

Declaration

I declare that this thesis has been composed by myself and that the work it contains is my own.

Contents

	Page
Contents	1
Abstract of Thesis	vi
Abbreviations	vii
Acknowledgements	ix
Chapter 1 <u>Introduction</u>	1
1.1 Adipose Tissue Metabolism	2
1.1.1 Lipogenesis	2
1.1.2 Lipolysis	3
1.2 Antagonists of Lipolysis	6
1.3 Lipolytic Agents	7
1.3.1 Activators of AdenylCyclase	8
1.3.1.1 The Catecholamines and the Sympathetic Nervous System	8
1.3.1.2 Glucagon and Related Peptides	9
1.3.1.3 Adrenocorticotropic Hormone and Melanocyte Stimulating Hormone	10
1.3.1.4 Pituitary Glycoprotein Hormones	11
1.3.2 Hormones with a Delayed Lipolytic Action	12
1.3.2.1 Thyroid Hormones and Glucocorticoids	12
1.3.2.2 Growth Hormone	14
1.3.3 Pituitary Lipolytic Peptides	15
1.3.3.1 The Lipotropins	18
1.3.3.2 Lipid Mobilizing Factor	19
1.3.3.3 Are the Lipolytic Peptides of Importance in the Body?	20
1.3.3.4 Inhibition of LMF	21
1.4 Aims of Present Study and Outline of Work	23

		Page
Chapter 2	<u>Preparation of Porcine Lipid Mobilizing Factor</u>	25
2.1	Introduction	25
2.2	Methods	26
2.2.1	Identification of Porcine Lipid Mobilizing Factor	26
2.2.1.1	<u>In Vivo</u> Assay of Adipokinetic Activity	26
2.2.1.2	<u>In Vitro</u> Assay of Lipolytic Activity	28
2.2.1.3	Polyacrylamide Disc Gel Electrophoresis	30
2.2.1.4	Electrophoresis on Sodium Dodecyl Sulphate Polyacrylamide Gels	32
2.2.1.5	Protein Estimation	34
2.2.2	Isolation of Porcine Lipid Mobilizing Factor	35
2.2.2.1	Chromatographic Procedures	35
2.2.2.2	Preparation of crude LMF	38
2.2.2.3	Extraction V of crude LMF	42
2.2.2.4	Extraction VIII of crude LMF	44
2.2.2.5	Extraction XI of crude LMF	45
2.2.3	Estimation of Molecular Weight of LMF on Sephadex G-50	46
2.3	Results	48
2.3.1	Identification of Porcine Lipid Mobilizing Factor	48
2.3.2	Isolation of Porcine Lipid Mobilizing Factor	52
2.3.3	Estimation of Molecular Weight of Porcine LMF on Sephadex G-50	69
2.4	Discussion	71
2.5	Summary	79

		Page
Chapter 3	<u>The Development of a Radio-immunoassay for Porcine Lipid Mobilizing Factor</u>	81
3.1	Introduction	81
3.2	Methods	83
3.2.1	Production of Antiserum	83
3.2.1.1	LMF in Freund's Complete Adjuvant as Immunogen	83
3.2.1.2	LMF Conjugated to Bovine Serum Albumin as Immunogen	84
3.2.1.3	LMF in Polyacrylamide as Immunogen	87
3.2.2	Identification of Cross-Reacting Antiserum by Binding to ^{125}I -labelled LMF	88
3.2.2.1	Labelling of LMF with Iodine-125	88
3.2.2.2	Removal of Free ^{125}I and Damaged Labelled Peptide from ^{125}I -labelled LMF	91
3.2.2.3	Assessment of Antiserum: Antiserum Titration Curves	93
3.2.2.4	Assessment of Antiserum: Standard Curve and Specificity of Antiserum	94
3.2.2.5	Assessment of Antiserum: Nature of Non-Specific Cross-Reactions	97
3.3.	Results	98
3.3.1	Identification of Cross-Reacting Antiserum by Binding to ^{125}I -labelled LMF	98
3.3.2	Assessment of Antisera for Radioimmunoassay	104
3.4	Discussion	116
3.5	Summary	129

		Page
Chapter 4	<u>The Effect of Serum on LMF Induced Lipolysis</u>	130
4.1	Introduction	130
4.2	Methods	133
4.2.1	Degradation of ^{125}I -labelled LMF	133
4.2.2	<u>In Vitro</u> Assay of Lipolytic Activity	134
4.2.2.1	Preparation of Buffer	135
4.2.2.2	Isolation of Rabbit Fat Cells	135
4.2.2.3	Assay Conditions	136
4.2.2.4	Glycerol Assay	137
4.2.2.5	Optimum Collagenase Con- centration	140
4.2.2.6	Statistical Methods	140
4.2.3	Application of <u>In Vitro</u> Assay: Investigation of the Serum Effect on LMF Induced Lipolysis	142
4.2.3.1	A Standard Procedure	142
4.2.3.2	Sera from Individual Animals	143
4.2.3.3	Dialysed Serum from Individual Animals	144
4.3	Results	145
4.3.1	Degradation of ^{125}I -labelled LMF	145
4.3.2	<u>In Vitro</u> Assay of Lipolytic Activity	145
4.3.3	Application of the <u>In Vitro</u> Assay	152
4.4	Discussion	164
4.5	Summary	173
Chapter 5	Discussion	174

	Page
Appendix 1 Nomenclature of LMF Preparations	185
Appendix 2 Materials	186
Appendix 3 Equipment	188
References	189

Abstract of Thesis

A lipolytic peptide Lipid Mobilizing Factor (LMF) has been isolated from deep frozen porcine pituitaries. The method was based on that for the isolation of human Lipid Mobilizing Factor but a number of modifications have been made to extract the peptide from porcine glands. Porcine LMF resembles porcine ACTH and ρ LPH but is distinguishable from both. An attempt was made to develop a radioimmunoassay for the peptide. A specific antiserum could not be raised in rabbits. The only reacting antisera cross-reacted with other pituitary hormones, notably the glycoprotein hormones. Three different immunization schedules were used for antibody production. An in vitro assay for lipolytic activity using isolated rabbit fat cells was developed, initially for identification of lipolytic fractions during the isolation of LMF. The assay was subsequently used to investigate the effect of porcine serum on the lipolysis induced by porcine LMF. Pooled serum potentiated the action of LMF. Sera from individual pigs were assayed to see if there was any relation between their potentiation of lipolysis and either the growth rate or backfat thickness of the pigs. No correlations were found between the total lipolysis and either of these characteristics. Dialysis of the serum samples did not produce a significant result.

Abbreviations

The abbreviations used in this thesis are those recommended by the editors of the Biochemical Journal (1976, 153, 1-21) with the following additions.

LMF	lipid mobilizing factor
cAMP	cyclic 3'-5'-adenosine monophosphate
bis	N,N'-methylenebisacrylamide
TEMED	N,N,N',N'-tetramethylethylenediamide
TRIZMA BASE	tris (hydroxymethyl) amino methane
CM	carboxymethyl
DEAE	diethylaminoethyl
ECDI	1-ethyl-3-(3 dimethylaminopropyl) carbodiimide hydrochloride
FCA	Freund's complete adjuvant
FFA	free fatty acids
GAD	general assay diluent
GAM	glycerol assay medium
PK	pyruvate kinase
LDH	lactate dehydrogenase
GK	glycerol kinase
TCA	trichloroacetic acid
o	ovine
p	porcine
C line	control line
HP line	high growth rate, low backfat line
LP line	low growth rate, high backfat line

viii.

ACTH	adrenocorticotropic hormone
FSH	follicle-stimulating hormone
GH	growth hormone
LH	luteinizing hormone
LPH	lipotropin
MSH	melanocyte-stimulating hormone
PRL	prolactin
TSH	thyroid-stimulating hormone
SDS	sodium dodecyl sulphate

Acknowledgments

The work to be described was carried out in the Department of Physiological Genetics of the Animal Breeding Research Organisation. I am grateful to the Director Dr. J.W.B. King for permitting me to use these facilities.

I would like to thank Mr. W.R. Carr and his assistant Mr. G. Baxter for continuing support and advice. I am also grateful to Dr. R.L. Spooner, Dr. M.R. Williams, Dr. A.J. Webb and Mr. D.L. Sales for further advice on immunology, animal breeding and statistics. I am indebted to Professor G.S. Boyd of the Department of Biochemistry of the University of Edinburgh for maintaining so much interest in the progress of this project.

I am grateful to Mr. D. McVitie of the Moredun Institute for providing and keeping experimental animals; to Dr. O. Trygstad of the Rikshospitalet, Oslo, Norway for the gift of porcine LMF; to Dr. L. Graf of the Research Institute of Pharmaceutical Chemistry, Budapest, Hungary for the gift of porcine ρ LPH; and to Dr. N. Standal of the Institute of Animal Genetics and Breeding, Aas-NLH, Norway for providing pig sera.

This project was carried out while in receipt of an Agricultural Research Council post-graduate scholarship for which I am most grateful.

CHAPTER 1

Introduction

It is only comparatively recently that adipose tissue was recognised as being of importance in the energy metabolism of the body (Wertheimer, 1965). This followed the realisation of the importance of plasma free fatty acids in energy homeostasis. Although they make up only about 5% of the total plasma fatty acids, the plasma free fatty acids have a rapid turnover rate (Gordon & Cherkes, 1956). The introduction by Dole (1956) of a new method for the estimation of plasma free fatty acids, provided a further boost to research in this area. The metabolism of adipose tissue has since been the subject of a number of reviews (Renold & Cahill, 1965; Rudman & Di Girolamo, 1967; Vague, 1969; Jeanrenaud & Hepp, 1970; Bjöntorp & Östman, 1971; Vague & Boyer, 1974).

In this review the metabolism of the adipose tissue will be briefly discussed. Lipolysis, the breakdown of storage triglycerides to glycerol and free fatty acids and their release from the fat cells, will then be dealt with in greater detail. Emphasis will be placed on the hormonal control of lipolysis. Finally, genetic variations in the body composition of man and swine, which may be related to adipose tissue metabolism, will be discussed.

Variations occur between species in their adipose tissue metabolism (Rudman & Di Girolamo, 1967). Most mammalian work has been carried out in rats and the following discussion is based on experiments with this animal, unless otherwise stated.

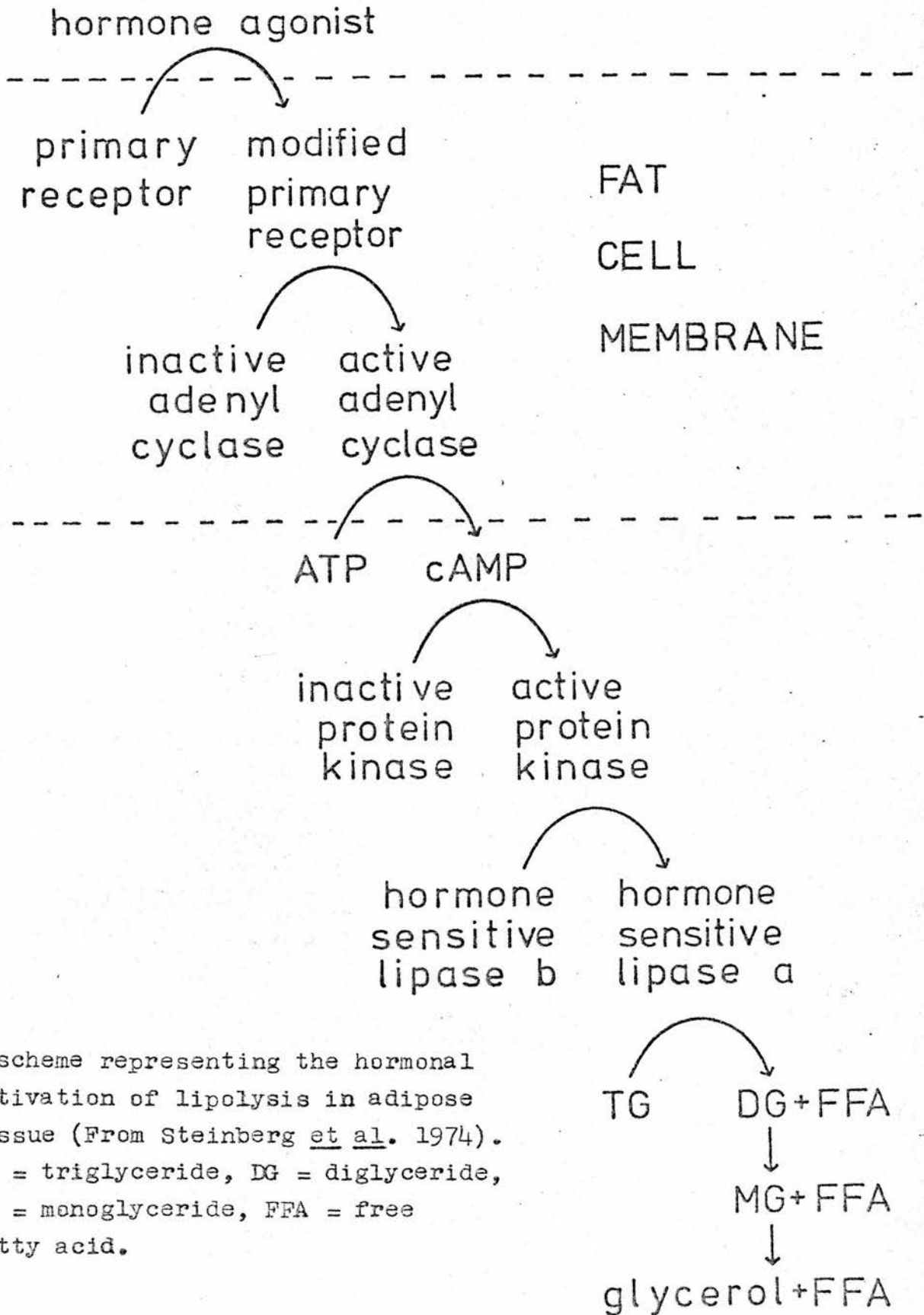
1.1 Adipose Tissue Metabolism

1.1.1 Lipogenesis

Adipose tissue is the largest energy reservoir in the body (Dole, 1965). Its major metabolic pathways reflect this function, being lipolysis and lipogenesis. The latter is the synthesis of triglycerides, which are the principal energy store in adipose tissue. The fat cell content of glycogen, an alternative energy store, is very low in normally fed animals, though it does rise when a starved animal is refed (Vaughan & Steinberg, 1965). The immediate precursors of triglycerides are α -glycerophosphate and free fatty acids. The former is derived from glucose or glycogen via the glycolytic intermediates glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Margolis & Vaughan, 1962). The major source of free fatty acids for triglyceride synthesis is from the plasma. In adipose tissue, plasma triglycerides, from the liver or directly from the gut, are hydrolysed by lipoprotein lipase, and the released free fatty acids, after entering the fat cells, are re-esterified to storage triglycerides (Robinson, 1965, 1970). Free fatty acids can be synthesised in the adipose tissue from glucose (Martin & Vagelos, 1965) via acetyl CoA. Of the glucose entering a normal fat cell, approximately 42% can be recovered as CO_2 , 13% as triglyceride fatty acid, 44% as triglyceride glycerol and 1% as glycogen (Jeanrenaud, 1961). The oxidation to CO_2 of a large proportion of glucose results from the production of NADPH used in fatty acid biosynthesis.

responsible for this step is stimulated by a number of agonists and is known as hormone sensitive or triglyceride lipase. Most of the glycerol produced by hydrolysis of triglyceride passes into the circulation, as adipose tissue glycerokinase activity is low (Robinson & Newsholme, 1967), and little or no glycerol is phosphorylated for subsequent re-esterification (Reshef & Shapiro, 1970). Other tissues, which have a higher glycerokinase activity, especially the liver, phosphorylate the glycerol, which is then either converted to glucose or undergoes glycolysis. Free fatty acids, released by lipolysis, can be re-esterified to triglyceride in the fat cells (Vaughan & Steinberg, 1963). However, lipolysis is normally stimulated when circulatory glucose is low, so there will be little α -glycerophosphate in the adipose tissue available for lipogenesis. Most of the released free fatty acids enter the circulation where they bind to albumin (White et al., 1968) and are transported to other tissues, except the brain, for the provision of energy. The brain normally meets its entire energy requirement by the oxidation of glucose (White et al., 1968).

The rate limiting enzyme, hormone sensitive lipase, is activated via a cascade mechanism (Steinberg et al., 1974). An activating hormone binds to a specific receptor and by modification of this, stimulates adenyl cyclase to convert ATP to cyclic AMP (or cAMP) (Butcher et al., 1965; Butcher, 1966; Butcher et al., 1972). Cyclic AMP activates a protein kinase which phosphorylates inactive hormone sensitive lipase "b" to the active form, hormone sensitive lipase "a" (Huttunen et al., 1970; Huttunen & Steinberg, 1971). The lipolytic cascade may be depicted as in Figure 1.1.

Figure 1.1 The lipolytic cascade.

The activity of hormone sensitive lipase can be affected in other ways. Once formed, cAMP is destroyed by phosphodiesterase, producing AMP (Butcher & Sutherland, 1968). During stimulation of fat cell adenylyl cyclase, inhibition of the phosphodiesterase by methyl xanthines, such as caffeine or theophylline, leads to a much greater increase in lipolysis than with the agonist alone (Butcher et al., 1968). Methyl xanthines, which alone are weakly lipolytic are synergistic with activators of adenylyl cyclase.

Certain hormones do not stimulate triglyceride breakdown in fat cells by increasing the cAMP pool. Growth hormone and glucocorticoids increase lipolysis, after a lag period of 2 hours, and also potentiate each others lipolytic activity (Goodman, 1970; Fain, 1973). Their actions are blocked by inhibitors of protein synthesis such as actinomycin D and puromycin. Growth hormone and glucocorticoids stimulate lipolysis by induction of the synthesis of a further protein (Fain, 1973). Thyroid hormones similarly require a lag time before their action on adipose tissue is seen (Bray and Goodman, 1965). The response of adipose tissue to adrenaline is potentiated by thyroid hormones but neither of these effects of thyroid hormones are a result of adenylyl cyclase activation or of increased protein synthesis (Goodman, 1970; Fain, 1973).

1.2 Antagonists of Lipolysis

Before a detailed examination of lipolysis stimulators, agents which inhibit the breakdown of triglyceride will be mentioned. Insulin is the most important of these and its

actions on adipose tissue were reviewed by Ball (1970). In the absence of glucose, insulin lowers the release of glycerol and free fatty acids from adipose tissue in response to lipolytic agents such as adrenaline and ACTH (Jungas & Ball, 1962). Butcher et al. (1966) showed that the rise in cAMP, induced by the lipolytic hormones in fat cells, was inhibited by the presence of insulin. The mechanism of this effect of insulin on cAMP is not clear. It could be either by inhibition of adenyl cyclase, or by activation of phosphodiesterase (Ball, 1970). Insulin is not antilipolytic in avian adipose tissue (Langslow & Hales, 1969). In the presence of glucose, the antilipolytic action of insulin disappears. The release of free fatty acids is still low, but this is due to increased re-esterification following insulin stimulated entry of glucose into the fat cell. Glycerol release, stimulated by adrenaline, is actually increased in the presence of both insulin and glucose in vitro (Ball, 1970).

Prostaglandin E inhibits lipolysis by reducing the cAMP concentration in the fat cell (Butcher et al., 1968). The basal rate of lipolysis is also inhibited and this antagonist probably acts via competitive inhibition of the binding of ATP to the adenyl cyclase complex (Stock & Westermann, 1968; Westermann & Stock, 1970).

1.3 Lipolytic Agents

Factors which stimulate lipolysis, both in vivo and in vitro, fall into two categories according to their mode of action. Some activate adenyl cyclase in the fat cell

membrane giving a rapid response. Others have a more delayed effect but have a permissive role in the 'rapid' lipolytic action of the former. Included in the first group are a number of pituitary lipolytic peptides one of which is Lipid Mobilizing Factor (LMF). These will be discussed in detail following a description of lipolytic hormones, which have additional actions outside the adipose tissue.

1.3.1 Activators of Adenyl Cyclase

1.3.1.1 The Catecholamines and the Sympathetic Nervous System.

The sympathetic nervous system plays an important part in the regulation of lipid metabolism. This is mediated by the catecholamines, which are potent lipolytic agents.

Noradrenaline is released in adipose tissue by stimulation of the sympathetic nerve endings (Himms-Hagen, 1967), While adrenaline, released by the adrenal medulla acts via the circulation. The sympathetic nervous system responds to changes in activity of the body by providing the necessary substrates for energy production. Part of this is the provision of free fatty acids, released from the adipose tissue. The release of noradrenaline in the adipose tissue provides sufficient energy, in the form of free fatty acids, for small increases in activity, whereas a larger demand for energy is met by the release of adrenaline into the circulation (Brodie et al., 1965).

Both in vivo and in vitro the catecholamines stimulate lipolysis, although considerable inter-species differences have been found (Himms-Hagen, 1967). Their effect is enhanced in vivo by their inhibition of insulin release from

the pancreas (Robison et al., 1972). Insulin inhibition of lipolysis is therefore reduced. Catecholamine stimulation of adenylyl cyclase activity and lipolysis involves the β -adrenergic receptors (Ahlquist 1948; Himms-Hagen, 1967; Fain, 1973). In the human fat cell, α -adrenergic receptors are also present. If these are specifically blocked by phentolamine, lipolysis, stimulated by adrenaline, is enhanced. The α -adrenergic receptors appear to have an inhibiting action on lipolysis but their purpose is obscure (Björntorp & Östman, 1971; Robison et al., 1972).

1.3.1.2 Glucagon and Related Peptides. Glucagon is a lipolytic hormone (Lefebvre, 1975), which also stimulates the breakdown of glycogen in the liver. It was several times (on a weight basis) less active, in rat fat in vitro, than adrenaline (Himms-Hagen, 1961). Its lipolytic action in vivo can be masked by glucagon stimulation of insulin release, which initially decreases serum free fatty acids. Following an initial fall, a considerable increase in serum free fatty acids was found in man 4 hours after glucagon injection (Lefebvre & Luyckx, 1969). Infusion of glucagon into man, at a concentration too low to stimulate insulin release, increased lipolysis (Marks, 1973). In avian adipose tissue, glucagon is the major lipolytic hormone and stimulates lipolysis at much lower concentrations than any other lipolytic hormone (Langslow & Hales, 1969).

Secretin, an intestinal hormone which is identical to glucagon in half of its amino acid sequence, is also active in adipose tissue (Lazarus et al., 1968). It was a more

potent stimulator of adenylyl cyclase in rat fat cell ghosts than glucagon (Rodbell et al., 1970). A number of other intestinal peptides (Grossman, 1974) have been isolated, some of which have an effect on lipolysis. One, from ducks, with glucagon like immunoreactivity (GLI) is antilipolytic in this species (Krug and Mialhe, 1976), whereas another, which has a vasodilatory action (vasoactive intestinal polypeptide) can stimulate lipolysis in rat fat cells (Frandsen & Moody, 1973). Different receptors on the fat cell membrane are specific for secretin and glucagon, and differ from the receptors of other lipolytic hormones such as adrenaline or ACTH (Rodbell et al., 1970; Kuo, 1970). The secretin receptor appears to be shared by the vasoactive intestinal polypeptide (Bataille et al., 1974).

1.3.1.3 Adrenocorticotrophic Hormone and Melanocyte-Stimulating Hormone. Of the pituitary lipolytic hormones, ACTH and α MSH may be classed together (Scott & Lowry, 1974). The latter has never been detected in human pituitaries and, by radioimmunoassay, consists of less than 1% of the biological MSH activity. As its total amino acid sequence differs, from the first 13 residue of ACTH, only by an acetylated N-terminal residue (Butt, 1967), it is probable that α MSH is actually the acetylated N-terminal fragment of ACTH.

