Young, V. Cancer Res. 37: 2336-2347 (1977).
39. Bland, K.I., Adcock, R.A., Ratcliffe, D.J. and Fry, D.E. J. Surg. Res. 28: 416-420 (1980).
40. Greenstein, J.P. J. Nat. Cancer Inst. 3: 397-404 (1943).
41. Toporek, M. Cancer Res. 33: 2579-2583 (1973).
42. Gropp, C., Havemann, K. and Scheuer, A. Cancer 46: 347-354 (1980).
43. Theologides, A. Ann. N.Y. Acad. Sci. 230: 14-22 (1974).
44. Nixon, D. et al. Am. J. Med. 68: 683-690 (1980).
45. Jelliffe, D.B. In "The assessment of the nutritional status of the community (with special reference to field surveys in developing regions of the world)", Geneva World Health Organization (1966).
46. Gurney, J. and Jelliffe, D.B. Am. J. Clin. Nutr. 26: 912-915 (1973).

47. Beck, J. In "Nutritional management of the cancer patient", J. Wollard. New York Raven Press. pp. 21-42 (1979).
48. Costa, G. Cancer Res. 37: 2419-2424 (1977).
49. Heymsfield, S., Olafson, R., Kutner, M. and Nixon, P. Am. J. Clin. Nutr. 32: 693-702 (1979).
50. Martorell, R., Yarborough, C., Lechtig, A., Delgado, H. and Klein, B. Am. J. Clin. Nutr. 29: 46-53 (1976).
51. Stini, W. Am. J. Phys. Anthropol. 36: 341-351 (1972).
52. Bistrian, B., Blackburn, G., Hallowell, E. and Heddle, R. JAMA 230: 858-861 (1974).
53. Jelliffe, E.F.P. and Jelliffe, D.B. J. Trop. Pediat. 15: 177-188 (1969).
54. Bistrian, B., Blackburn, G., Sherman, M. and Scrimshaw, N. Surg. Gynaecol. Obstet. 141: 512-516 (1975).
55. Zelen, M. Cancer Chemother. Rep. 4: 31-42 (1973).
56. Fuisancho, A. Am. J. Clin. Nutr. 27: 1052-1058 (1974).
57. Hankin, J. and Rawlings, J. Am. J. Clin. Nutr. 31: 2005-2016 (1978).
58. Sloan, A. and Koeslag, J. S. Afr. Med. J. 47: 125-127 (1973).
59. Durnin, J.V.G.A. and Rahaman, M.M. Br. J. Nutr. 21: 681-689 (1967).
60. Kent, S. Geriatrics 34: 83-90 (1979).
61. Wynder, E. Cancer 46: 899-904 (1980).
62. De Waard, F., Baanders-Van Halewiyn, E. and Huizinga, J. Cancer 17: 141-151 (1964).
63. De Waard, F. Cancer Res. 35: 3351-3356 (1975).
64. Cole, P. Cancer 46: 865-867 (1980).
65. Beer, A. and Billingham, R. Lancet 2: 296 (1978).
66. Adibi, S.A. Metabolism 25: 1287-1302 (1976).
67. Holt, L.E. Jr., Snyderman, S.E., Norton, P.M., Roitman, E. and Finch, J. Lancet 2: 1343-1348 (1963).
68. Saunders, S.J., Truswell, A.S., Barbezat, G.O., Wittman, W. and Hansen, J.D. Lancet 2: 795-797 (1967).
69. Mann, H.B. and Whitney, D.R. Ann. Math. Statist. 18: 50 (1947).
70. Adibi, S.A. Am. J. Physiol. 221: 829-838 (1971).
71. Smith, S.R., Pozefsky, T. and Chnetni, M.K. Metabolism 23: 603-618 (1974).

72. Goldberg, A.L. and Dice, J.F. Jr. Ann. Rev. Biochem. 42: 835-869 (1974).
73. Fulks, R.M., Li, J.B. and Goldberg, A.L. J. Biol. Chem. 250: 290-298 (1975).
74. Waalkes, T.P. and Udenfriend, S. J. Lab. & Clin. Med. 50: 733-736 (1957).
75. Goldberg, A.L., Martel, S.B. and Kushmerick, M.J. Meth. in Enzymology 39: 82-94 (1975).
76. Tomas, F.M., Munro, H.N. and Young, V.R. Biochem. J. 178: 139-146 (1979).
77. Dice, J.F., Walker, C.D., Byrne, B. and Cardiel, A. Proc. Nat. Acad. Sci. (USA) 75: 2093-2097 (1978).
78. Goldberg, A.L. In "Plasticity of Muscle", Dirk Pette. Walter de Gruyter & Co., Berlin, New York (469-492) (1980).
79. Shoji, S. and Pennington, J.T. Mol. Cell Endocrinol. 6: 159-169 (1977).
80. Bates, P.J. Pathways of intracellular protein degradation in cultured muscle cells. M.Sc. Thesis, UCT 1981.
81. Mallette, L.E., Exton, J.H. and Park, C.R. J. Biol. Chem. 244: 5113-5723 (1969).
82. Goldberg, A.L., Tischler, M., De Martino, G. and Griffin, G. Fed. Proc. 39: 31-36 (1980).
83. Mayer, M., Amin, R. and Shafrir, E. Arch. Biochem. Biophys. 161: 20-25 (1974).
84. Jungass, R.L. and Ball, E.G. Biochem. 2: 383-388 (1963).
85. Vaughan, M. J. Biol. Chem. 237: 3353-3358 (1962).
86. Steinberg, D. In "Control of Lipid Metabolism", ed. J.K. Grant. Academic Press, London, New York pp. 111-138 (1963).
87. Olefsky, J.M. J. Lipid Res. 18: 459-464 (1977).