ACTH is lipolytic in rats in vivo and in vitro (White & Engel, 1958; Hollenberg et al., 1961), but it is more active in rabbits, a species less sensitive than rats to adrenaline. Human adipose tissue does not respond to ACTH

(Rudman, 1963; Rudman & Di Girolamo, 1967). Adenyl cyclase in the rat fat cell membrane was activated by ACTH implicating the lipolytic cascade in its lipolytic action (Butcher et al., 1968). The fat cell receptor for ACTH can be differentiated from those for adrenaline and other hormones (Birnbaumer & Rodbell, 1969; Rodbell et al., 1970; Kuo, 1970). Stimulation in vitro of lipolysis by ACTH, but not by adrenaline, had been shown to require Ca^{++} ions for maximal response (Lopez et al., 1959; Bally & Tilbury, 1968). Kuo (1970), found that Ca^{++} ions were required for ACTH to activate cAMP formation in fat cells. This indicated that Ca^{++} ions were needed for the stimulation of adenyl cyclase, possibly for the binding of the hormone to its receptor. Yanagi et al. (1967) suggested that ACTH stimulated lipolysis by increasing the intracellular Ca^{++} ion content and for this reason did not activate lipolysis in their absence. The Ca^{++} requirement of ACTH induced lipolysis is not the only ionic factor which can influence lipolysis. When the Na^+/K^+ pump of fat cell membranes was inhibited by ouabain, lipolysis was also inhibited, suggesting a connection between these ions and lipolysis (Mosinger, 1970).

1.3.1.4 Pituitary Glycoprotein Hormones

Other pituitary hormones have been reported to be lipolytically active. Burns et al. (1967) found that a TSH containing fraction of human pituitaries was the most lipolytic on human adipose tissue. Initially further purification could not separate the lipolytic from the thyroidotropic activity (Storring et al., 1972). Later work

did indicate that the activities were separable. Stevenson & Fernie (1973) suggested that the lipolytic activity associated with human TSH was due to a contaminant. A similar result was reported by Weisweiler & Schwandt (1973), who isolated and identified a lipolytic contaminant of porcine TSH as a lipotropin.

The gonadotropins were considerably less lipolytic (between 3 and 200 times less) than TSH, which itself was about 200 times less potent than ACTH on rat fat cells (Farmer et al. 1972). As the lipolytic effect of TSH is probably due to contamination, it seems unlikely that the gonadotropins have any physiological effect on lipolysis.

1.3.2 Hormones with a Delayed Lipolytic Action

1.3.2.1 Thyroid Hormones and Glucocorticoids

The actions of the thyroid hormones and the glucocorticoids on lipid mobilization are similar, in that, in the absence of either, the response of adipose tissue to adenylyl cyclase activators is diminished. Adrenalectomised dogs failed to show an increase in serum free fatty acids on injection of adrenaline, but cortisone treatment restored the response (Shafrir & Steinberg, 1960). Adipose tissue from hypothyroid rats gave a diminished response to adrenaline compared to tissue from euthyroid rats. Adipose tissue from hyperthyroid rats gave an exaggerated response (Debons & Schwartz, 1961; Deykin & Vaughan, 1963).

Both thyroid hormones and glucocorticoids require a lag time for their effects to be detected (Fain, 1973) and alone, neither of them are strongly lipolytic in normal rat adipose tissue in vitro (Debons & Schwartz, 1961; Fain et al.,

1965). The impairment of lipolysis following hypophysectomy is partly due to the lack of TSH and ACTH needed to stimulate thyroid hormone and glucocorticoid secretion.

The mechanisms of their permissive role in lipolysis are different. The permissive effect glucocorticoids have on adrenaline induced lipolysis in rats, was inhibited by actinomycin D, indicating that protein synthesis was involved (Goodman, 1970). When the components of hormone sensitive lipase activation were examined, protein kinase activity increased on pretreatment of adipose tissue with glucocorticoids. It was postulated that the induction of protein kinase by glucocorticoids was responsible for their action on lipolysis (Lamberts et al., 1975). The permissive (and potentiating) effect of the thyroid hormones is not dependent on protein synthesis, as actinomycin D did not suppress the action of triiodothyronine on adipose tissue (Vaughan, 1967; Goodman, 1970). Fain (1973) has suggested that the permissive effect of the thyroid hormones on lipolysis is secondary to their calorogenic action on the fat cell. Triiodothyronine either decreases the efficiency of adipose tissue mitochondrial oxidative phosphorylation or increases the utilization of the produced energy. However, Fain (1973) did not postulate how such alterations could potentiate lipolysis. Correze et al. (1974) found that thyroidectomy did not reduce the activation of adenyl cyclase by adrenaline. However, protein kinase was not activated by the cAMP so they suggested that the thyroid hormones influenced the lipolytic cascade between cAMP and protein kinase. They postulated that the presence of thyroid hormones normally inhibited

phosphodiesterase, but in their absence cAMP was degraded before it could activate the protein kinase.

1.3.2.2. Growth Hormone

Growth hormone at one time appeared to have two actions on lipolysis. One of these was a stimulation of lipolysis which required a lag time of 2 hours and involved protein synthesis as the hormone was inhibited by puromycin and actinomycin D (Fain et al., 1965; Fain, 1973).

Glucocorticoid was required for maximum stimulation and was synergistic with growth hormone. The actions of growth hormone and glucocorticoids differed, in that alone the former potentiated the lipolytic activity of theophylline, a phosphodiesterase inhibitor, but not adrenaline (Goodman, 1969). Glucocorticoids potentiated adrenaline stimulated lipolysis but did not affect the lipolysis induced by theophylline (Goodman, 1970).

Growth hormone has also been reported to have a 'rapid' lipolytic action similar to that of adrenaline, ACTH and other agonists, which produce an immediate increase in lipolysis by activation of hormone sensitive lipase. Lipolysis, stimulated by bovine growth hormone, though at a high concentration, was detected within the 2-hour lag time required for protein synthesis (Hamid et al., 1965; Swislocki et al., 1971). However, Lee et al., (1974) successfully separated a quick acting lipolytic activity from bovine growth hormone, leaving the parent molecule with a much reduced lipolytic activity. TSH was suggested as a possible lipolytic contaminant but as the lipolytic activity of TSH

can be removed, it was more likely that the growth hormone was contaminated with lipotropin or similar lipolytic peptide. Trygstad & Foss (1968) separated a novel lipid mobilizing factor (LMF) from a human growth hormone preparation. Somatotropic activity remained with the parent molecule, which lost its lipolytic activity.

1.3.3 Pituitary Lipolytic Peptides

Trygstad's lipid mobilizing factor (LMF) is one of a number of pituitary lipolytic peptides (Table 1.1) isolated from various species by different research teams (Rudman et al., 1970; Chrétien, 1973; Schwandt, 1974). Interest in the pituitary as a possible source of a lipid metabolism regulating factor, was aroused by the impairment of fat mobilization on hypophysectomy and by the increase in serum free fatty acids produced, among other effects, by injection of pituitary extracts (Astwood, 1965; Anselmino & Hoffman, 1931). The pituitary was further implicated as the source of a lipolytic factor, by the appearance in urine from fasted rats of a Fat Mobilizing Substance of low molecular weight, which depended on the presence of the pituitary or hypothalamus (Chalmers, 1965).

The relationships between the various peptides cannot be entirely known until their amino acid sequences have been determined. Using the information available some may be grouped together (Table 1.1). A major distinction is between the neurophysins, which are large cystine rich proteins derived from the posterior pituitary and the smaller lipolytic peptides of the anterior pituitary. The

Table 1.1. Lipolytic peptides that have been extracted from pituitary glands.

a) Lipolytic peptides possibly related to ACTH, MSH or MSH

Peptide	Species of Origin	Molecular Weight	Reference
↑LPH xLPH	ovine, porcine, bovine ovine, porcine, bovine	9-11,000 6-7,000	} reviewed by Chrétien (1973)
Peptide A Peptide B	porcine porcine	5,700 11,400	
LMF	human porcine	5,400 or 2,100 -	} reviewed by Schwandt (1974)
peptide L	ovine	5,400	
peptide I	porcine	5,000	Trygstad (1968 a,b) Trygstad et al. (1972)
			Birk & Li (1964b)
			Astwood et al. (1961)

b) Neurophysins and other cystine rich peptides

Peptide	Species of Origin	Molecular Weight	Reference
neurophysin I, II	bovine	19-21,000	Hollenberg & Hope (1968)
neurophysin	human	10,000	Foss <u>et al.</u> (1973)
neurohypophyseal peptide	bovine	5,500	Preddie & Saffran (1965)
neurohypophyseal peptide	porcine	9,900	Wuu & Saffran (1969)
fraction L	porcine	-	Rudman <u>et al.</u> (1961)
peptide II	porcine	10-20,000	Astwood <u>et al.</u> (1961)
7D6	porcine	8,900	Rudman <u>et al.</u> (1970)
7D7	porcine	5,500	Rudman <u>et al.</u> (1970)
fettstoffwechselhormon	porcine	-	Schleyer <u>et al.</u> (1974)

c) Other pituitary lipolytic peptides

Peptide	Species of Origin	Reference	Isolated By
'new' lipolytic peptide	human	Graf <u>et al.</u> (1974)	Graf <u>et al.</u>
'lipotropin'	ovine, porcine, bovine	Chrétien (1973) Schwandt (1974)	Ryshka & Khokhlov
adiposin	porcine bovine	Chrétien (1973) Schwandt (1974)	Leites & Davtyan
lipid mobilizer	porcine	Schwandt (1974)	Seifter & Baeder

neurophysins bind strongly to either oxytocin or vasopressin and it seems likely that their primary function is involved in the storage or transport of these hormones (Rudman et al., 1970). The reason for their lipolytic activity remains to be explained.

1.3.3.1 The Lipotropins

The most fully characterized of the lipolytic peptides are the lipotropins (LPH) (Chrétien, 1973). Found in a number of species, they consist of β LPH, γ LPH and β MSH, and are structurally related to ACTH. They also resemble ACTH in biological activity, in that rabbit adipose tissue is particularly sensitive to the lipolytic action in vitro of the lipotropins and Ca^{++} ions are required for a maximum response (Lis et al., 1972). Chrétien (1973) has suggested that β LPH is the precursor of γ LPH and β MSH in much the same way as proinsulin is the precursor of insulin (Steiner et al., 1967). In sheep, the 58 amino acid residues of γ LPH are identical to residues 1-58 of β LPH, while the 18 residues of β MSH correspond to residues 41-58 of both β and γ LPH.

Recent work has indicated that fragments of β LPH may have a function in neurotransmission. A peptide, met-enkephalin (Hughes et al., 1975) which binds to opiate receptors in the brain, has the same sequence as residues 61-65 of β LPH. Larger fragments of β LPH, containing this sequence, bind more strongly to the opiate receptors (Graf et al., 1976). Whether this occurs in the body and, if so, for what purpose remains unknown.

Lipotrophic peptides A and B of Schwandt (1974), extracted from porcine pituitaries, showed a number of similarities to LPH. Rabbit adipose tissue was the most sensitive to peptides A and B (Weisweiler & Schwandt, 1975). They also stimulated lipolysis from rat adipose tissue, which was unresponsive to ovine γ LPH and ρ MSH, but did respond to a large amount of ovine ρ LPH. Peptides A and B were lipolytic in mouse and guinea pig adipose tissue.

1.3.3.2 Lipid Mobilizing Factor

Lipid mobilizing factor (LMF) was one of the first lipolytic peptides to be isolated from human pituitary glands (Trygstad, 1968 a,b). Deep frozen rather than acetone dried glands had to be used. The human peptide would not cross-react in a radioimmunoassay for ovine ρ LPH (Chrétien, 1973) indicating that immunologically there was a difference in structure between these two. Species difference could account for this. LMF resembled the lipotropins in the sensitivity of different species to its lipolytic action. Both LMF and LPH were most active in vitro in rabbit, with a minimum effective dose in the nanogram range. LMF resembled ρ MSH and γ LPH in being inactive in normal rats, where a large dose of ρ LPH was required to stimulate lipolysis. In rabbits, ρ LPH had a hypocalcaemic action in vivo (Chretien, 1973), which it shared with lipotrophic peptides A and B. One preparation of LMF did exhibit this effect but a further preparative step removed the hypocalcaemic activity (Trygstad, 1968b). In contrast, LMF and peptides A and B in rabbits caused hyperglycaemia in vivo, which did not follow injection with LPH. Porcine LMF was isolated by Trygstad

et al. (1972) but was not extensively investigated. Both porcine and human LMF were active in vitro on porcine adipose tissue, which until recently had been considered insensitive to any hormonal lipolytic stimulus (Rudman, 1963). Porcine ρ LPH was active in vivo in swine (Tamasi et al., 1969).

1.3.3.3 Are the Lipolytic Peptides of Importance in the Body?

A major problem with any of the pituitary lipolytic peptides is in ascribing to them a physiological significance. Do any of them play a major role in lipid metabolism or are they fragments of other hormones produced by the preparative procedures? A radioimmunoassay for ovine ρ LPH has shown a basal concentration of 2 ng/ml for this peptide in sheep serum (Chrétien, 1973). This is the only direct evidence for the existence of a lipolytic peptide in the circulation. Biological assays for lipolytic activity cannot be used to identify a peptide in the serum because they are not specific and because the likely serum concentration of a lipolytic peptide would be outside the range of a biological assay. Extraction of a lipolytic peptide from serum or plasma does not confirm its physiological role as it may still be an artefact of the extraction procedure. Without a sensitive and specific assay for the peptides it is difficult to correlate their concentration with the physiological state of the animal. Changes, in serum concentrations of a peptide, which correlated with increased or decreased lipid mobilization, would help to confirm its physiological role.

The existence of a system regulating the lipolytic activity of a peptide, for instance by inhibition or potentiation, would also suggest a physiological role for the peptide,

especially if the regulation varied with the state of the animal. The lipolytic activity of ACTH in rat adipose tissue was potentiated by the serum of certain species (Benuzzi-Badoni et al., 1968), but no attempt was made to see if the potentiation varied between serum from animals in different physiological conditions. ACTH and other pituitary peptides were inactivated by extracts of rat (though not rabbit) adipose tissue (Rudman et al., 1964 a,b) probably by a proteolytic reaction. Ho et al. (1975) have extracted another lipolytic peptide inactivating factor from adipose tissue. This was non-diffusible on dialysis and bound strongly to albumin. Trygstad & Foss (1972) had earlier partially purified an inhibitor of LMF from human serum, which was associated with the albumin fraction. Possibly this corresponded to the inactivating factor of Ho(1975). Adrenaline was not affected by any of these inhibitory factors.

1.3.3.4 Inhibition of LMF

Trygstad's partial purification of a serum inhibitory factor (Trygstad & Foss, 1972) followed the observation that human serum could inhibit LMF induced lipolysis (Trygstad & Foss, 1967) in vivo in rabbits. Variations were found in the inhibition caused by serum from normal patients, from some obese patients and from children with lipodystrophy. The last two groups have disturbed lipid metabolism. Serum from lipodystrophic children gave about 5% inhibition of LMF induced lipolysis while serum from some obese patients gave nearly 100% inhibition. Normal patient's serum and serum from a second group of obese patients gave from 20-70% inhibition.

The extent of inhibition appeared to reflect the ability of the patients to mobilize fat.

In swine, animals bred for several generations, using backfat thickness and growth rate as selection criteria, provided a suitable model for the examination of variation in the regulation of LMF activity. The pigs were in a control line (C-line), a high growth rate, low backfat line (HP-line) and a low growth rate, high backfat line (LP-line) (Vangen, 1974a). Adipose tissue, taken at slaughter, from the lean pigs (HP-line) gave the greatest lipolytic response to both porcine LMF and adrenaline. Fat pig (LP-line) adipose tissue showed the least response (Trygstad et al., 1972; Standal et al., 1973). Serum, taken at slaughter, from the lean pigs had very little inhibitory effect on the adipokinetic activity of human LMF in rabbits, in vivo, while serum from the fat pigs was strongly inhibitory. Samples from pigs in the control line gave intermediate results in the experiments, both with adipose tissue and with serum. Apparently a correlation existed between an animal's leanness and the inhibitory activities of its adipose tissue and serum on LMF induced lipolysis. An estimate of a young animal's future leanness and growth rate, obtained by examination of its serum for inhibition of LMF, would be useful as an early selection criterion (Standal et al., 1973). At present a pig is several months old before it is selected for further breeding. The development of a system to produce an earlier estimate of an animal's breeding potential was a major objective of this thesis.

1.4 Aims of Present Study and Outline of Work

Before any investigation could be made into the physiological role of LMF or the development of an assay to help predict the growth of pigs based on serum inhibition of LMF, a ready source of the peptide was required. The isolation of LMF from deep frozen porcine pituitaries was attempted. At first, the method of Trygstad (1968 a,b) was followed but a homogeneous preparation could not be isolated following this procedure. As Trygstad's method was designed for human pituitaries, the lack of homogeneity of the isolated preparation could be due to the use of porcine glands. Modifications were made to his method over a number of extractions. Three separate extractions are described in the following Chapter, illustrating the development of an alternative isolation procedure. The basic modifications made were the replacement of dialysis for desalting and the introduction of an ion-exchange column on which LMF was absorbed and then desorbed by an increase in buffer concentration.

Verification of a physiological role for LMF would be provided by evidence of its presence in serum. An attempt was made to do so by radioimmunoassay. For this a specific antiserum was required. The inoculation of rabbits with human LMF had previously failed to stimulate the production of anti-LMF antiserum (Norman & Turter, 1968). In fact anti-growth hormone was raised. With LMF fractions isolated here, three attempts were made to produce anti-LMF antiserum using different immunization procedures. Initially the simplest method was used. LMF was emulsified in an oil based adjuvant

(Freunds Complete Adjuvant) and a series of injections given to 3 rabbits. For the second attempt at antibody production, LMF was conjugated to a large immunogenic molecule (albumin) by the method used by Goodfriend et al. (1964) for the conjugation of bradykinin to rabbit serum albumin. The conjugate was emulsified in Freunds Complete Adjuvant and injected at regular intervals into another three rabbits. For the last attempt, LMF was purified by polyacrylamide disc gel electrophoresis, and the polyacrylamide containing LMF sliced out. The gel was homogenized and injected into three rabbits, where the polyacrylamide acted as an adjuvant (Weintraub & Raymond, 1963). Serum from all the inoculated rabbits was assessed for binding to ^{125}I -labelled LMF.

LMF, prepared by the method described in Chapter 2 was used in an in vitro assay for lipolytic activity to investigate the effect of the addition of serum on LMF induced lipolysis. After standard conditions for the inclusion of serum were established, sera from individual pigs were examined for any difference in effect. The sera came from pigs in all three lines of the animals bred by selecting for high growth rate and low fat, or low growth rate and high fat (Vangen, 1974a). The samples were therefore from a wide range of animals. This enabled correlations to be investigated between an animal's growth data and the effect of its serum on LMF induced lipolysis. If significant correlations had been found, the assay could have been used to predict a young animal's eventual growth and leanness.

CHAPTER 2.Preparation of Porcine Lipid Mobilizing Factor2.1. Introduction

Trygstad (1968a) first isolated LMF from deep frozen human pituitary glands utilizing a procedure similar to those used for other pituitary peptides (Schally et al., 1962; Birk & Li, (1964a); Stockell Hartree, 1966; Graf et al., 1969). After precipitation of impurities from a pituitary homogenate, by pH adjustment or organic solvents, the required protein was isolated by gel filtration and ion-exchange chromatography.

At first Trygstad's method was followed closely, but was then modified as the porcine LMF isolated by his method was not homogeneous. A major modification was to dispense with dialysis, to ensure that LMF was not lost through the dialysis membrane. Using ammonium acetate as the buffer, enabled protein in the fractions to be recovered by freeze drying without dialysis. When necessary, desalting was performed by gel filtration. LMF was finally purified by Trygstad on a diethylaminoethyl (DEAE) cellulose anion exchange column, on which LMF was not absorbed but eluted in the breakthrough peak. However, any other basic peptides in the sample, would also elute in the breakthrough peak. To eliminate this possibility chromatography on a carboxy methyl (CM) cellulose cation exchanger was introduced. LMF was absorbed onto this, to be desorbed by a change in the ionic environment. The development of an alternative procedure for the isolation of porcine LMF is demonstrated by a detailed description of 3 extractions (V, VIII, XI) which

illustrate the modifications made. Even though these methods enabled LMF to be isolated, further modifications could still be introduced to improve the simplicity and ease of use of the procedure.

LMF was at first identified by biological assays of lipolytic activity. An in vivo assay, which measured adipokinetic activity was soon replaced by an assay of lipolysis in vitro. The former method proved to be wasteful in peptide and expensive in rabbits. The latter, used also for investigation of serum effect on LMF induced lipolysis (chapter 4), provided a much simpler method for the estimation of the activity of a lipolytic agent. Polyacrylamide disc gel electrophoresis at pH8.9 became the usual method of identification of porcine LMF. A sample of porcine LMF from Trygstad served as a standard. Electrophoresis on sodium dodecyl sulphate polyacrylamide gels helped to confirm that Trygstad's LMF and the isolated peptide were similar and probably identical.

2.2. Methods

2.2.1. Identification of Porcine Lipid Mobilizing Factor

2.2.1.1. In Vivo Assay of Adipokinetic Activity

To determine the adipokinetic activity, in vivo, of LMF preparations, a method based on that of Trygstad (1967) was used. The increase in serum free fatty acids (FFA) was measured after injection into rabbits of a sample of the preparation dissolved in saline.

0.1mg or 1.0mg of the preparations to be tested were made up to 5ml with 0.9% (W/V) saline and injected

subcutaneously between the shoulder blades of male or female New Zealand White rabbits. The rabbits, which were fed ad libitum, were between 4 and 6 months old. Prior to injection they had been bled of 7ml of blood from the peripheral ear vein. 90 minutes later another 7ml of blood was taken from each rabbit. After coagulation the blood was centrifuged at 2,500g for 10 minutes, and the serum aspirated. The serum was stored at -18°C unless assayed immediately for FFA content.

Serum FFA concentration was estimated using a modification of Dole's method (Dole & Meinertz, 1960; Patterson, 1963). A stock standard solution of 1.0mM palmitic acid in n-heptane was dispensed, in duplicate into boiling tubes in volumes of 0, 0.25, 0.50, 1.0, 1.5 and 2.0ml. The standards were evaporated to dryness under nitrogen in a water bath at 60°C . 2.0ml of distilled water was added to each tube. 2.0ml aliquots of unknown sera were dispensed, in duplicate, to similar boiling tubes. 10ml of an extraction mixture, consisting of isopropanol : n-heptane : 1.0M HCl in the ratio 40:10:1, was added to standards and unknowns. After a 15 second mix on a vortex mixer, 4.0ml of double glass distilled water followed by 6.0ml of n-heptane were added to each tube. After a further 15 second mix the upper, n-heptane layers were transferred to 25ml conical flasks and evaporated to dryness, under nitrogen, on a hot plate. The residues were each dissolved in a mixture of 2.5ml of 95% (W/V) ethanol and 0.5ml of 0.02% (W/V) ethanolic Nile Blue indicator solution which had

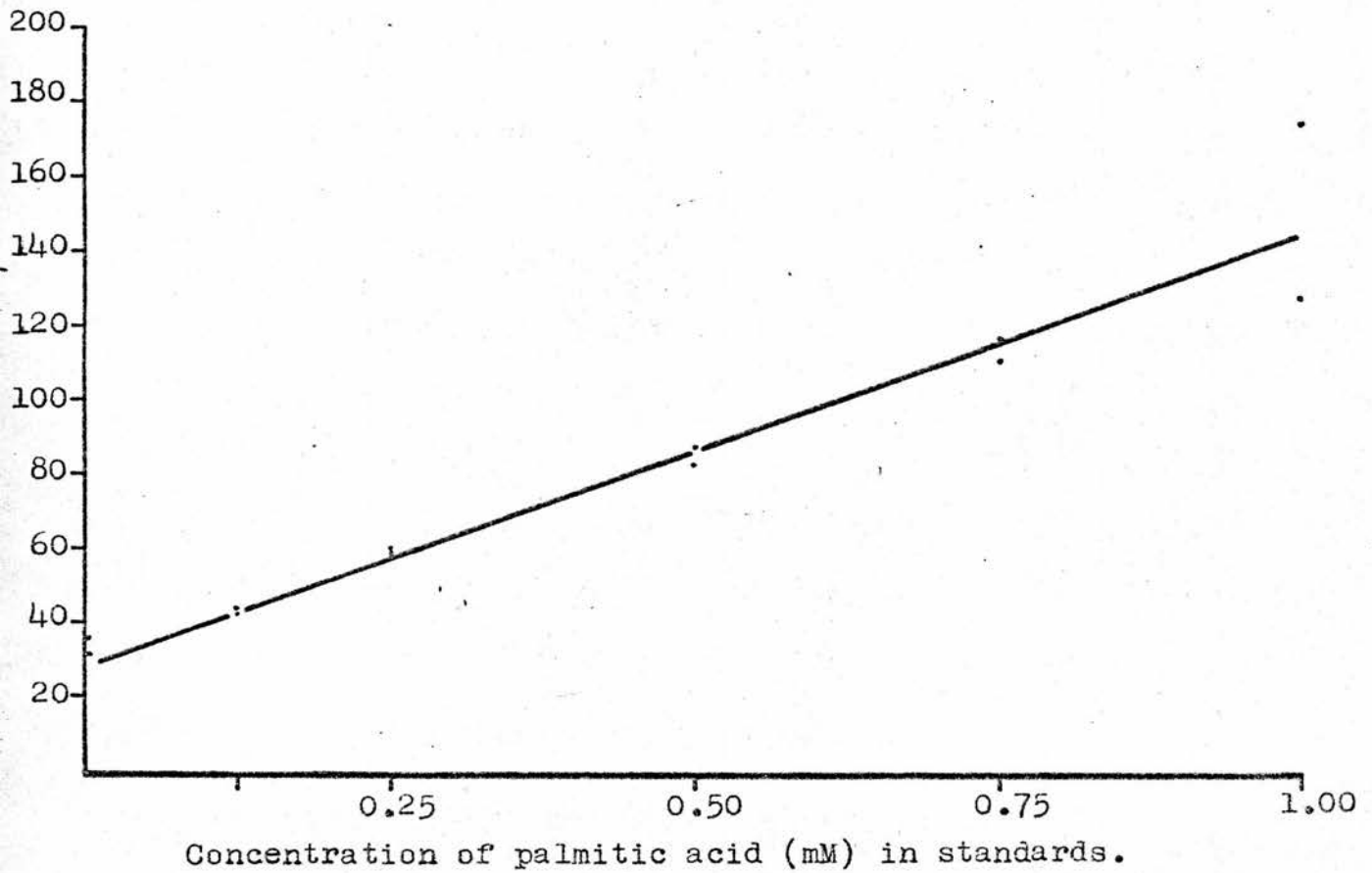
been heated in a 37°C water bath. The standards and unknowns were titrated against 0.01M NaOH under nitrogen using a 'Conway' micro-burette. Using a calibration curve (Figure 2.1.) of the amount of palmitic acid in the standards against the volume of NaOH titrated, the amount of free fatty acid in the unknown sera could be estimated. The difference in serum FFA concentration before and 90 minutes after the injection of a preparation into the rabbit gave a measure of the preparation's adipokinetic activity. As only one rabbit was injected with each sample, this assay gave a qualitative rather than quantitative measure of the peptides' activities.

2.2.1.2. In Vitro Assay of Lipolytic Activity

The in vitro assay of lipolytic activity, which measures the increase in the rate of glycerol release from isolated rabbit fat cells, on incubation with standard or unknown preparations, is fully described in section 4.2.2. The assay was used here to identify lipolytic fractions during the isolation of LMF and to establish dose-response relationships for some of the purer preparations.

For identification of lipolytically active fractions the preparations, before or after lyophilization, were diluted to either 0.01, 0.02 or 0.05 mg/ml in Krebs-Ringer bicarbonate buffer and 0.1ml aliquots were dispensed, in quadruplicate, to the incubation vials. Each vial then contained 1.0, 2.0 or 5.0 µg of unknown peptide per 1.1ml of incubate. For the dose-response relationships, the LMF preparations were usually dispensed at three different

Figure 2.1 Calibration Curve for Estimation of Free Fatty Acid Concentration



Samples and standards extracted into isopropanol:
n-heptane: 1.0M HCl (40:10:1), evaporated to dryness and dissolved in 95% ethanol. Titrated against 0.01M NaOH using 0.02%(W/V) ethanolic Nile Blue as indicator.

concentrations giving a three-point assay between 0.02 and 5.0µg of peptide in 1.1ml of incubate. Three different preparations could be assayed in one experiment. Alternatively one preparation could be assayed at 8 or 9 concentrations.

2.2.1.3. Polyacrylamide Disc Gel Electrophoresis

Polyacrylamide disc gel electrophoresis was carried out according to Davis (1964) except that the spacer and sample gels were omitted. The samples were applied in a concentrated sucrose solution. A 'Shandon' electrophoresis kit was used.

The following stock solutions were made up in distilled water. A:28.0g of acrylamide + 0.735g of N,N'-methylenebisacrylamide (BIS) made up to 100ml. B:36.6g of tris (hydroxymethyl) aminomethane (TRIZMA BASE) + 48.0ml of 1.0M HCl + 0.23ml of N,N,N',N'-tetramethylethylenediamine (TEMED) made up to 100ml (pH8.9). C:6.0g of TRIZMA BASE + 28.8g of glycine made up to 1000ml (pH8.3). These were stored at 4°C and brought to room temperature before use.

8 clean glass tubes, 7cm x 0.6cm (internal diameter), were tightly stoppered at the lower end and positioned vertically. 3ml of A + 1.5ml of B + 7.5ml of distilled water were mixed and de-aerated. 0.1ml of 14% (W/V) ammonium persulphate in water was added to the mixture and the tubes filled up to a 6cm mark. Distilled water was carefully layered on top and the acrylamide allowed to polymerize, taking up to 30 minutes. The stock running buffer (C) was diluted 10 times and the lower compartment of the electrophoresis apparatus filled. A drop of 0.01% (W/V)

bromophenol blue dye in water was added to the remaining running buffer for the upper reservoir.

After the polyacrylamide had gelled in the tubes, the upper aqueous layer was removed and replaced by 50 μ l of an approximately 1.0mg/ml solution of the peptide sample mixed with 20 μ l of 80%(W/V) sucrose. Running buffer for the upper reservoir was layered over the peptide-sucrose mixture. The gel tubes were removed from their stoppers and placed in the electrophoresis apparatus so that the lower end of polyacrylamide was in contact with the buffer in the bottom reservoir. Care was taken not to disturb the peptide layer. The top of the gel tubes projected into the upper reservoir, which was then filled with running buffer. The cathode of a D.C. output power pack was connected to a platinum electrode in the upper reservoir, while the anode was connected to similar electrode in the lower reservoir. Electrophoresis was carried out at a constant current of 4mA per gel for approximately 1 hour, until the leading edge of bromophenol blue dye approached the lower end of the gels. The gels were flushed from their tubes with 10%(V/V) acetic acid using a narrow gauge needle on a 5ml syringe. They were stained and fixed in 1%(W/V) amido black in 10%(V/V) acetic acid for 1 hour. The gels were destained overnight in 10%(V/V) acetic acid.

The mobility of the protein bands (distance of protein migration \div distance of anion front migration) could be calculated because a narrow band of stained protein was usually apparent at the position where the bromophenol blue

dye had migrated. The protein had probably associated with the dye. However, this did give a means of finding the distance of anion front migration and thence the mobility.

2.2.1.4. Electrophoresis on Sodium Dodecyl Sulphate
Polyacrylamide Gels

Whereas separation on polyacrylamide disc gel electrophoresis at pH8.9 is dependent on both the charge and the dimension of the molecules, electrophoresis on sodium dodecyl sulphate (SDS) polyacrylamide gel is solely dependent on the molecular dimensions of the sample (Weber & Osborn, 1969). It is assumed that SDS anions bind to proteins rendering them all negatively charged. Separation then depends on the size of the molecule and their mobilities give an indication of molecular weight. Below 10,000 mol.wt. the method is not reliable for determination of molecular weight. It was used here to compare LMF preparations with other pituitaries hormones and Trygstad's porcine LMF.

The following stock solutions were made up in distilled water. Solution A was also used in electrophoresis at pH8.9 (section 2.2.1.3). A : 28.0g of acrylamide + 0.735g of N,N'-methylenebisacrylamide made up to 100ml. B : 1.0 litre of 0.2M Na_2HPO_4 and C : 1.0 litre of 0.2M NaH_2PO_4 . Solutions B and C were mixed, prior to electrophoresis to give approximately 250ml of 0.2M phosphate buffer pH7.0. 8 clean glass tubes (7cm x 0.6cm i.d.) were stoppered at the lower end and positioned vertically. 2.5ml of A + 4ml of 0.2M phosphate buffer pH7.0 + 3.5ml of distilled water + 10 μ l of TEMED (N, N, N', N'-tetramethylethylenediamine) were

added to 0.01g of SDS and de-aerated. 0.1ml of 14% (W/V) ammonium persulphate in water was added, the gel tubes were filled to a 6cm mark, water layered on to the surface and the acrylamide allowed to polymerize.

The protein samples were prepared by incubating 50 μ l of a 1mg/ml solution of the sample with 40 μ l of 0.25% (W/V) SDS in 0.2M phosphate buffer pH7.0, 10 μ l of 80% (W/V) sucrose in bromophenol blue (0.01%, W/V) and approximately 1 μ g of dithioerythritol, at 37°C for 2 hours. Running buffer was prepared by adding 200ml of 0.2M phosphate buffer pH7.0 to 0.5g of SDS and making up to 500ml with water. The gels, samples and running buffer were at a final concentration of 0.08M phosphate buffer pH7.0 with 0.1% (W/V) SDS.

Prior to application of protein samples the gels were pre-run at a constant current of 4mA per gel for 15 minutes. Buffer from the upper and lower reservoirs was then mixed to redistribute the ions. The protein samples were layered onto the polyacrylamide gels, the buffer replaced in the reservoirs and electrophoresis was carried out at a constant current of 8mA per gel, until the bromophenol blue dye approached the lower end. This was usually about 2 hours. The gels were removed, fixed, stained and destained as described in the previous section.

In calculating the mobility of the protein bands, allowance had to be made for the swelling of the gels which takes place in 10% (V/V) acetic acid.

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after staining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

For proteins of known molecular weight, above 10,000, the mobility plotted against the log of molecular weight should give a linear relationship.

2.2.1.5. Protein Estimation

a) Spectrophotometrically at 280nm. In the column chromatography described in the following sections the presence of protein in a fraction was revealed by measurement of its absorbance at 280nm ($E_{280\text{nm}}$). A 0.5ml aliquot of the fraction was placed in a microcell with a path length of 1 cm and the absorbance taken at 280nm in a spectrophotometer. Column buffer was used as a blank. Only proteins or peptides containing tyrosine or tryptophan residues absorb at this wavelength (Goodwin & Morton, 1946). For a rough estimate of protein concentration it was assumed that a 1mg/ml solution of protein gave an absorbance of about 1.0. This is the average absorbance of serum proteins (Chase and Williams, 1967).

b) By the Folin-Ciocalteu Reaction. More accurate estimations of protein content were made using the Folin-Ciocalteu's phenol reagent in the method described by Lowry et al. (1951).

A copper alkali solution was prepared fresh each day as follows. 100ml of 0.2M NaOH + 100ml of 4%(W/V) Na_2CO_3 + 2ml of 2%(W/V) Sodium potassium tartrate + 2ml of 1%(W/V) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ were mixed. The Folin-Ciocalteu's phenol reagent

was diluted 1:1 with distilled water. A standard solution of bovine serum albumin (BSA) was made up at a concentration of 250 μ g/ml.

The BSA standard was diluted to give 0, 50, 100, 150, 200 and 250 μ g/ml solutions. Quadruplicate 0.5ml aliquots were dispensed into 7.0ml test tubes. To these and to 0.5ml of the protein samples in quadruplicate, 2.5ml of copper alkali solution was added and mixed. After 10 minutes at room temperature 0.25ml of diluted Folin-Ciocalteu's reagent was added. After mixing, the tubes were left at room temperature for 1 hour and the absorbance taken at 700nm against water blanks. A calibration curve was drawn (Figure 2.2.) of protein (BSA) concentration against absorbance and from this the protein content of unknowns was found.

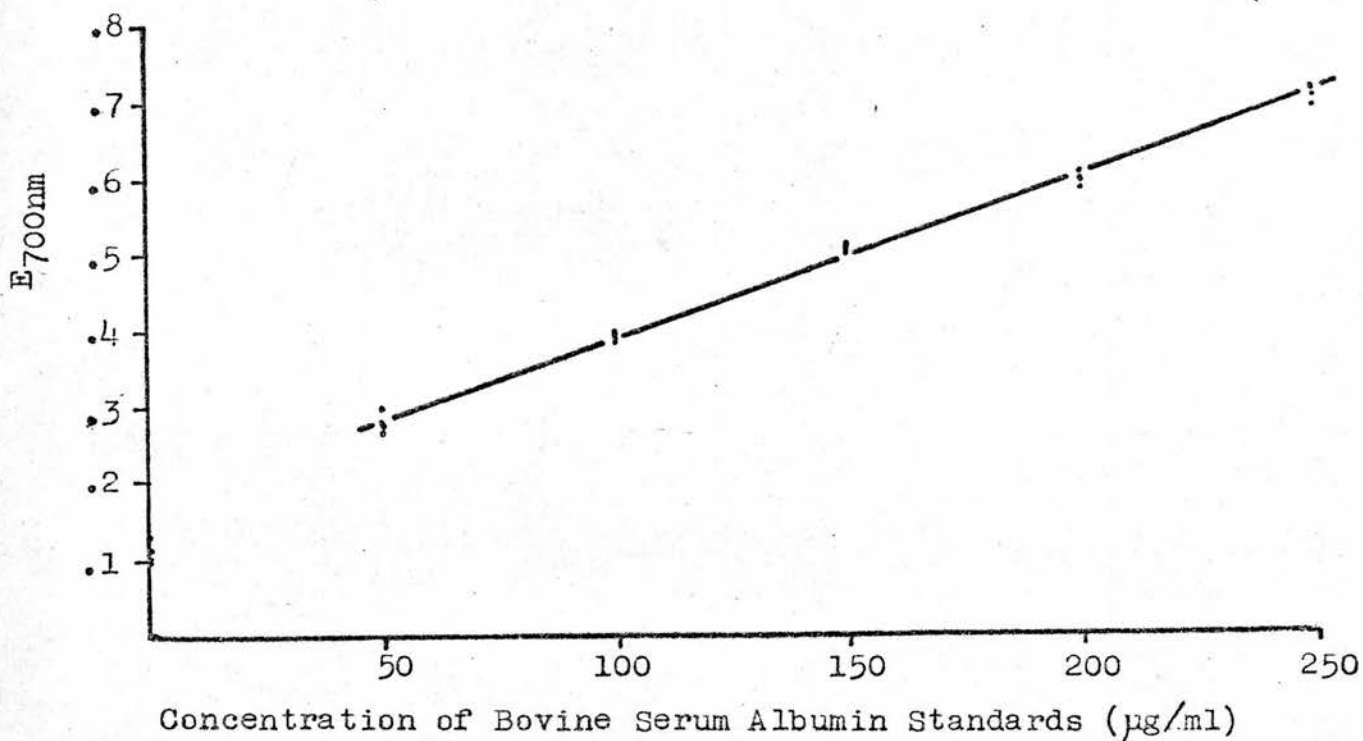
2.2.2. Isolation of porcine Lipid Mobilizing Factor

2.2.2.1. Chromatographic Procedures

For both gel filtration on Sephadex cross-linked dextran and ion-exchange chromatography on ion-exchange celluloses, the procedures described in the manufacturers' handbooks, were followed. These are 'Gel filtration in Theory and Practice' published by Pharmacia Fine Chemicals, and Whatman's 'Advanced Ion-Exchange Celluloses - Laboratory Manual' respectively.

In both cases, protein samples were applied directly on to the surface of the gel or cellulose. With gel filtration, settling of the gel created dead space between the gel surface and the upper end-piece. If the sample was

Figure 2.2. Calibration curve for protein estimation by the Folin-Ciocalteu Reaction.



The absorbance of protein standards and unknown samples was taken at 700nm against water blanks after reaction with copper alkali solution and Folin-Ciocalteu's phenolic reagent.

pumped onto the column with the dead space occupied by buffer, dilution of the sample and broadening of eluted peaks would occur. Instead, the buffer above the gel was removed, the protein sample applied, without disturbing the gel surface and allowed to run into the column. A layer of buffer was then replaced. An air gap was usually present between this buffer and the top of the tube, which was sealed with a rubber bung. Running buffer was introduced into the column through the bung, by a needle and directed on to the side of the tube by a short piece of plastic tubing. The same method was used for ion-exchange chromatography as often the total length of the tube was not full of cellulose. There was usually an air gap between the cellulose surface and the rubber bung.

Wright's columns were used throughout the preparation of LMF and apart from the rubber bung replacing the upper end piece their fittings were also used. Operating pressure was maintained using a variable peristaltic pump. Fractions were collected on a fraction collector, which was adjustable for the collection time of each fraction. For ion-exchange chromatography both step-wise and gradient elutions were employed. In the former, the buffer was completely changed for one of a higher concentration. In the latter, the buffer strength was gradually increased using a gradient maker.

The eluted buffer concentration was monitored during ion-exchange chromatography by measuring its conductivity. The conductivity did not give a direct reading of molarity,

but the buffer concentration of column eluates could be estimated by comparison to the conductivity of standard buffer solutions of various concentrations. The conductivity of the eluate was taken during desalting on Sephadex G-15 to identify salt-free fractions, which were those with the same conductivity as the buffer.

As the buffer for most columns was the volatile ammonium acetate (NH_4Ac), protein could be recovered from solution by freeze drying. Protein containing fractions were pooled in large volume round bottom flasks. These were frozen at -18°C . By occasionally altering their position the solutions froze round the flasks. They were sealed into the chamber of a pre-cycled freeze drier and the pressure reduced to 0.1 Torr (13.3Pa). After 24-48 hours the lyophilized proteins were collected from the sides of the flasks. Not all the ammonium acetate evaporated, but the buffer could be completely removed by dissolving the protein in distilled water and repeating the freeze drying until the weight of lyophilized protein was constant. The freeze dried preparations were weighed and stored at -18°C . Electrophoretic mobilities were found for all fractions. Lipolytic activity was found for some and especially for those with the LMF like mobility. Lipolytic dose-response relationships were found only for the purer samples.

2.2.2.2. Preparation of crude LMF

a) Precipitation of Residue 4. The isolation of LMF differed only slightly from that described by Trygstad (1968a), up to the isolation of residue 4. He removed salts

from residue 4 by dialysis but if, as he reported, the molecular weight of LMF is 2,100 (Trygstad 1968b), LMF should be lost through the dialysis membrane. To prevent any loss of peptide, desalting was carried out by Sephadex G-15 gel filtration, which excludes proteins above 1,500 mol.wt. After desalting, crude LMF was further purified by a number of different procedures in attempts to improve the method. Three of these will be described in detail. All procedures were carried out at 4°C. The isolation of crude LMF is illustrated in figure 2.3.

100g of deep frozen porcine pituitaries were homogenized in 0.1M Na_2HPO_4 pH8.5 for 2 minutes in an MSE 'Atomix' mixer. The homogenate was extracted in 400ml of this buffer (4ml per g of pituitary) for 4 hours. The extraction mixture was maintained at pH8.5 with 1.0M NaOH. The homogenate was centrifuged at 20,000g for 20 minutes. Supernatant 1 was decanted and the residue washed twice with 100ml of 0.1M Na_2HPO_4 pH8.5. The resulting supernatants were added to supernatant 1. Residue 1 was lyophilized.

1.0M HCl was added to supernatant 1 until pH5.5 was reached. After extraction for 10 minutes at this pH the mixture was centrifuged at 20,000g for 20 minutes. Supernatant 2 was decanted and residue 2 was lyophilized. Filtered acetone was added dropwise to supernatant 2 until it was 55% (V/V) saturated (1,222ml of acetone per 1,000ml of supernatant 2). After a further centrifugation at 20,000g for 20 minutes, supernatant 3 was decanted and

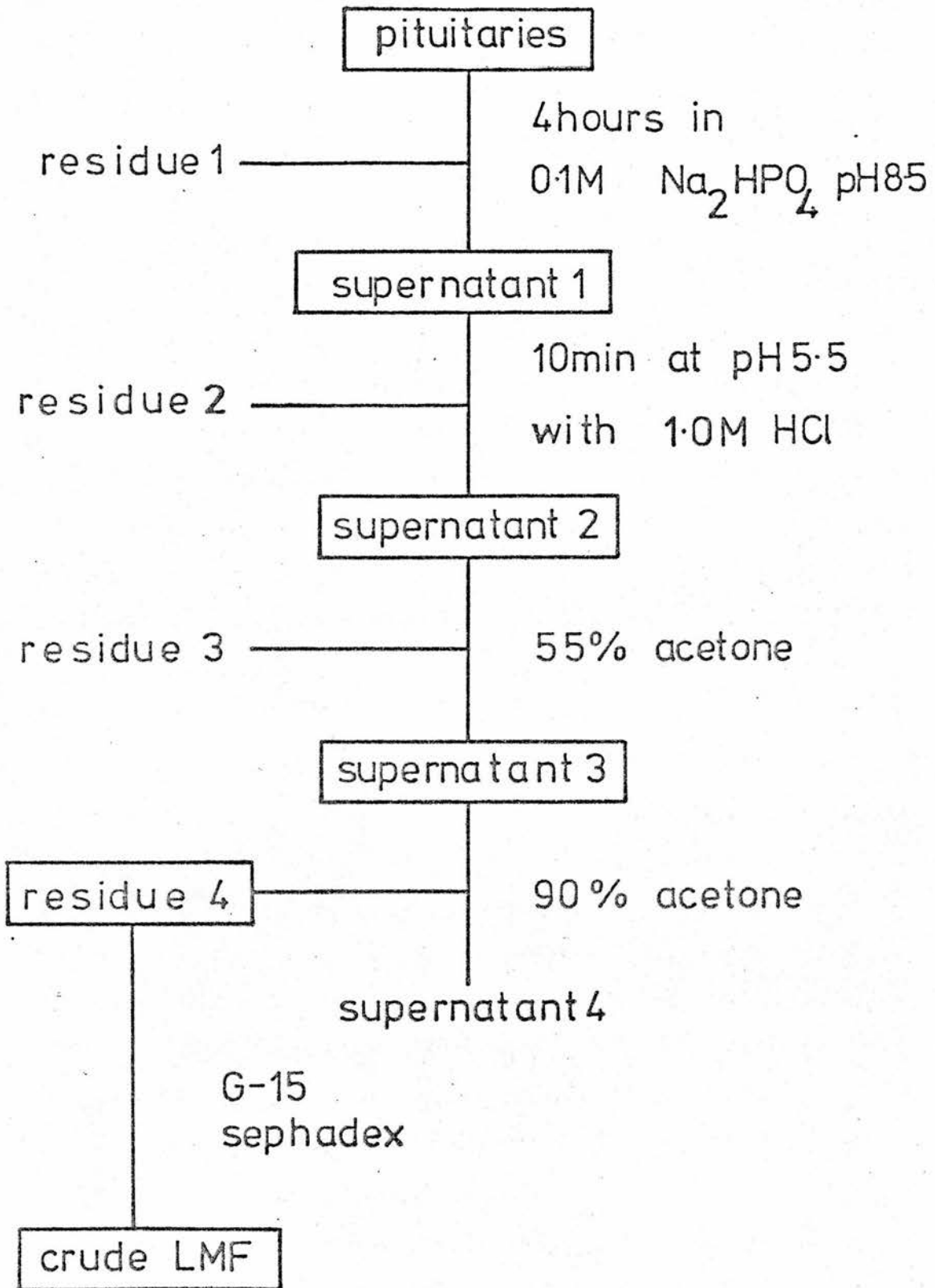


Figure 2.3. The preparation of crude LMF from porcine pituitaries.

residue 3 was lyophilized. More acetone was added dropwise to supernatant 3 until 90% (V/V) saturation was reached (3,500ml acetone per 1,000ml of supernatant 3). Residue 4 settled out overnight. Most of supernatant 4 was decanted. Centrifugation at 20,000g for 20 minutes collected residue 4 together and the remaining supernatant was decanted.

b) Desalting of residue 4. Approximately 10g (wet weight) of residue 4 came from 100g of pituitaries. There was a high proportion of salt in the residue and this was removed by gel filtration. Residue 4 was dissolved in 15ml of 0.01M ammonium acetate (NH_4Ac) pH5.4. Insoluble material was removed by centrifugation at 35,000g for 10 minutes, followed by filtration through Whatman grade 1 filter paper. The sediment was washed twice with 5ml of 0.01M NH_4Ac pH5.4 and after further centrifugation and filtration the supernatants were combined.

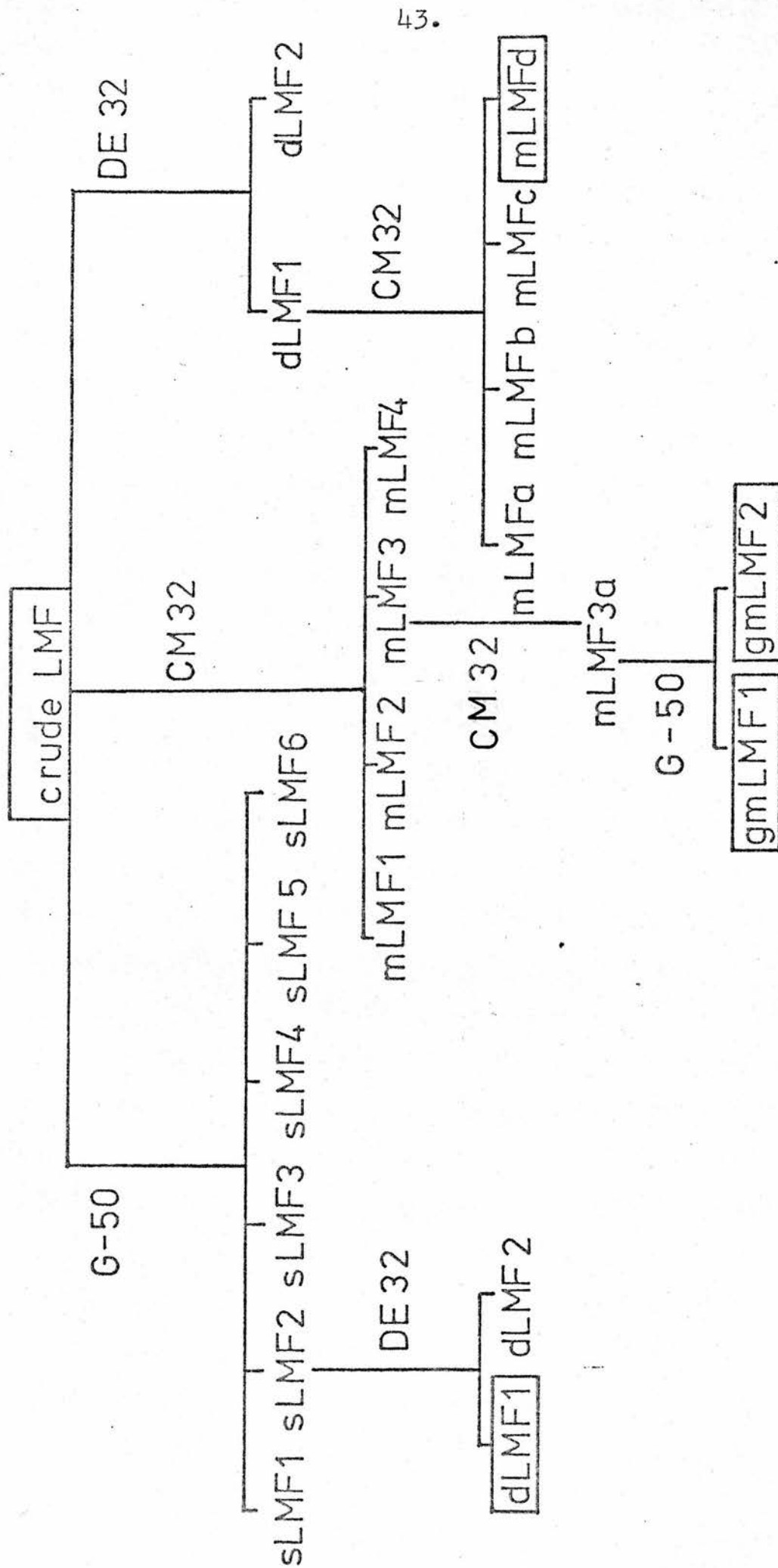
A Sephadex G-15 column ($4\text{cm}^2 \times 55\text{cm}$) was equilibrated with 0.01M NH_4Ac pH5.4. Because the volume of residue 4 (25ml) was $>10\%$ of the column volume, it was desalted in separate batches of 12.5ml each. The samples were applied in turn to the column and buffer was pumped through at a flow rate of 18ml/h. 6ml fractions were collected. Salt free protein fractions from both fractionations, identified by low conductivity and high absorbance at 280nm, were pooled and either lyophilized or immediately chromatographed on ion-exchange cellulose. Fractions, with a high conductivity and therefore salt rich, were pooled and lyophilized.

From the crude preparation, LMF was isolated in a number of different extractions, which were labelled with Roman numerals from I to XIII. Of these, three will be described to illustrate the development of an alternative isolation method. These will be extractions V, VIII and XI. They are illustrated in figure 2.4. For the nomenclature of the various fractions see appendix 1.

2.2.2.3. Extraction V of crude LMF

a) Sephadex G-50 1.0g of freeze dried crude LMF, originally from 200g of pituitaries, was dissolved in 2.0ml of 0.1M NH_4Ac pH7.0 and applied to a Sephadex G-50 column ($8\text{cm}^2 \times 60\text{cm}$) equilibrated with the same buffer. The flow rate was 20ml/h and 5ml fractions were taken. The lipolytic activity of 1.0 μg of material from every tenth fraction from tubes 45 to 115 was assayed in vitro. The following fractions were pooled and lyophilized, 35-60, 61-70, 71-80, 81-90, 91-100, 101-120 and called sLMF₁, sLMF₂ etc. The lyophilized material was assayed for lipolytic activity and electrophoresed on polyacrylamide gels.

b) Diethylaminoethyl Ion-Exchange Cellulose An $8\text{cm}^2 \times 12.5\text{cm}$ column of Whatman's microgranular DEAE ion-exchange cellulose (DE 32) was equilibrated with 0.01M NH_4Ac pH5.4. sLMF₂ from G-50 gel filtration of crude LMF was dissolved in 5.0ml of this buffer. pH and conductivity were checked to ensure they remained constant. The solution was applied to the column which was eluted with a flow rate of 60ml/h. 5ml fractions were collected.



V

VIII

XI

Figure 2.4. The purification of IMF from crude LMF by 3 different procedures (Extractions V, VIII & XI). Fractions in boxes represent the preparations which contain LMF.

Unabsorbed protein came through the column in a breakthrough peak. When the absorbance at 280nm of the eluate fell towards zero following this peak, the buffer was replaced by 0.2M NH_4Ac pH5.4. Later the column was cleaned with a mixture of 0.01M NH_4Ac pH5.4 plus 1.0M NaCl.

Protein containing peaks were pooled and lyophilized and called dLMF₁, dLMF₂, etc.

2.2.2.4. Extraction VIII of crude LMF

a) Carboxy Methyl Ion-Exchange Cellulose. The salt free protein peaks from both gel filtrations of residue 4 on Sephadex G-15 were combined (62ml) and pumped onto a 2cm² x 17cm column of Whatman's microgranular CM ion-exchange cellulose (CM 32). This crude LMF was derived from 167g of pituitaries. The column had been equilibrated with 0.01M NH_4Ac pH5.4, the buffer used for G-15 desalting. The flow rate was 30ml/h and 5ml fractions were collected. After the absorbance at 280nm, due to the unabsorbed proteins of the breakthrough peak, had fallen towards zero a buffer gradient to 0.1M NH_4Ac pH5.4 was introduced. Following the gradient the buffer was changed to 0.5M NH_4Ac pH5.4 by a step-wise increase in concentration. Protein peaks were pooled and lyophilized and called mLMF₁, mLMF₂ etc.

After examination of the purity of the fractions by electrophoresis, mLMF₃ was run again on the same CM 32 column. The column was equilibrated and the sample dissolved (2ml) in 0.05M NH_4Ac pH5.4. A gradient to 0.1M NH_4Ac pH5.4 was established and the main protein peak on elution was pooled and lyophilized (mLMF_{3a}).

b) Cleaning on sephadex G-50. The lyophilized protein from rechromatography on CM 32 was run on a small G-50 gel filtration column ($1.8\text{cm}^2 \times 13.5\text{cm}$) in 0.2M acetic acid. Dissolved in 1ml of buffer the peptide solution was run on the column at a flow rate of 60ml/h with 1ml fractions being collected. The main peak and a trailing edge were pooled separately, lyophilized and called gmLMF₁ and gmLMF₂.

2.2.2.5. Extraction XI of crude LMF

a) Diethylaminoethyl Ion-Exchange Cellulose. A $2\text{cm}^2 \times 10\text{cm}$ column of DE 32 was equilibrated with 0.01M NH₄Ac pH5.4. A solution of crude LMF, from 130g of pituitaries, had a volume of 80ml after desalting on Sephadex G-15 in 0.01M NH₄Ac pH5.4. This solution was pumped onto the DE 32 column at 40ml/h with 6.7ml fractions being collected. The column was eluted with starting buffer until the absorbance of the eluate at 280nm fell towards zero. A step-wise change in buffer was made to 0.5M NH₄Ac pH5.4. The unabsorbed protein containing fractions of the breakthrough peak (dLMF₁) were pooled for further fractionation and the fractions of the later peak (dLMF₂) were pooled for lyophilization.

b) Carboxy Methyl Ion-Exchange cellulose. A $2\text{cm}^2 \times 17\text{cm}$ column of CM 32 was equilibrated with 0.01M NH₄Ac pH.5.4. The pooled fractions of unabsorbed protein (dLMF₁) from the DE 32 column (described above) was pumped on to the column at 36ml/h. 3.6ml fractions were collected. The sample volume was 100ml. The column was eluted with starting buffer until the absorbance of the eluate fell towards zero. A gradient of increasing buffer strength to 0.1M NH₄Ac pH5.4

was applied to the column. On completion of the gradient a step-wise increase in running buffer concentration was made to 0.5M NH_4Ac pH5.4 to clean the cellulose. Protein peaks were pooled, lyophilized and called mLMF_a , mLMF_b , mLMF_c and mLMF_d .

2.2.3. Estimation of Molecular Weight of LMF on Sephadex G-50

As the separation by gel filtration of protein mixtures is based on the size of the protein molecules, the method can be adapted to estimate the molecular weight of an unknown peptide. (Andrews, 1964). A column of a suitable grade of sephadex is first calibrated with proteins of known molecular weight. The volume of eluate in which they are eluted (V_E) is plotted against the log of their molecular weight and from the elution volume of the unknown sample, its molecular weight may be estimated.

a) Molecular Weight Estimation on a Calibrated Column of Sephadex G-50. A $2\text{cm}^2 \times 90\text{cm}$ column of Sephadex G-50 was equilibrated with 1.0M acetic acid. The column was calibrated with glucagon, lysozyme and chymotrypsinogen. 1-2mg of two of these were dissolved in 1.8ml of 1.0M acetic acid and applied to the column. Chymotrypsinogen can form aggregates but at the concentration used (1mg/ml) is almost entirely in the monomeric form (Andrews 1964). The column was eluted with a flow rate of 6ml/h and 3ml fractions were taken. Over a number of runs the elution volume (V_E) of each standard protein was determined at least twice. V_E was measured to the nearest ml by extrapolation of the slopes of each peak. 5mg of mLMF_d from extraction XI, was

dissolved in 1.8ml of buffer, applied to the column and its elution volume found under identical conditions to the standards. The molecular weight was estimated from comparison to the calibration curve of V_E of the standards against their molecular weight. LMF was recovered by freeze drying.

b) Molecular Weight of ^{125}I -labelled LMF on a G-50 column.

The molecular weight of a sample of LMF labelled with ^{125}I (Section 3.2.2.1) could be estimated from a direct comparison against proteins run on a sephadex column at the same time. 2.0mg of bovine insulin, 2.0mg of lysozyme and 2.0mg of carbonic anhydrase were dissolved in 0.8ml of 1.0M acetic acid. 0.2ml of ^{125}I -labelled LMF (working label) was added. This mixture was applied to a 2 cm² x 87cm column of G-50 equilibrated with 1.0M acetic acid. The flow rate was 9ml/h and 3ml fractions were collected. The fractions were counted for γ -radiation in a γ -spectrometer as well as having their absorbance at 280nm taken. The relative position of the peak of radioactivity to the protein peaks gave an indication of the molecular weight of the labelled peptide. The elution positions of insulin and lysozyme were confirmed by running them separately under identical conditions to those used for the main fractionation. For these runs 3.0mg of the proteins were dissolved in 0.5ml of buffer and eluted as before.

The molecular weights of the standard proteins and peptides is shown in table 2.1.

2.3. Results

2.3.1. Identification of Porcine Lipid Mobilizing Factor

2.3.1.1. In Vivo Assay of Adipokinetic Activity

The increase in serum free fatty acids, in rabbits, 90 minutes after injection of 0.1mg of protein from fractions from the Sephadex G-50 gel filtration of crude LMF is shown in table 2.2. These fractions were from extraction I and resembled sLMF₁, sLMF₂ etc. of extraction V. Fractions 1,2,3 and 6 had little or no effect on the release of free fatty acids into the circulation. Fraction 5 had the highest activity while 4 was also active.

2.3.1.2. In Vitro Assay of Lipolytic Activity

Assessment of the validity of the rabbit fat cell in vitro assay for lipolytic activity is described in section 4.3.2. When this assay was utilized to identify lipolytic fractions during the isolation and purification of LMF, the results are described with the relative fractionation. Dose-response relationships, which can be compared to an ACTH standard dose-response curve (figure 4.1), were investigated for a number of purified LMF fractions. These results are in section 2.3.2. which describes the purification of the fractions.

2.3.1.3. Polyacrylamide Disc Gel Electrophoresis

Electrophoresis at pH8.9 of a number of pituitary hormones and peptides is shown in plate 2.1 as an example of the method. The ovine hormones GH, PRL, LH, FSH and TSH, showed a varying degree of purity. FSH was impure while TSH, PRL and LH appeared to be homogeneous. GH did not show a heavily stained band, probably because the sample applied

Table 2.1 Molecular Weights of Standard Proteins

Protein or Peptide	Molecular Weight	Reference
Carbonic anhydrase	29,000	Weber & Osborn(1969)
Chymotrypsinogen	25,700	Weber & Osborn(1969)
Lysozyme	14,300	Weber & Osborn(1969)
Insulin	5,700	Carnegie(1965)
Glucagon	3,500	Andrews(1964)

These proteins were used to calibrate Sephadex G-50 columns for the molecular weight determination of LMF.

Table 2.2 Adipokinetic Activity of sLMF-I Fractions

Fractions	Increase in serum FFA μmol/ml
sLMF-I ₁	0.08
sLMF-I ₂	0
sLMF-I ₃	0.31
sLMF-I ₄	1.82
sLMF-I ₅	2.45
sLMF-I ₆	0.23

0.1mg of fractions from the G-50 filtration of crude LMF (extraction I) were made up to 5.0ml in 0.9%(W/V) saline and injected subcutaneously between the shoulder blades of rabbits. Blood was taken from each rabbit prior to the injection and 90 minutes afterwards. The increase in serum free fatty acids 90 minutes after injection was measured.

Plate 2.1. 50 μ g of proteins run at 4mA per gel for 1 hour in 7%(W/V) gels at pH8.9, stained in amido black in 10%(V/V) Acetic acid and destained in 10% acetic acid.

Plate 2.2. 50 μ g protein run at 8mA per gel for 2 hours in 7%(W/V) gels at pH7.0 containing 0.1%(W/V) sodium dodecyl sulphate. Stained and destained as above.

Plate 2.1. Polyacrylamide Disc Gel Electrophoresis of Pituitary Hormones and Peptides

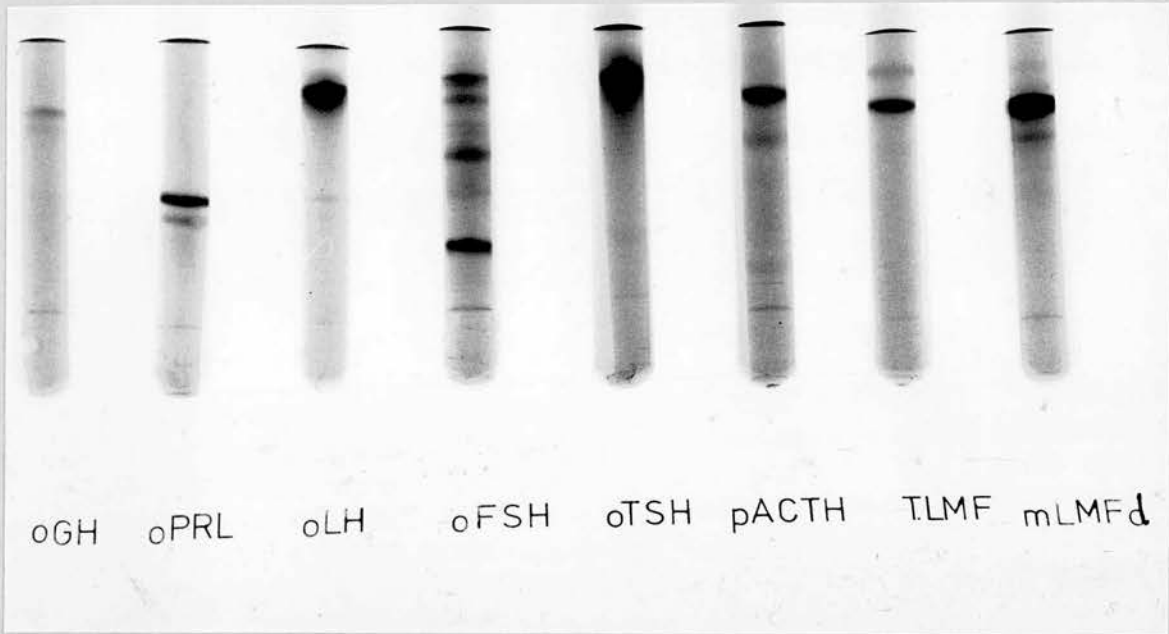
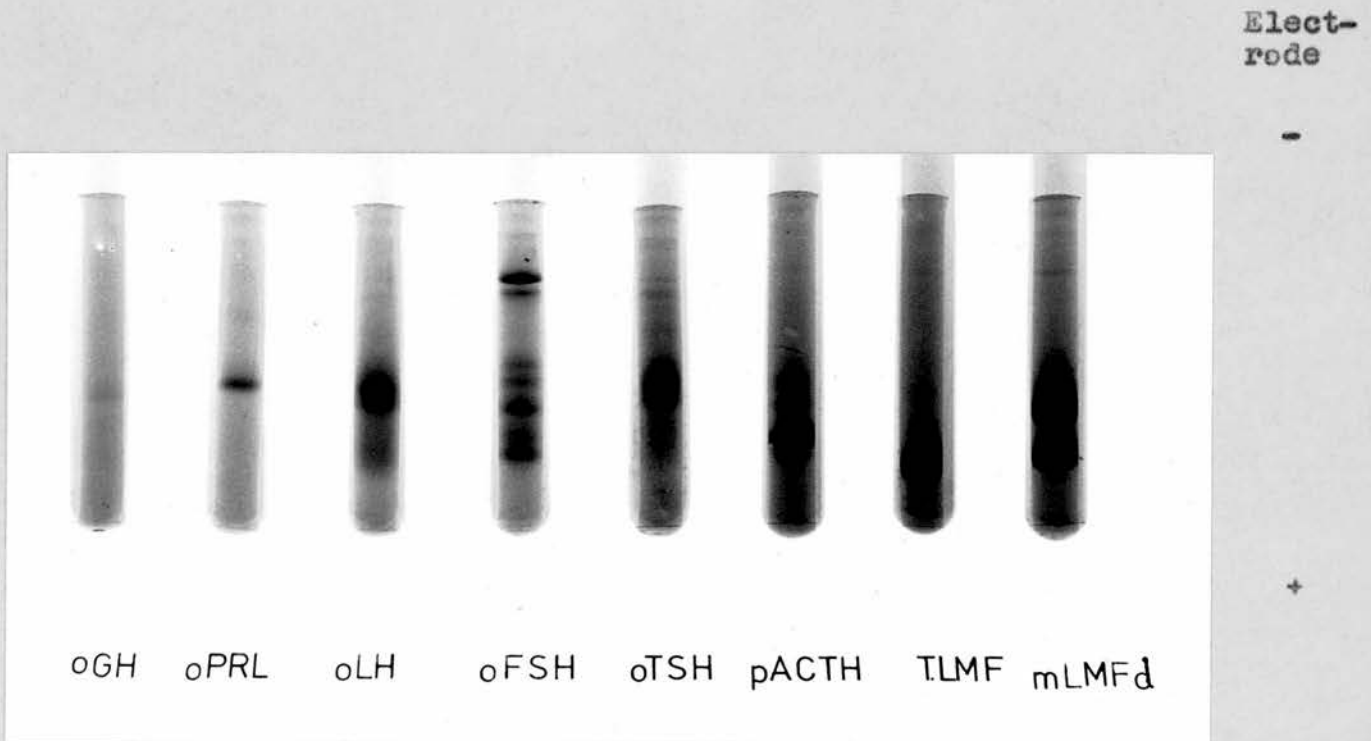


Plate 2.2. Pituitary Hormones & Peptides Electrophoresed on S.D.S. Polyacrylamide Gels



was too small. Porcine ACTH gave a band with similar mobility to both Trygstad's porcine LMF (T.LMF) and one of my purer preparation, mLMFd. This band had a mobility of 0.28. Examples of electrophoresis of fractions during the isolation of LMF are given in the relevant sections. Stained protein bands with a mobility of approximately 0.28 were taken to be LMF. During isolation the presence of LMF in a fraction was indicated by such a protein band on electrophoresis.

2.3.1.4. Electrophoresis on Sodium Dodecyl Sulphate Polyacrylamide Gel

The pituitary hormones and peptides, whose electrophoresis was described in the previous section, were also run on SDS polyacrylamide gels (Plate 2.2). The differing purities of the ovine hormones was again illustrated in that FSH had a large number of impurities of varying size while LH, PRL and TSH were more homogeneous. mLMFd had a band with a similar mobility to Trygstad's LMF, which had a higher mobility than porcine ACTH. mLMFd also had a more diffuse second band with lower mobility.

2.3.1.5. Protein estimation

Comparison of Absorbance at 280nm and Folin-Ciocalteu's Method. A standard curve for Folin-Ciocalteu's method of protein estimation was shown in figure 2.2. From this the protein content of two solutions of LMF preparations, mLMF_{3a}, gmLMF₂, from extraction VIII were found. They contained 156 and 118µg/ml respectively. The absorbance at 280nm of 10 times more concentrated solutions were 1.20



and 1.60 respectively, which showed that the absorbance at 280nm gave a rough estimate of protein concentration.

The protein content of samples during isolation was routinely assayed by absorbance at 280nm but for in vitro assays and radioimmunoassay the concentration was checked by Folin-Ciocalteu's method.

2.3.2. Isolation of Porcine Lipid Mobilizing Factor

2.3.2.1. Preparation of crude LMF

a) Precipitation of Residue 4. The following results from the precipitation of LMF came from extraction IX in which the wet weight of the deep frozen porcine pituitaries extracted was 100g (approximately 400 pituitary glands). The yields of lyophilized residues 1, 2 and 3 were 4.5g, 2.7g and 10.4g respectively. From the absorbance at 280nm the yield of protein in residue 4 was 350mg.

Residues 1-3 of this extraction were assayed for lipolytic activity in the in vitro assay (Table 2.3). Residue 4 had been desalted and fractionated on CM32 ion-exchange cellulose. The fraction resembling $mLMF_3$ of extraction VIII, described below, contained most LMF and was called $mLMF-IX_3$. This was included in the in vitro assay. At a level of 1.0 μ g in the incubation volume of 1.1ml, residues 1-3 increased the release of glycerol by 0.36, 0.42 and 0.36 μ mol/g fat/hour respectively. The same amount of $mLMF-IX_3$ increased lipolysis by 1.54 μ mol/g fat/hour.

b) Desalting of residue 4. An example of the desalting of crude LMF on Sephadex G-15 is shown in figure 2.8a as the first stage of extraction VIII. Half of residue 4 in

Table 2.3. Lipolytic Activity of Fractions from Extraction IX of LMF

Fraction	Glycerol Production rate $\mu\text{mol/g fat/h}$
Res. 1	1.58 \pm 0.08''
Res. 2	1.64 \pm 0.05'''
Res. 3	1.58 \pm 0.04''''
mLMF-IX ₃	2.76 \pm 0.29'''''
Basal	1.22 \pm 0.07

1.0 μg of the lyophilized fractions were incubated with 1.0ml of rabbit fat cell suspension for 2 hours in quadruplicate. The glycerol production rates were compared to basal rate by Student's 't' test. ''''0.001>P, ''''0.01>P>0.001, ''0.02>P>0.01, (n=4).

12.5ml gave a salt free peak and a second peak of 280nm absorbing material, which corresponded to the high salt containing fractions. On lyophilization the latter peak weighed about 90mg per 100g of pituitaries. The yield of salt free crude LMF, was about 350mg of protein per 100g of pituitaries.

2.3.2.2. Extraction V of crude LMF

a) Sephadex G-50. An assay of the lipolytic activity of every tenth fraction from G-50 fractionation of 1.0g of crude LMF revealed that most were able to stimulate the release of glycerol from rabbit adipocytes (Figure 2.5). Fractions 105 and 115 were the exceptions. After lyophilization, the pooled fractions (sLMF₁₋₅) were found to have approximately equivalent lipolytic activities (Table 2.4). By polyacrylamide disc gel electrophoresis fractions sLMF₂ and sLMF₃ were shown to contain peptides with an LMF like mobility of 0.28 (Plate 2.3). The yields of fractions 1-5 were 550, 169, 70.3, 31.9 and 53.7mg respectively.

b) Diethylaminoethyl Ion-Exchange Cellulose. On eluting the column with starting buffer unabsorbed protein was eluted in a breakthrough peak dLMF₁, which had a small subsidiary peak dLMF_{1a} (Figure 2.6). The main peak had an LMF like peptide band on electrophoresis (Plate 2.3). The subsidiary peak had more impurities (not illustrated). The protein desorbed by an increase in buffer concentration (dLMF₂) was of acidic nature with a high mobility on polyacrylamide (Plate 2.3). The lyophilized yields of

Figure 2.5 Extraction V: Sephadex G-50 Fractionation of crude IMF

Buffer: 0.1M NH_4Ac pH7.0 Flow Rate 20ml/h

Fraction Volume: 5ml Column: 8cm² x 60cm of G-50

Sample: 1g of crude IMF Sample volume: 2.0ml

Vertical bars represent the glycerol production rates (mean \pm SEM) when 5.0 μ g of fractions 45, 55, etc. were incubated in quadruplicate with 1.0ml of rabbit fat cell suspension. Basal rate is also included.

280nm

4.0

3.0

2.0

1.0

55.

Glycerol
Production
Rate
($\mu\text{mol}/\text{g fat}/\text{hour}$)

3

2

Ba

120

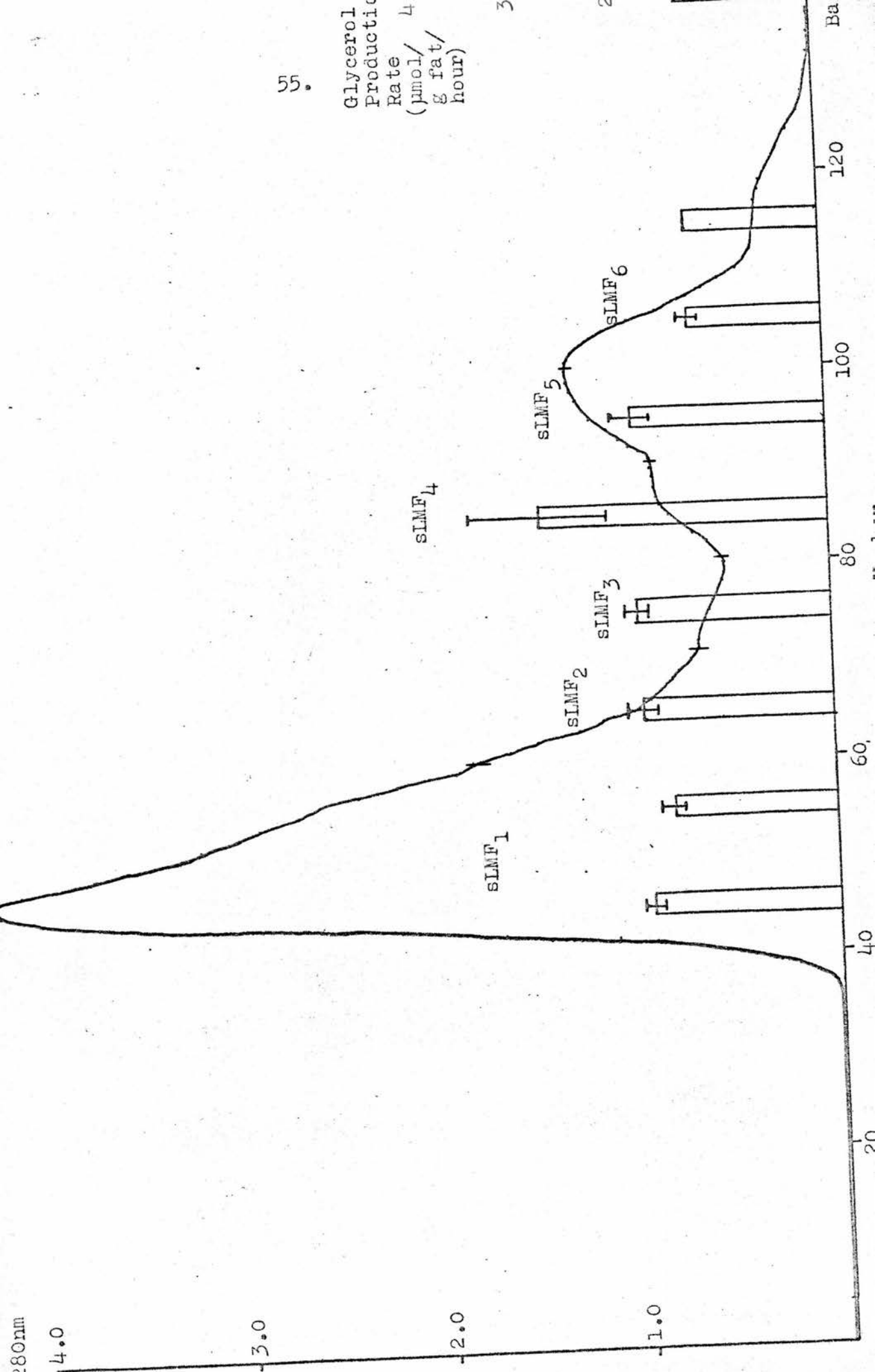
100

80

60

40

20



SLMF₄

SLMF₅

SLMF₆

SLMF₁

SLMF₂

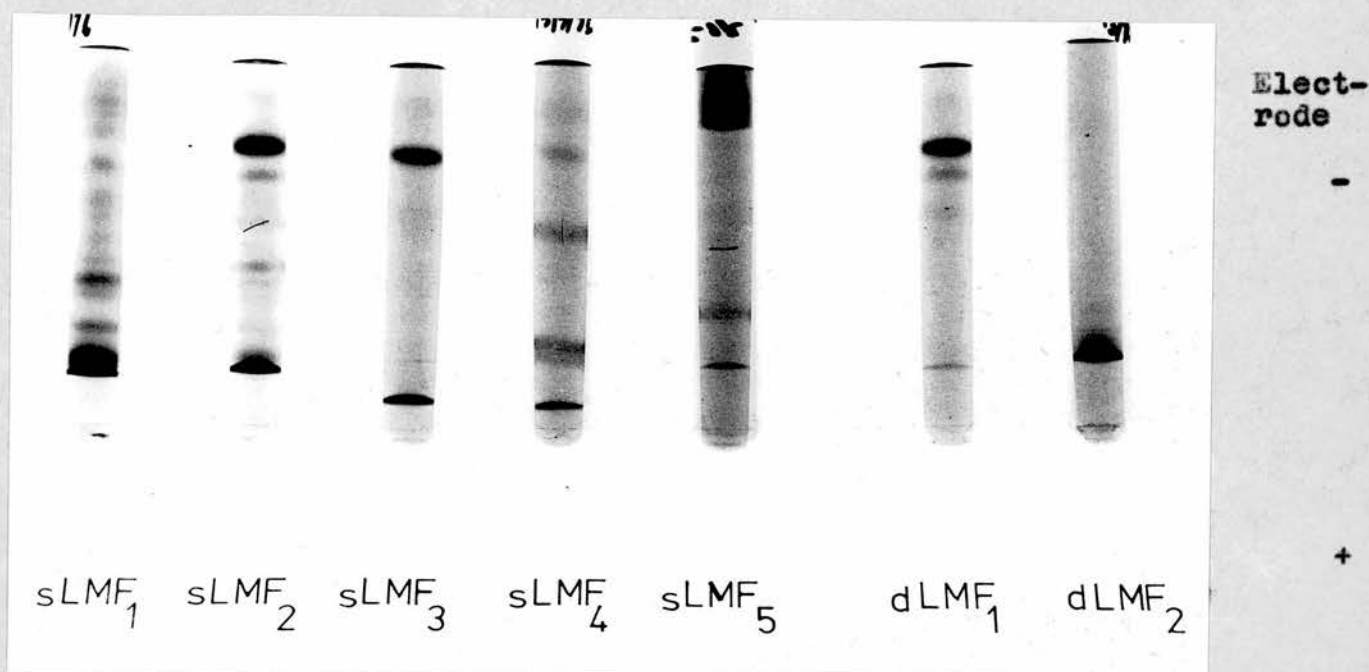
SLMF₃

Table 2.4 Lipolytic Activity of Lyophilized fractions from the Gel Filtration of crude LMF in Extraction V

Fraction	Glycerol Production rate $\mu\text{mol/g fat/h}$
SLMF ₁	4.70 \pm 0.36
SLMF ₂	3.97 \pm 0.64
SLMF ₃	3.77 \pm 0.26
SLMF ₄	4.00 \pm 0.36
SLMF ₅	4.20 \pm 0.32
Basal	0.75 \pm 0.04

5.0 μg of lyophilized fractions from Sephadex G-50 gel filtration of crude LMF from extraction V, was incubated in quadruplicate with 1.0ml of rabbit fat cell suspension and the glycerol production rate measured. Results are reported as mean \pm SEM (n=4).

Plate 2.3 Electrophoresis Gels of fractions from
Extraction V of Crude LMF



Fractions sLMF₁₋₅ were from sephadex G-50 gel filtration of crude LMF; dLMF₁ and dLMF₂ were from DEAE ion-exchange chromatography of sLMF₂. Approximately 50 μ g of each sample was electrophoresed on polyacrylamide gels at pH8.9.

Figure 2.6 Extraction V: DEAE cellulose Fractionation of sLMF₂.

Buffers: I 0.01M NH₄Ac pH5.4

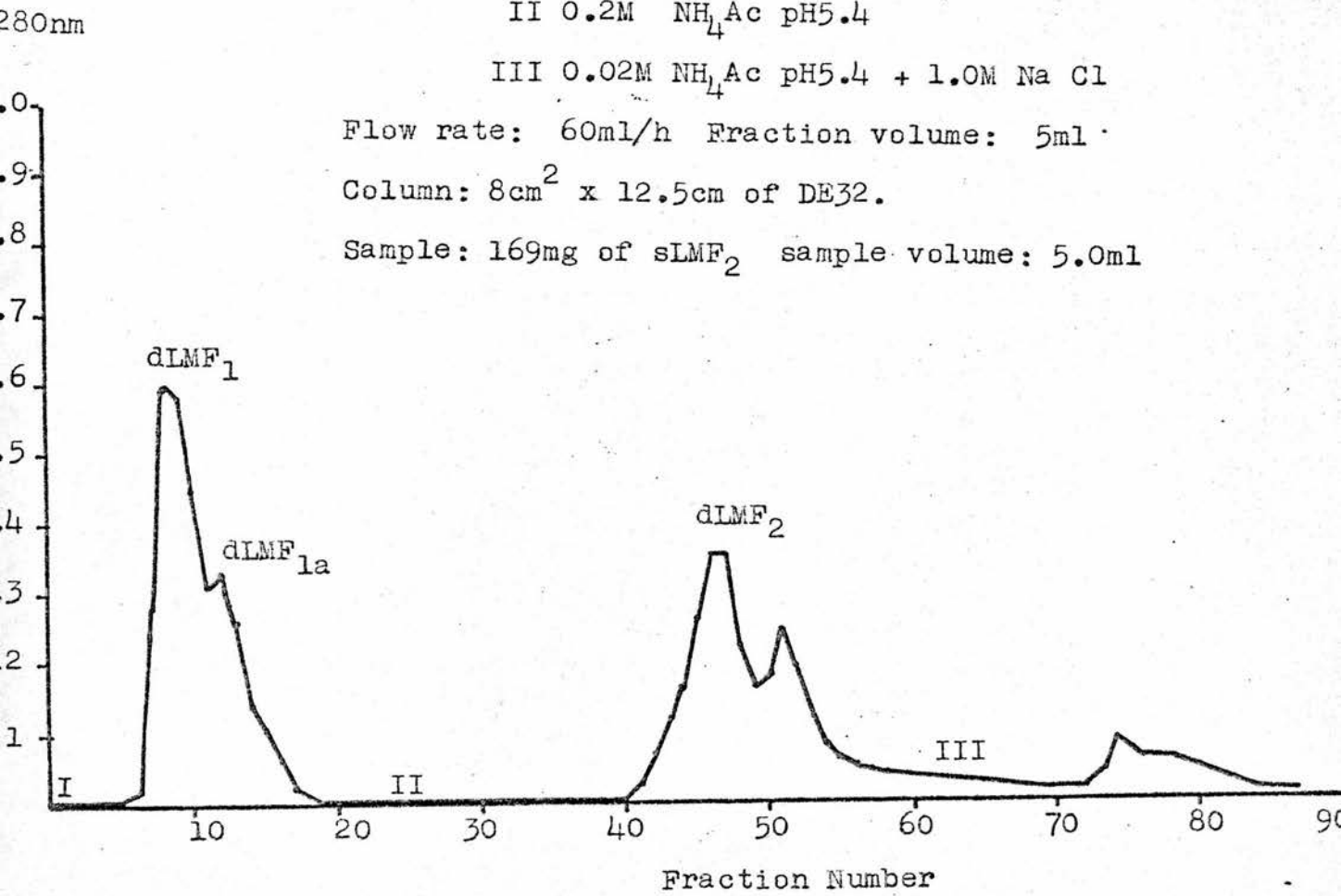
II 0.2M NH₄Ac pH5.4

III 0.02M NH₄Ac pH5.4 + 1.0M Na Cl

Flow rate: 60ml/h Fraction volume: 5ml

Column: 8cm² x 12.5cm of DE32.

Sample: 169mg of sLMF₂ sample volume: 5.0ml



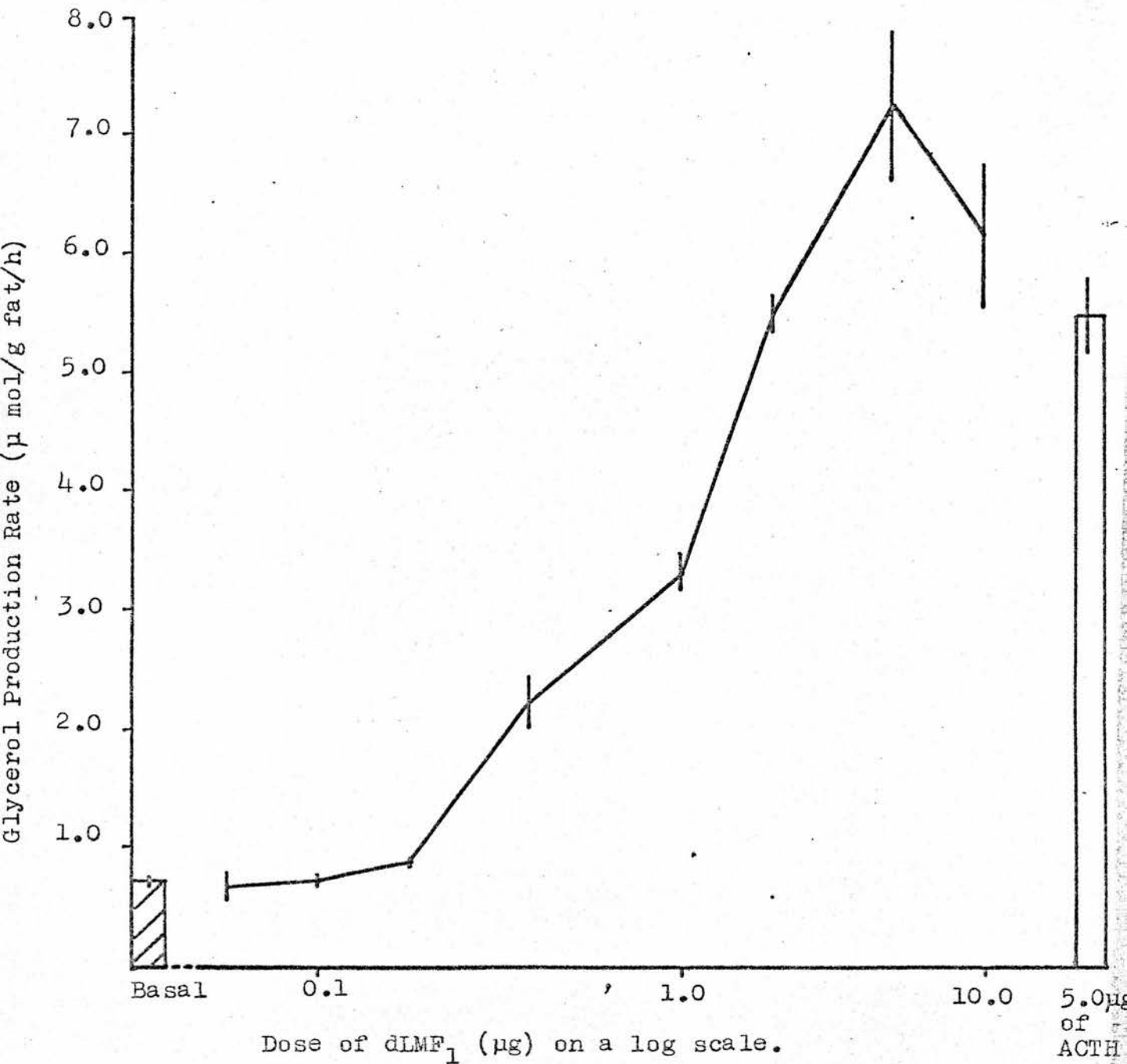


Figure 2.7. Lipolytic activity in vitro of dLMF₁ from extraction V of crude LMF

Each point represents mean \pm S.E.M. of quadruplicate samples. Basal and ACTH induced lipolysis are included.

dLMF₁, dLMF_{1a} and dLMF₂ were 16.4mg, 4.5mg and 18.4mg respectively.

c) Lipolytic Activity of dLMF₁. dLMF₁ was assayed for lipolytic activity in an 8-point dose-response curve over the range 0.05 - 10µg of peptide in 1.1ml of incubate (figure 2.7). 0.2µg of dLMF₁ was required before a significant release of glycerol was found. 5.0µg of dLMF₁ was more active than an equivalent amount of ACTH.

A fraction similar to dLMF₁ was obtained by anion exchange chromatography (on DEAE cellulose) of sLMF₃.

2.3.2.3. Extraction VIII of crude LMF.

a) Carboxy Methyl Ion-Exchange Cellulose. As can be seen from their electrophoretic mobilities on polyacrylamide gels, fractions were eluted from the CM cellulose column in order of increasing basicity (Figures 2.8b and plate 2.4).

Fraction mLMF₃ with a stained band on electrophoresis of mobility 0.28 was run again on CM cellulose (figure 2.8c).

The main peak from this fractionation, mLMF_{3a}, showed a decrease in impurities on electrophoresis. The yield of lyophilized material for these fractions were 258, 11.3, 14.70 and 22.8mg for mLMF₁, mLMF₂, mLMF₃, mLMF₄ respectively and 6.5mg for mLMF_{3a}.

b) Sephadex G-50 On running mLMF_{3a} through a G-50 column (figure 2.8d) both the main peak gmLMF₁ and trailing edge gmLMF₂ appeared to be homogeneous on polyacrylamide disc gel electrophoresis (plate 2.4). The lyophilized weights of gmLMF₁ and gmLMF₂ were 3.8mg and 1.5mg.

Figure 2.8 Extraction VIII: crude LMF after desalting on G-15(a) was fractionated on CM ion-exchange cellulose (b) with a buffer concentration gradient. The LMF containing fraction, mLMF₃, was rerun on CM ion-exchange cellulose (c) with a more concentrated initial buffer and finally was gel filtrated on Sephadex G-50 (d).

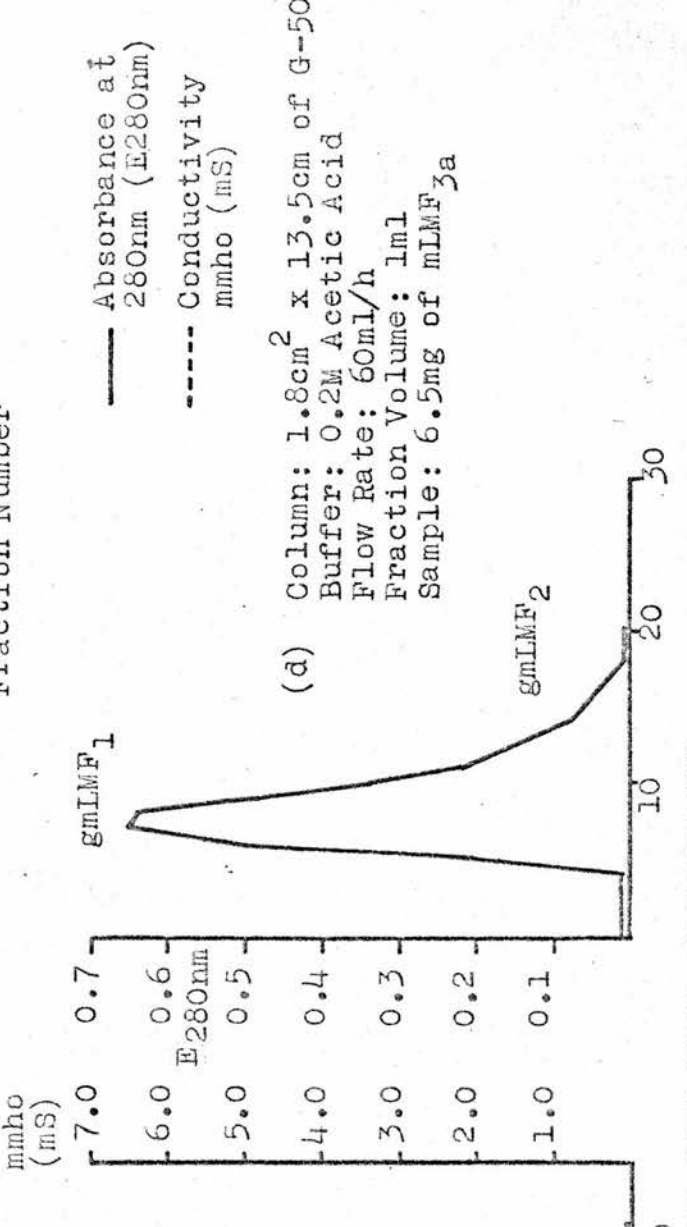
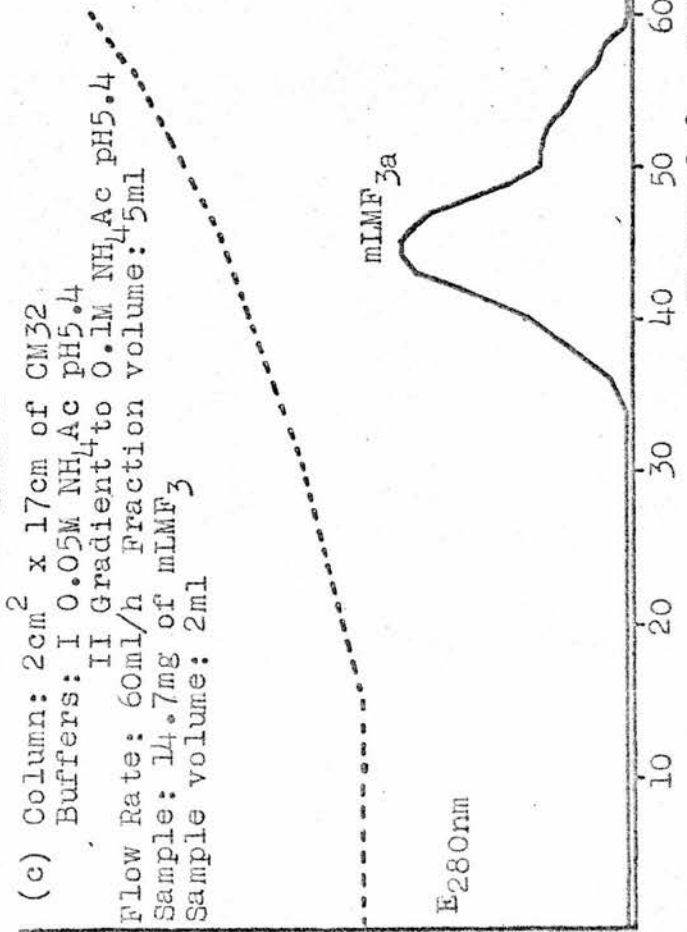
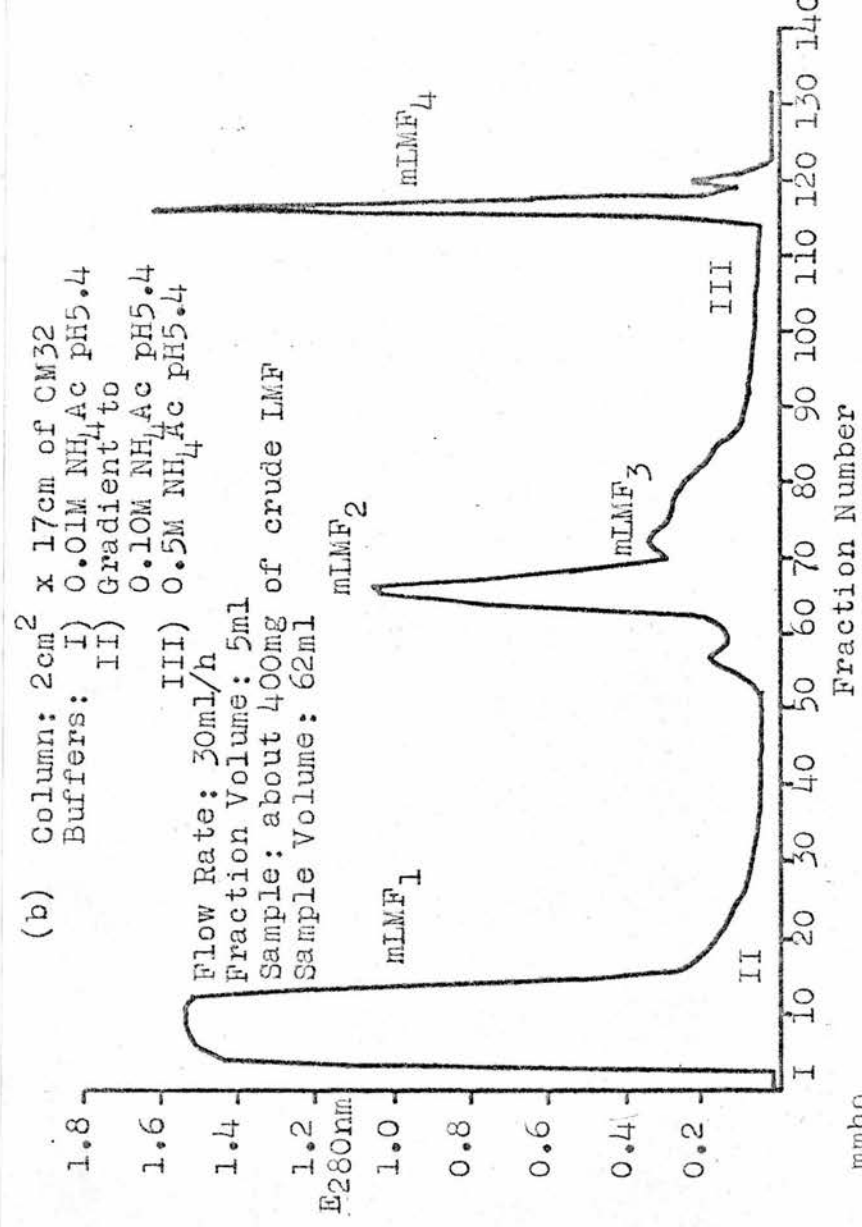
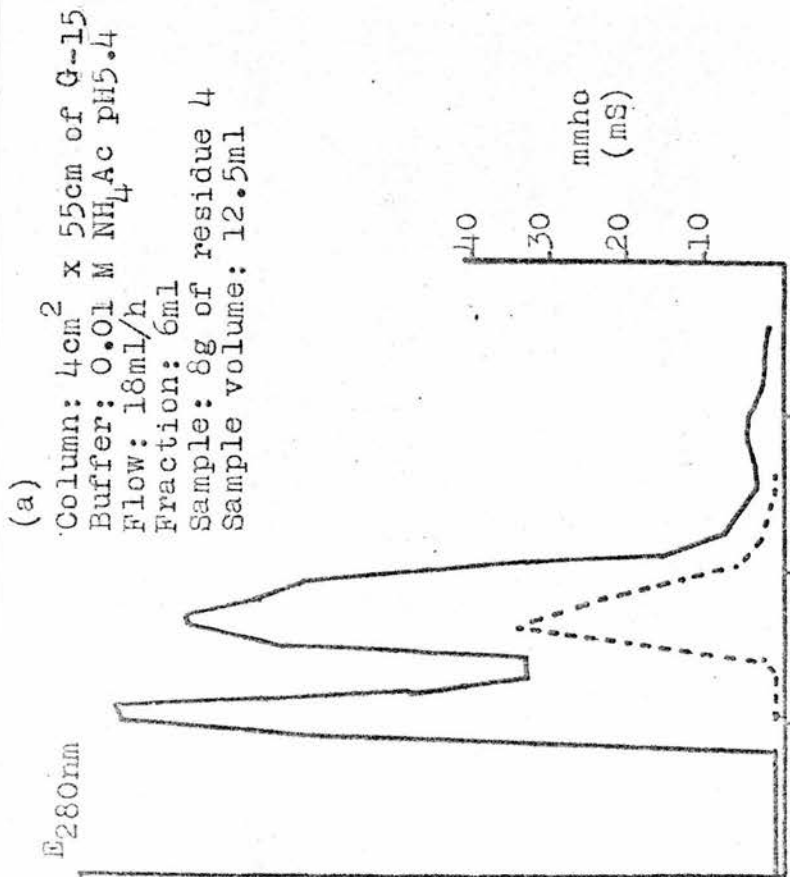
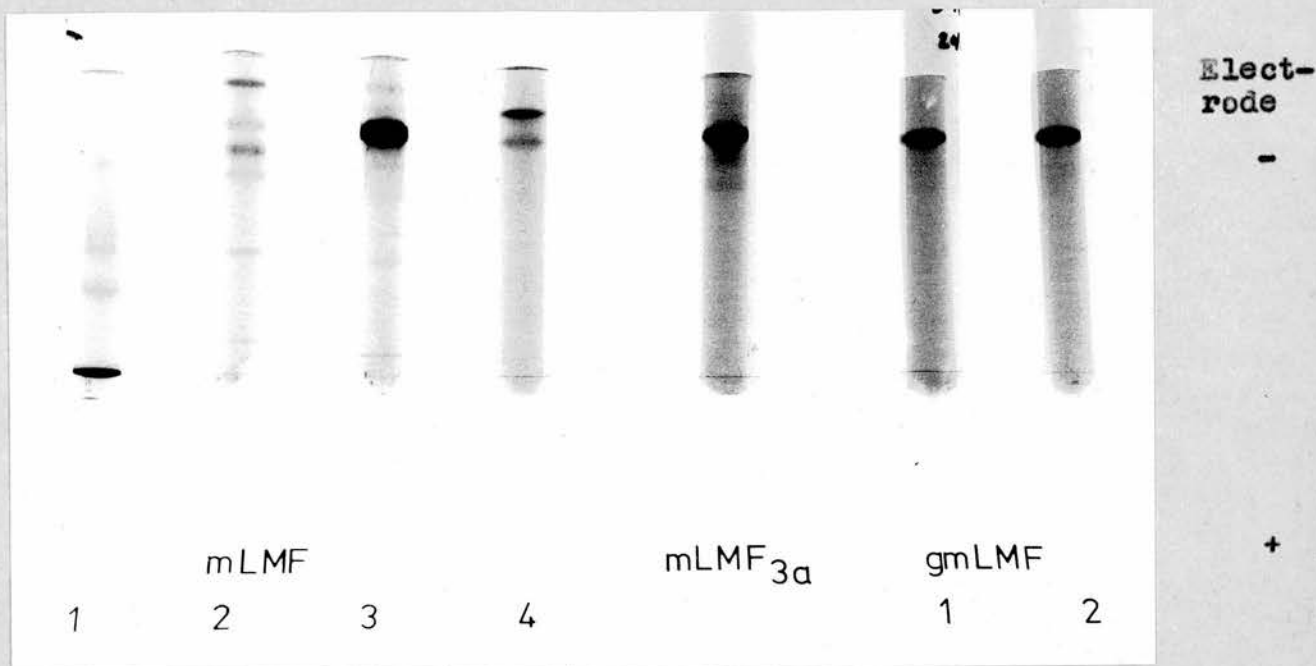


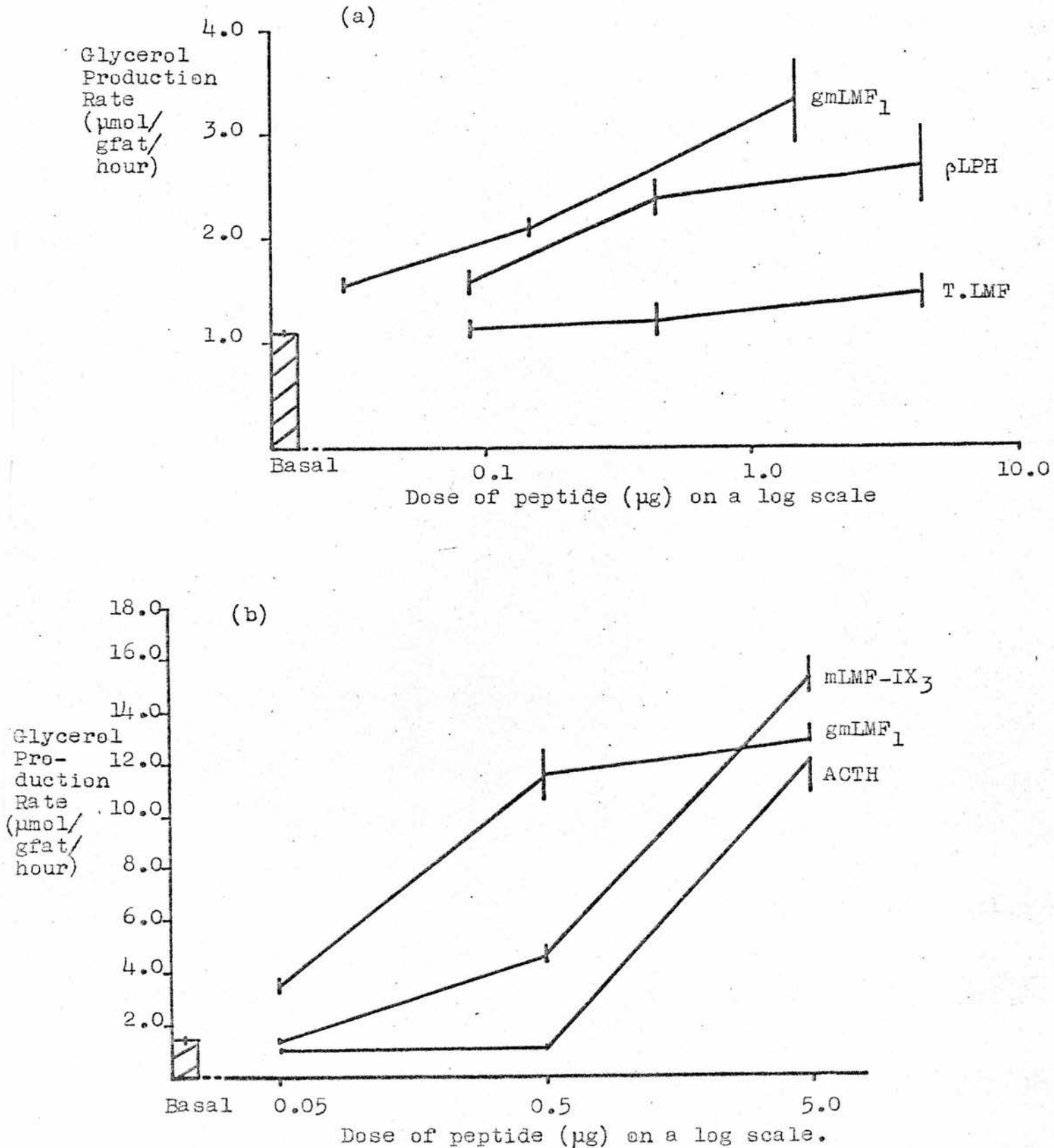
Figure 2.8 Extraction VIII of Crude LMF

Plate 2.4 Electrophoresis gels of fractions from
Extraction VIII of Crude LMF



Fractions mLMF₁₋₄ are from CM ion-exchange chromatography of crude LMF, mLMF₃ was re-chromatographed on CM to give mLMF_{3a} and gmLMF₁ and gmLMF₂ are from G-50 sephadex gel filtration of mLMF_{3a}. Approximately 50µg of each sample was electrophoresed on polyacrylamide gels at pH8.9.

Figure 2.9. Lipolytic activity, In Vitro, of gmLMF₁ from Extraction VIII, compared to other Peptides



Each point is the mean \pm SEM of quadruplicate samples, basal lipolysis is shown in both figures.

c) Lipolytic activity of gmLMF₁. In two separate dose-response experiments, gmLMF₁ was shown to stimulate lipolysis over the range of 0.05 to 5.0µg in 1.1ml of incubate (figure 2.9 a, b). This fraction was more active than porcine ρ LPH, ACTH, a similar fraction from extraction IX and a sample of Trygstad's porcine LMF (T.LMF).

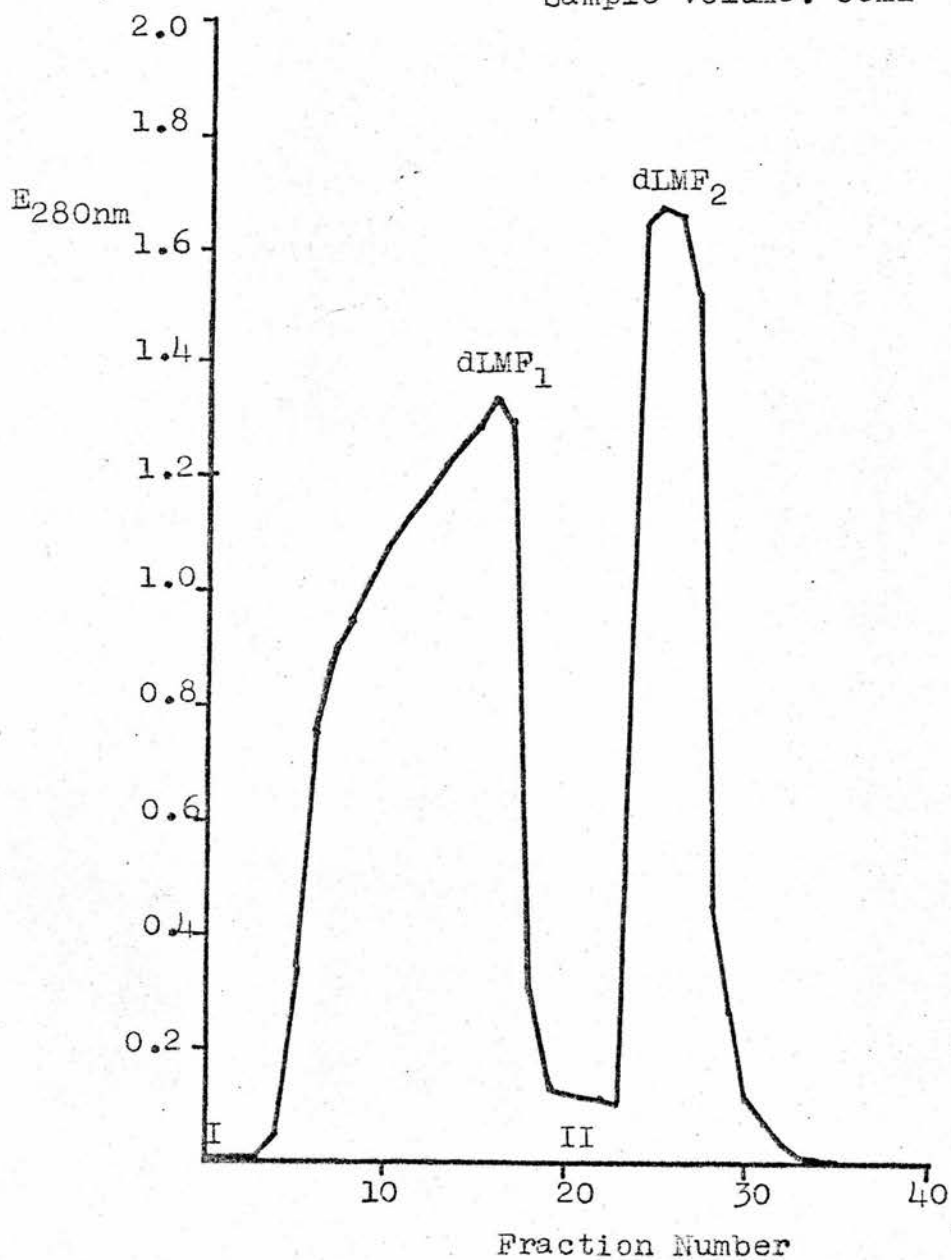
2.3.2.4. Extraction XI of Crude LMF

a) Diethylaminoethyl Ion-Exchange Chromatography. The unabsorbed protein dLMF₁ (figure 2.10) which eluted in the starting buffer was subsequently fractionated on CM cellulose without lyophilization. The remaining protein which desorbed on increasing the buffer concentration to 0.5M NH₄Ac pH5.4 (dLMF₂) was lyophilized and weighed 212mg. On electrophoresis (plate 2.5) it was shown to consist of acidic proteins and resembled the previously isolated dLMF₂ of extraction V (figure 2.6 & plate 2.3).

b) Carboxy Methyl Ion-Exchange Chromatography. On a cation exchanger, the unabsorbed protein from DEAE cellulose, gave a breakthrough peak mLMF_b with initial shoulder mLMF_a, both of which eluted with the starting buffer (figure 2.11). When the buffer strength was gradually increased, two further peaks were desorbed, mLMF_c and mLMF_d. The latter contained a high proportion of LMF. The electrophoretic mobilities of these fractions are shown in plate 2.5. The yield of lyophilized peptides were 93.5mg, 102.5mg, 8.1mg, 7.2mg for mLMF_a, mLMF_b, mLMF_c and mLMF_d respectively.

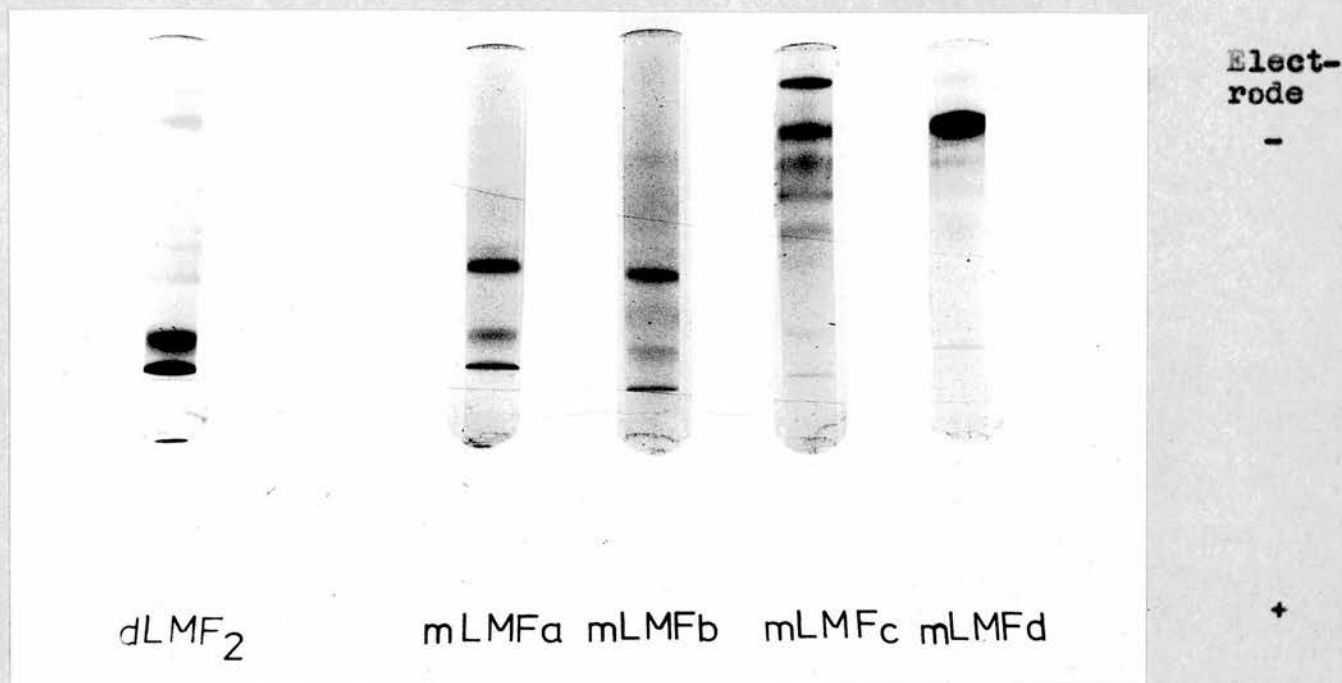
Figure 2.10 Extraction XI:
Crude LMF on DEAE Cellulose

Column: 2cm² x 10cm of DE32
 Buffers: I) 0.01M NH₄Ac pH5.4
 II) 0.5M NH₄Ac pH5.4
 Flow Rate: 40ml/h
 Fraction Volume: 6.7ml
 Sample: about 500mg of
 crude LMF
 Sample volume: 80ml



Crude LMF was pumped onto DE32 ion-exchange cellulose following desalting on sephadex G-15.

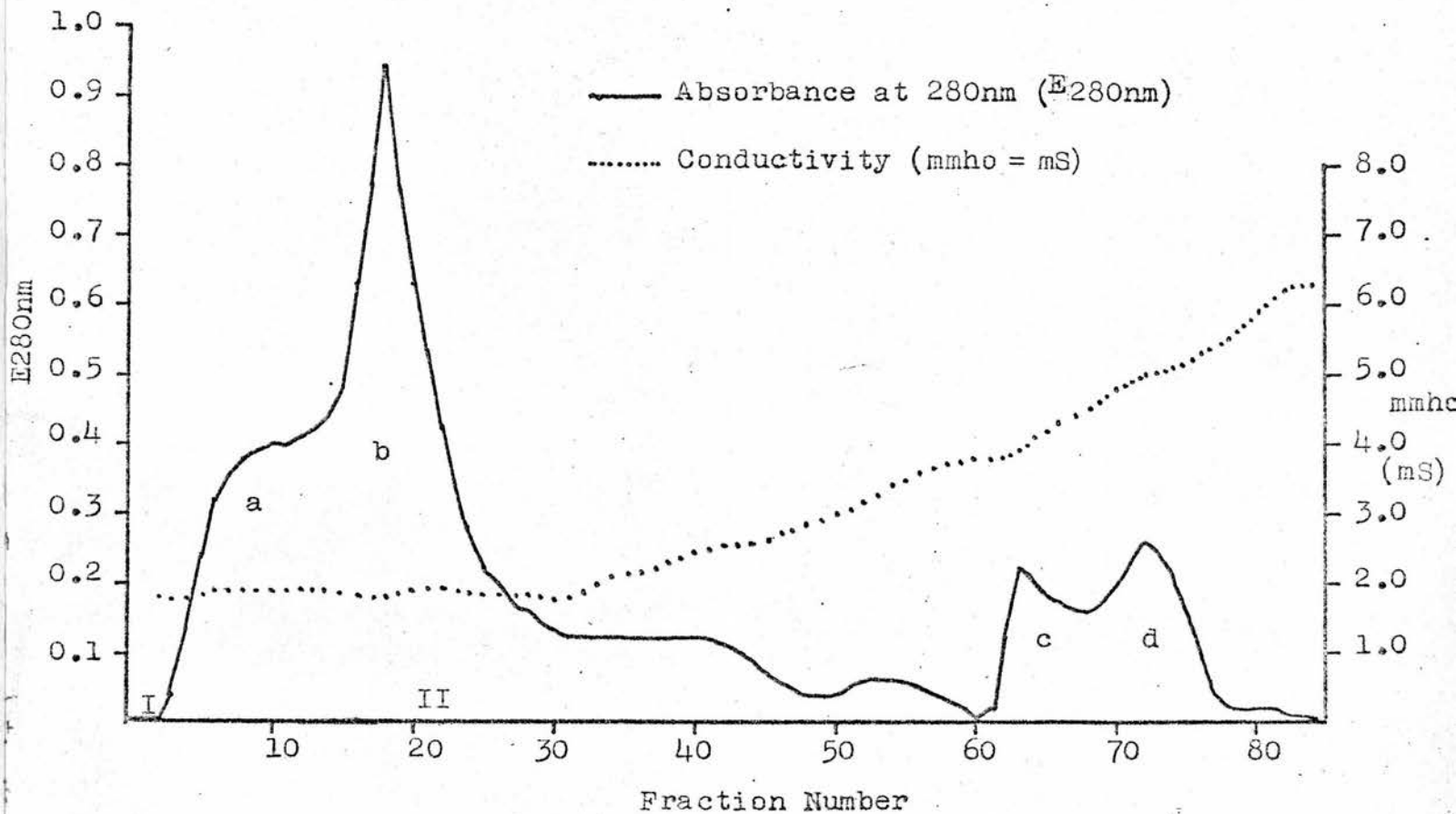
Plate 2.5. Polyacrylamide Gel Electrophoresis of fractions from Extraction XI of Crude LMF.



dLMF₂ is the protein initially absorbed when crude LMF was run on a DEAE cellulose column. The unabsorbed proteins, on CM cellulose chromatography with a buffer gradient gave mLMFa-d. Approximately 50 μ g of each sample was electrophoresed on polyacrylamide gels at pH8.9.

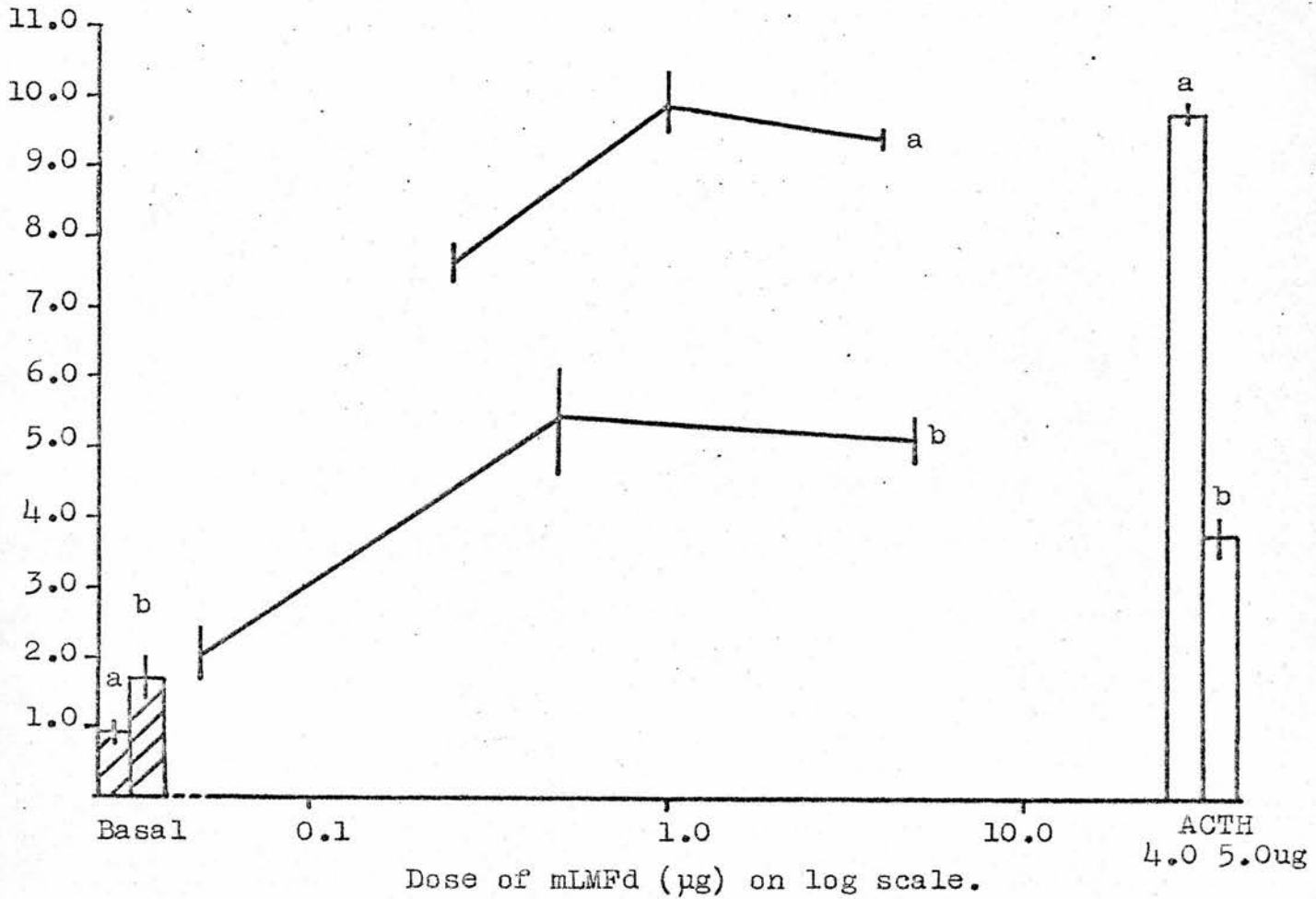
Figure 2.11. Extraction XI: Unabsorbed Protein from Anion Exchanger on CM Cellulose

Column: $2\text{cm}^2 \times 17\text{cm}$ of CM32
 Buffers: I) $0.01\text{M NH}_4\text{Ac pH}5.4$
 II) Gradient to $0.10\text{M NH}_4\text{Ac pH}5.4$
 Flow Rate: 36ml/h
 Fraction Volume: 3.6ml
 Sample: about 250mg of dLMF_1
 Sample Volume: 100ml



Unabsorbed protein from DEAE cellulose was pumped directly onto CM32 ion-exchange cellulose and a gradient of increasing buffer strength applied.

Figure 2.12. Lipolytic Activity, in vitro of mLMF_d from Extraction XI



a) mLMF_d assayed at 0.25, 1.0 and 4.0 μg

b) mLMF_d assayed at 0.05, 0.5 and 5.0 μg

Each point is the mean \pm SEM of quadruplicate samples.

Basal and ACTH induced lipolysis are included from both assays.

Assays (a) and (b) were performed on separate days with different fat cell suspensions.

c) Lipolytic Activity of mLMFd. mLMFd was assayed for a dose-response relationship in two separate assays (a & b) between doses of 0.5 and 5.0 μ g in 1.1ml of incubate (figure 2.12). It was active above a dose of 0.25 μ g but appeared to have a maximal effective dose between 0.5 and 1.0 μ g as in both cases a higher level of peptide did not cause a further release of glycerol. With ACTH at an equal concentration mLMFd was as active as the hormone at 4.0 μ g in assay (a) and in (b) was more active than ACTH at 5.0 μ g

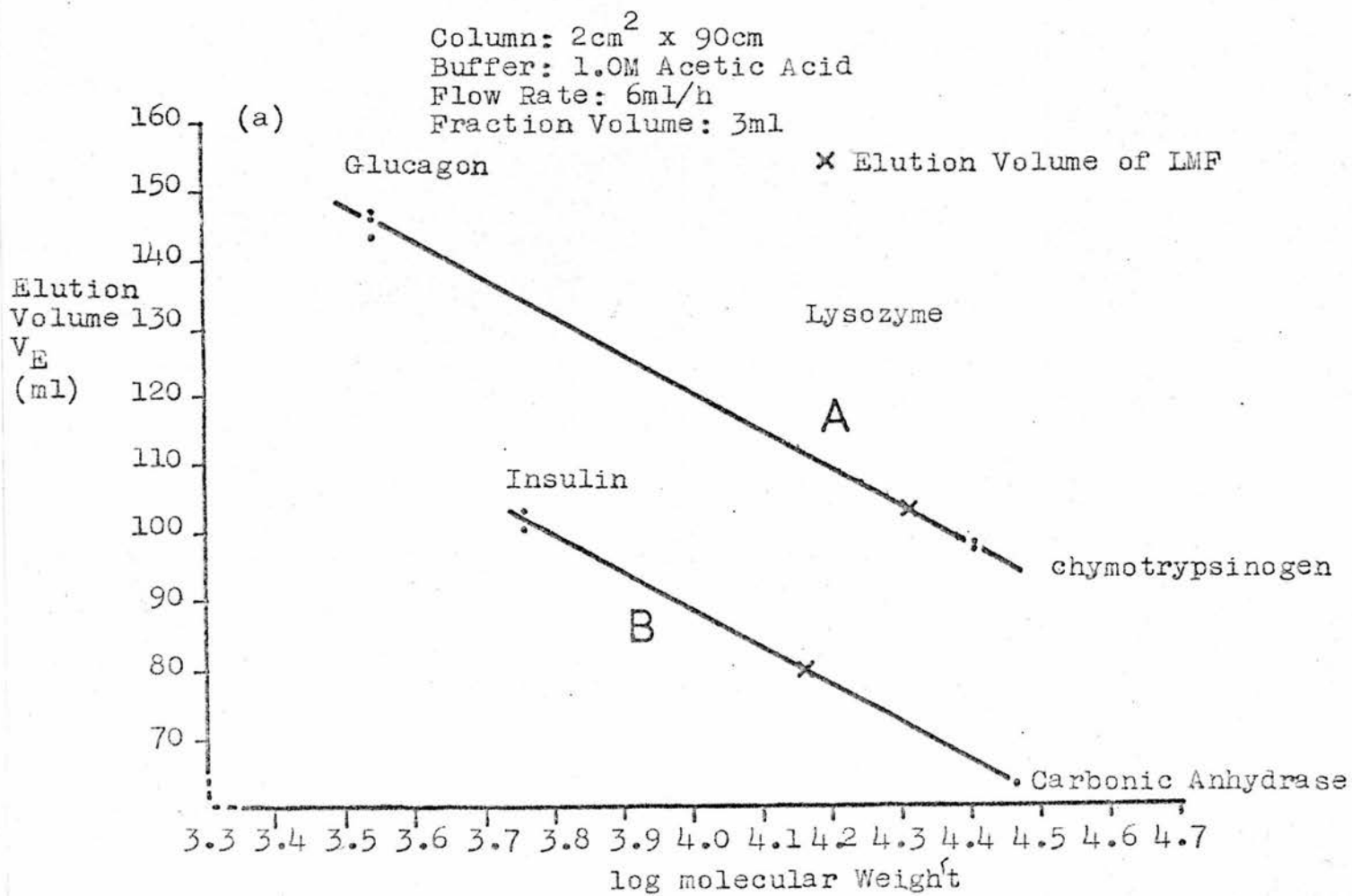
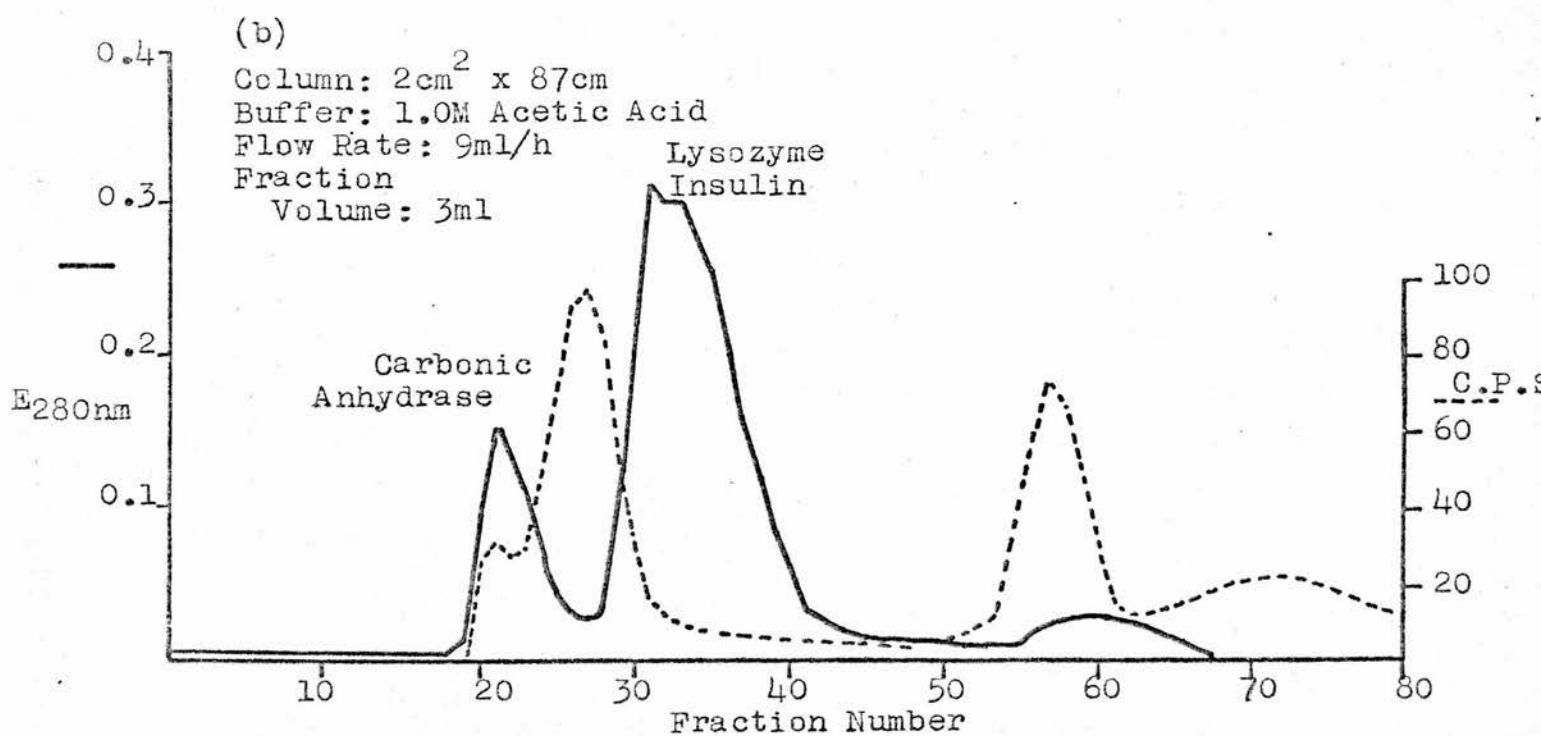
2.3.3. Estimation of Molecular Weight of Porcine LMF on Sephadex G-50

Lysozyme, one of the proteins used to calibrate the G-50 column in both determinations of the molecular weight of LMF, has an anomalous behaviour on gel filtration. Whitaker (1963) found that lysozyme, which has a chemically determined molecular weight of 14,300, eluted from a Sephadex G-100 column with an apparent molecular weight of 7,700. He suggested that the enzyme was forming weak complexes with the gel and was being retarded by this interaction with the sephadex. Lysozyme was therefore not suitable as a standard for molecular weight determination on a G-50 column and was omitted from the calibration curves.

a) On a Calibrated G-50 column. The graph of V_E against log of molecular weight is shown for glucagon and chymotrypsinogen in figure 2.13a, line A. mLMFd when run under similar conditions gave an elution volume which corresponded to a molecular weight of 20,000.

Figure 2.13a: Line A represents the calibration of a Sephadex G-50 column with glucagon and chymotrypsinogen and the elution volume of LMF under identical conditions. Line B is explained below.

Figure 2.13b: The elution from a Sephadex G-50 column of a mixture of carbonic anhydrase, lysozyme, insulin and ^{125}I -labelled LMF. The elution volumes were plotted in 2.13a above, (line B). Molecular weight estimates for LMF were made from both experiments.

Figure 2.13. Molecular Weight Determination on Sephadex G-50

b) By Estimation of ^{125}I -labelled LMF on a G-50 column. The elution diagram for carbonic anhydrase, insulin, lysozyme and ^{125}I -labelled LMF is shown in figure 2.13b. The main peak of radioactivity fell between carbonic anhydrase and a double peak of insulin and lysozyme, which had a V_E of 90-102ml. On running these proteins separately, elution volumes of 91ml for lysozyme and 103ml for insulin were found. V_E was plotted against log of molecular weight for insulin and carbonic anhydrase (figure 2.13a, line B). From this calibration curve the molecular weight of ^{125}I -labelled LMF was estimated as 14,500. It was assumed that the bovine insulin used as standard was present as a monomer under the elution conditions.

2.4. Discussion

Identification of LMF

The in vivo assay for adipokinetic activity was little used. Unless rabbits were injected at least in triplicate only a qualitative measurement of adipokinesis was possible. 18 or more rabbits would be necessary for a quantitative measure of the adipokinetic activity of fractions in table 2.2. Rabbits can develop resistance to human LMF (Trygstad, personal communication) which would cause a high turnover of experimental animals, with each one only being injected a few times. The relatively large amount of peptide required for injection into each rabbit (0.1mg) is a further disadvantage of the in vivo method. In fractionations with low yields of LMF, 5-10% of the peptide could be used in one assay. The alternative method in vitro was developed to overcome these disadvantages of measuring the biological activity of LMF in vivo.

In Vitro Assay of Lipolytic Activity

The assay of lipolytic activity in vitro, using rabbit isolated adipocytes was used here mainly to establish dose-response relationships for the more homogeneous samples of LMF. It was originally intended to determine lipolytic fractions during isolation with this assay. However, LMF containing fractions could not be distinguished by in vitro assay after G-50 gel filtration, such as in extraction V (figure 2.5 table 2.4). All the fractions assayed after lyophilization were equivalent in lipolytic activity. Polyacrylamide disc gel electrophoresis had to be used to determine which of these fractions contained LMF. As the biological assay was similarly unable to determine LMF in other fractionations, electrophoresis became the method of choice for LMF detection. The in vitro method did show that little lipolytic activity remained in residues 1, 2, 3 (table 2.3) compared to a purified sample of LMF.

Purified LMF from each of the three extractions was equivalent in activity or more active than other pituitary lipolytic peptides (ACTH, β LPH and T.LMF) which were included in the same assays (figures 2.7, 2.9 and 2.12). Trygstad's porcine LMF (T.LMF), which hardly stimulated lipolysis, even at 5.0 μ g, could have lost some activity in transit from Norway, when the sample was neither desiccated nor maintained below 0°C. While isolated porcine LMF did not have as low a minimum effective dose as that reported for human LMF (Trygstad 1968b), it was usually more active than the other lipolytic peptides tested in the in vitro assay. Possibly the assay used here was not as sensitive as the

assays used by other researchers. The incubation of a rabbit fat cell suspension, rather than slices of adipose tissue as used by Trygstad, may have reduced the sensitivity of the in vitro assay. Perhaps the receptors for LMF on rabbit fat cells were adversely affected by incubation with collagenase. Inconsistencies were found between fat cell suspensions. For example, the assays for the lipolytic activity of gmLMF₁ (figure 2.9a,b) were carried out one after the other, but gave maximal glycerol production rates of 3.29 and 15.28 $\mu\text{mol/g fat/h}$. Such large differences between preparations of suspended fat cells are further discussed in chapter 4.

Polyacrylamide Disc Gel Electrophoresis

By polyacrylamide disc gel electrophoresis, fractions with the same electrophoretic mobility as a standard sample of Trygstad's porcine LMF were identified. Compared to the other pituitary hormones electrophoresed at pH8.9 (plate 2.1), LMF had a similar mobility to ACTH. In a later experiment LMF had a similar mobility to a subsidiary band of β LPH (plate 3.1). LMF was distinguished from ACTH by electrophoresis on SDS polyacrylamide gels (plate 2.2) on which the latter had a lower mobility. β LPH is larger than ACTH so it would also have a lower mobility than LMF on SDS gels. However, as LMF resembled only the minor band of β LPH, which is probably an impurity, it was possible that LMF was the same peptide as this contaminant of β LPH. From its mobility on SDS polyacrylamide gels, the molecular weight of LMF would appear to be lower than that of ACTH (4,600).

The 7%(W/V) polyacrylamide gels used for SDS electrophoresis had 2.6% cross-linker (i.e. bis acrylamide as a

percentage of total acrylamide) which was the same percentage of crosslinker as the 10%(W/V) acrylamide gels used by Weber and Osborn (1969). This enabled the same stock acrylamide to be used for electrophoresis at pH8.9 and for SDS gels. The 7%(W/V) gels were more flexible than 10%(W/V) gels and more easily removed from the tubes with less likelihood of damage.

As mentioned earlier, LMF containing fractions were usually identified by electrophoresis on polyacrylamide disc gels. This did have to rely on the purity of the standard porcine LMF obtained from Dr. Trygstad. However, the purity of the fractions were simultaneously assessed and heterogeneous preparations of LMF could rapidly be selected for further purification. The lipolytic activity of purer LMF preparations was confirmed by biological assay in vitro before being used for antiserum production or metabolic studies. Eventually the elution position of LMF could be recognised on columns of both ion-exchange celluloses, but electrophoresis of the fractions was still employed to confirm the presence of LMF.

Electrophoresis on SDS polyacrylamide gels was introduced as a precaution against the erroneous identification of a fraction as LMF. At pH8.9 electrophoresis could not distinguish between two peptides with identical mobilities. It was unlikely for dissimilar peptides to have the same mobility at pH8.9 and in the presence of SDS ions. Thus the major band of mLMFd was almost certainly the same peptide as Trygstad's porcine LMF (plates 2.1, 2.2). Other LMF

preparations with these mobilities on the two types of gel, were also likely to be the same as Trygstad's porcine LMF.

Preparation of crude LMF

In the precipitation of porcine LMF the pH of supernatant 1 was reduced to pH5.5, not 4.5 as in the preparation of human LMF. The latter was to reduce the pH below the isoelectric point of human growth hormone which is pH4.9 (Trygstad, 1968a). As porcine GH has a higher pI of 6.3 (Li & Liu, 1964) then for porcine material the pH did not have to be lowered so far. Trygstad reported the molecular weight of human LMF to be in the region of 5,400 (1968a) by gel filtration or 2,100 (1968b) by ultracentrifugation and his porcine LMF did show a low molecular weight on SDS polyacrylamide electrophoresis. Theoretically a molecule of this size should be able to pass through a dialysis membrane so, even though Trygstad found otherwise, dialysis was avoided in preparation of porcine LMF. Desalting of residue 4 was achieved by Sephadex G-15 gel filtration. The salt peak from the desalting, which contained some 280nm absorbing material, was once run on a CM cellulose ion-exchange column after dialysis. No protein eluted in the usual position for LMF, confirming that there was none in the low molecular weight fraction from G-15 gel filtration.

Extraction V

Fractionation of crude LMF on Sephadex G-50 (extraction V) was not an effective purification measure. LMF was not confined to one peak but was found mostly in the

descending portion of the first, major peak. LMF containing fractions were purified by DEAE cellulose where the acidic protein seen on electrophoresis of sLMF₂ (plate 2.3) was absorbed by the cellulose and basic peptides eluted in the breakthrough peak. Anion exchange chromatography could not remove the band of impurity seen in both sLMF₂ and dLMF₁ which had a slightly higher mobility than LMF. Extraction V gave the purest LMF of a number of similar extractions. In others, fractions from gel filtration contained more basic peptides, which DEAE cellulose chromatography was unable to remove. After extraction V, carboxy methyl cellulose which can fractionate these basic proteins replaced DEAE cellulose. CM cellulose had been used by Gilardeau and Chrétien (1970) in the isolation of porcine β LPH. LMF did not absorb to DEAE cellulose in 0.01M NH₄Ac pH5.4 but it did absorb to CM cellulose under the same conditions (extraction VIII). By desalting on Sephadex G-15 in the buffer used for subsequent cation exchange, it was not necessary to freeze dry fractions and re-equilibrate them for ion-exchange. The eluate from desalting was pumped directly on to the CM column.

Extraction VIII

Extraction VIII produced a preparation (mLMF₃) which, after cation exchange, had a high proportion of LMF but also had five minor bands (Plate 2.4 figure 2.8). After rerunning on CM cellulose and cleaning on Sephadex G-50 apparently homogeneous preparations were obtained (gmLMF₁ and gmLMF₂). No difference could be seen on electrophoresis between the main peak from the G-50 column and its trailing

edge. These were the purest preparations obtained in any quantity. When $mLMF_3$ was re-chromatographed on CM cellulose, the starting buffer was more concentrated than usual (0.05M NH_4Ac pH5.4), but LMF was still absorbed by the cation exchanger. The buffer concentration had to be raised slightly before the LMF desorbed. A comparison of the behaviour of LMF on CM cellulose ion-exchange columns, with the fractionation of other pituitary lipolytic peptides on the same material, is made in the general discussion (chapter 5).

Extraction XI

The introduction of a DEAE cellulose step prior to cation exchange on CM cellulose, removed most of the acidic proteins ($dLMF_2$). Some of these were not absorbed at this first stage and formed the breakthrough peaks seen on CM cellulose fractionation of $dLMF_1$ (figures 2.10, 2.11, plate 2.5). Possibly the DEAE cellulose column had been overloaded. The LMF containing peak ($mLMFd$) eluted from the CM cellulose column when the buffer conductivity rose to approximately 5.0mmho. This corresponded to the conductivity of the buffer at which LMF eluted in extraction VIII. Further purification of $mLMFd$ could be made by rechromatography on a CM cellulose column followed by sephadex gel filtration as in extraction VIII.

A disadvantage of extraction XI was the large volume (100ml) of the sample applied to the CM cellulose ion-exchange column. The procedure would be improved by lyophilization either before, or after the DEAE cellulose

step, to reduce the volume. The freeze dried sample would then be equilibrated with starting buffer for the next ion-exchange fractionation on a small gel filtration column. With such a lyophilization step, extraction XI would be the method of choice for the isolation of LMF.

Molecular Weight Estimation

With two points on each calibration curve, after the omission of lysozyme, only a qualitative estimate of the molecular weight of LMF could be made. For a more accurate determination, the gel filtration should be repeated a number of times and more protein standards should be used. The estimate of the molecular weight of mLMFd obtained from the gel filtration (between 14,000 and 20,000) disagreed with both Trygstad's (1968b) results and with the electrophoresis of mLMFd on SDS polyacrylamide gels. Trygstad found a molecular weight of 2,100 for human LMF by ultracentrifugation, while on SDS gels porcine mLMFd had a higher mobility than porcine ACTH (plate 2.2), indicative of a molecular weight below that of ACTH (4,600). An impurity of higher molecular weight was seen in the SDS gel of mLMFd. Possibly the proteins were associating together under the conditions of the gel filtration, causing the apparent high molecular weight on elution. 1.0M acetic acid as buffer was designed to reduce any such association between protein molecules and has been reported to successfully prevent the formation of insulin dimers (Grant & Reid, 1968). However, from the elution volume of mLMFd, the buffer did not prevent

this peptide from associating with the large molecular weight impurity or, alternatively, aggregating with itself. Possibly the introduction of a buffer such as a phenol-acetic acid-water mixture which has been used to prevent the absorption of aromatic peptides onto Sephadex, (Carnegie 1965) could reduce protein association and help to separate LMF from the larger contaminant.

The LMF preparation used in the investigation of molecular weight by gel filtration (mLMFd) was used because it was available at the time in sufficient quantity to give a detectable peak after elution from the G-50 column. The preparation was unfortunately not further purified by the gel filtration, as the large contaminant remained.

2.5. Summary

- 1) A heterogeneous preparation of crude LMF was isolated from a porcine pituitary homogenate by pH adjustment followed by acetone saturation. LMF was purified from the crude preparation by gel filtration and ion-exchange chromatography.
- 2) Sephadex G-50 gel filtration followed by DEAE cellulose ion-exchange chromatography did not produce a homogeneous LMF preparation.
- 3) On CM cellulose ion-exchange chromatography, LMF was initially absorbed and was desorbed by an increase in buffer strength. A homogeneous LMF preparation was purified by re-chromatography on CM cellulose followed by gel filtration on Sephadex G-50.
- 4) A DEAE cellulose step, before fractionation on a CM cellulose column helped to remove acidic proteins from the sample.

5) The recommended procedure for LMF isolation from a crude preparation would be: crude LMF → DEAE cellulose → CM cellulose (once or twice) → Sephadex G-50 in a buffer which prevents protein association.

6) Polyacrylamide disc gel electrophoresis was the most useful method for identification of LMF.

CHAPTER 3.The Development of a Radioimmunoassay for
Porcine Lipid Mobilizing Factor3.1 Introduction

In recent years the use of radioimmunoassay has markedly increased in endocrinological investigations. It has the advantages of greater sensitivity, specificity and ease of use over comparable biological methods. Radioimmunoassay is one type of saturation analysis (Ekins, 1970) and was first introduced by Yalow and Berson (1960) for the estimation of insulin. Since then the method has been extended to cover other molecular species and a number of reviews have described the procedure, its mathematical analysis and its applications (Greenwood, 1967; Berson & Yalow, 1964; Yalow & Berson, 1968; Yalow & Berson, 1970; Kirkham & Hunter, 1971; Jaffe & Behrman, 1974; Felber, 1974).

As described by Ekins (1970), the theory of a saturation analysis to determine the concentration of compound P in solution is as follows. Initially radioactive P is added to the solution and allowed to equilibrate with endogenous unlabelled P. Compound P is then extracted, if necessary. A specific reagent Q is added in sufficient amount to react with, or bind to, only a proportion of P, leaving some unreacted (or free) P. The ratio of reacted to unreacted radioactive P, under certain conditions, gives a sensitive measure of the concentration of P in the original solution. This ratio can be determined by a number of techniques. The concentration of P in unknown samples is determined by comparison to a set of standards.

In developing a possible radioimmunoassay for LMF, peptide purified as described in Chapter 2 provided the compound (P) for labelling and for use as standard. The major problem was the lack of a specific reagent (Q), which in a radioimmunoassay is the specific immunoglobulin present in antiserum. To overcome this problem, 9 rabbits were immunized with LMF, in 3 different ways, in an attempt to produce antiserum specific for this peptide. Serum from these rabbits was assessed for suitability for use in a radioimmunoassay.

A method for the separation of reacted compound (P-Q) from unreacted compound (free P) was readily available, if Q was a rabbit immunoglobulin specific for LMF (P in this case). A goat antiserum was used which was specific for rabbit immunoglobulins. By adding it to the mixture of P and Q after these had reacted, the goat antiserum precipitated all the rabbit immunoglobulins including those which had bound compound P. The ratio of bound P to free P was derived and subsequently the concentration of unlabelled P in the original solution was calculated. The calculations were performed as described by Rodbard et al. (1969). This 'double-antibody' method (Hunter & Ganguli, 1971) was used during the assessment of various sera samples for their suitability for radioimmunoassay.

An earlier attempt had been made by Norman & Turtur (1968) to raise antiserum against human LMF. None of the treated rabbits had produced serum which cross-reacted with the peptide. However, some sera were found to bind to

growth hormone indicating either that human LMF had a similar immunological structure to growth hormone, or that the preparation used as immunogen had been contaminated with growth hormone.

This chapter describes the production and assessment of antisera specific for LMF.

3.2 Methods

3.2.1 Production of Antiserum

3.2.1.1 LMF in Freund's Complete Adjuvant as Immunogen

For the first attempt at the production of anti-LMF antiserum, dLMF₁ was emulsified in Freund's Complete Adjuvant (Freund, 1951, Chase, 1967) prior to injection into rabbits. It is thought that when an immunogen is injected as such an emulsion it is slowly released in the immunized animal continually stimulating the immune response. The response can be increased by the inclusion of dead *Mycobacterium butyricum* in the adjuvant and the simultaneous injection of *Bordetella pertussis* vaccine.

2.0ml of Freund's Complete Adjuvant (FCA), consisting of 1 part Arlacel A: 7 parts liquid paraffin + 2 mg/ml *Mycobacterium butyricum*, was placed in the rotor of an MSE Homogeniser. 0.75mg of dLMF₁ was dissolved in 2.0ml of 0.9% (W/V) saline. 0.2ml aliquots of this were added in turn to, and emulsified in the adjuvant. The emulsion was complete when a drop on the surface of water did not disintegrate. The viscosity of the emulsion was reduced by its dispersion in an equal volume (4.0ml) of a 2% (V/V) solution of Tween 80 in 0.9% (W/V) saline, forming a second emulsion.

25ml of blood was taken from the peripheral ear vein of each of three New Zealand White rabbits (R294, R296 and R297). After bleeding, 1.2ml of the double emulsion was injected intramuscularly into each hind leg of the rabbits, followed by 0.2ml of the B. pertussis vaccine injected subcutaneously into each hind leg. 25ml of blood was taken from each rabbit at 2-weekly intervals. Following the bleed at 4 weeks, the rabbits received a booster injection of dLMF₁ in a double emulsion of FCA and Tween 80. 1mg of dLMF₁ in 1.5ml of 0.9% (W/V) saline was first emulsified in 1.5ml of FCA and subsequently in 3.0ml of 2% (V/V) Tween 80 in 0.9% (W/V) saline. 1ml of the double emulsion followed by 0.2ml of B. pertussis vaccine were injected into each hind leg of the rabbits as before.

The rabbits were given a second booster injection after the bleed at 8 weeks. 1mg of dLMF₁ in 1.5ml 0.9% (W/V) saline was dispersed, first in 1.5ml of FCA and then in 3.0ml of Tween 80 in 0.9% (W/V) saline. The double emulsion and B. pertussis vaccine were injected as before. The rabbits were bled finally after a further 2 weeks.

3.2.1.2 LMF Conjugated to Bovine Serum Albumin as Immunogen

The immunogenicity of a substance, which alone does not stimulate the production of antibodies, can be increased by its conjugation to a larger, more immunogenic molecule (Lekhite & Sehon, 1967). LMF was conjugated to bovine serum albumin (BSA) by the carbodiimide method of Goodfriend et al. (1964) (Figure 3.1) in an attempt to increase its immunogenicity.

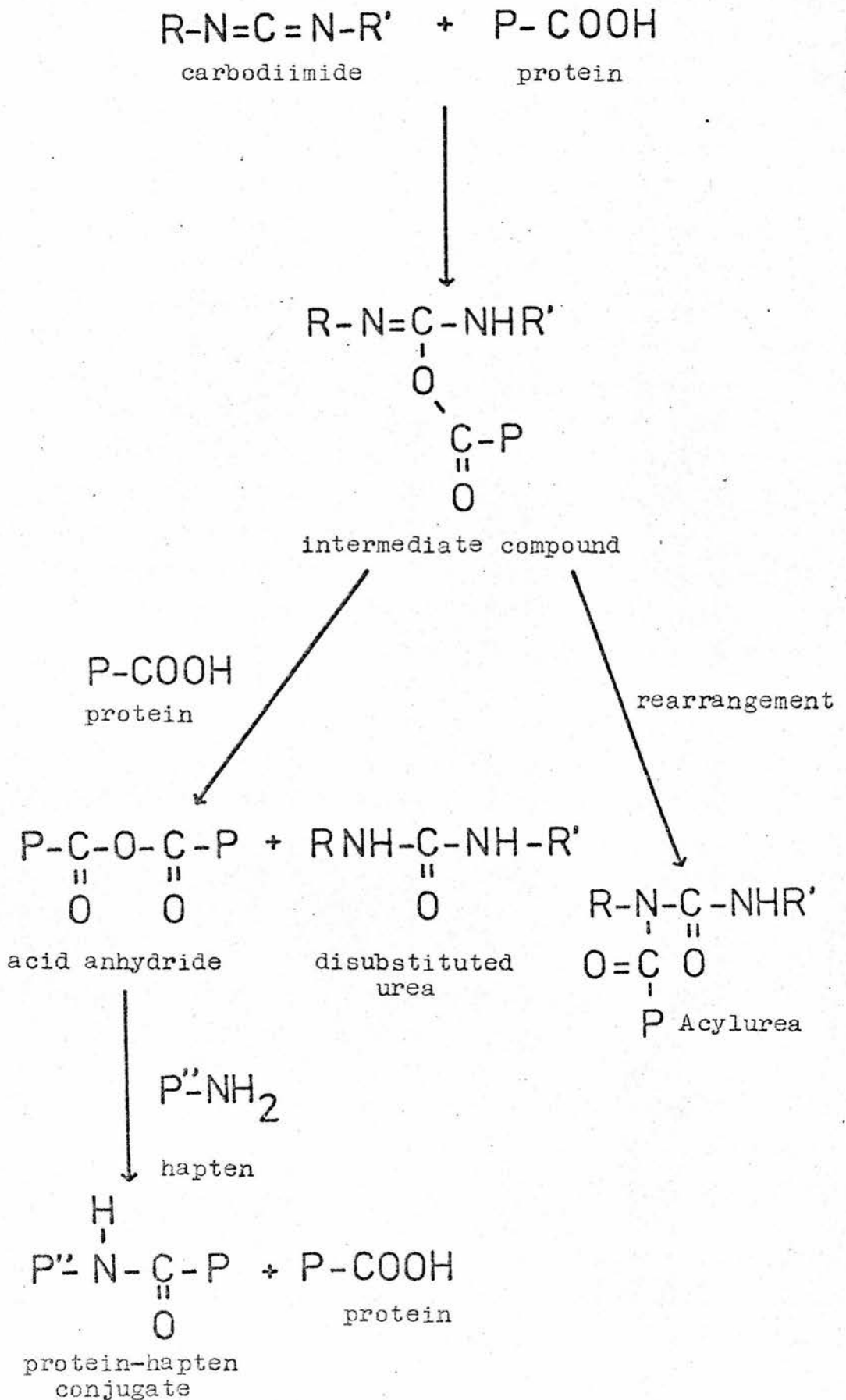


Figure 3.1 Formation of a protein-hapten conjugate by the carbodiimide reaction (from Lekhite & Sehon 1967).
 The hapten is a non-immunogenic protein or peptide.

For conjugation to BSA a preparation of LMF from extraction VII was used. This preparation resembled mLMFd (extraction XI Sect. 2.2.2.5) in purity and method of isolation and will be referred to as mLMF-VIId. As LMF was more scarce than peptides previously conjugated to BSA, such as bradykinin or angiotensin (Lekhite & Schon, 1967), the quantities of reactants were reduced and the time of reaction increased.

1.88mg of mLMF-VIId was dissolved with 2.48mg of BSA in 0.5ml of distilled water. 20.4mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) was dissolved in 0.25ml of distilled water and added to the protein mixture. The reaction proceeded overnight at room temperature. The reaction mixture was then dialysed against 0.9% (W/V) saline at room temperature for 3 days during which the external saline (500ml) was changed twice daily. The volume of immunogen solution was 1.2ml after dialysis. The dialysis tubing was washed with 0.8ml of 0.9% (W/V) saline and the washings added to the conjugate solution. This was emulsified in 2ml of FCA and then in 4ml of 2% (V/V) Tween 80 in 0.9% (W/V) saline. Rabbits R180, R198 and R199, which had previously been bled of 25ml were inoculated, with 1.2ml of the double emulsion, injected intramuscularly into each hind leg. A subcutaneous injection of 0.2ml B. pertussis vaccine followed. 25ml of blood was taken from the rabbits at intervals of 2 weeks.

The third bleed was followed by a booster injection of LMF-BSA conjugate in FCA-Tween 80 double emulsion. 1.52mg of mLMF-VIId had been conjugated to 1.77 mg of BSA by 24.2mg

of ECDI in a total volume of 0.4ml distilled water. The preparation of the double emulsion and its injection were carried out as before. A second booster injection was performed after the eighth bleed. 1.3mg of mLMF-VIIId was conjugated to 2.1mg of BSA by 22.4mg of ECDI. Emulsification and injection were as before.

3.2.1.3 LMF in Polyacrylamide as Immunogen

Polyacrylamide gel can be an effective adjuvant for the production of antiserum against purified immunogen (Weintraub & Raymond, 1963; Green et al., 1974). The immunogen is purified by electrophoresis on polyacrylamide gel which is then homogenized and injected. The immunogen slowly diffuses into the circulation of the immunized animal, where it stimulates antibody production.

A preparation of LMF from extraction X (mLMF-X₃), which was similar to mLMF₃ of extraction VIII (Section 2.2.2.4), was used as immunogen. On polyacrylamide disc gel electrophoresis (Section 2.2.1.3) this preparation had been found to move between 11 and 16mm towards the anode, allowing for the swelling of the gel in 10% acetic acid. 4 x 100 μ l aliquots of a 0.8mg/ml solution of the preparation (mLMF-X₃) were electrophoresed and the gels subsequently sliced into 2mm sections using a perspex die and scalpel blade. The sixth, seventh and eighth sections of all 4 gels were combined in the rotor of an MSE Homogeniser. 1.5ml of FCA was added and during homogenization 1.7ml of 0.9% saline was added in 0.2ml aliquots. The first emulsion plus homogenized polyacrylamide was suspended in 3.2ml of

2% (V/V) Tween 80 in 0.9% (W/V) saline. 1ml of the double emulsion was injected intramuscularly into each hind leg of 3 rabbits (R1298, R1299 and R1300) from which 25ml of blood had previously been taken. 0.2ml of B. pertussis vaccine was injected subcutaneously into each hind leg. 25ml of blood was taken from each rabbit at intervals of 2 weeks.

After the second of these bleeds the immunization was repeated with homogenized polyacrylamide gel from the electrophoresis of 4 x 100 μ l aliquots of mLMF-X₃ at a concentration of 1.0mg/ml.

Later, the rabbits were immunized with mLMF-X₃ which had not been electrophoresed but was dispersed in a double emulsion of FCA and Tween 80 in 0.9% (W/V) saline. The rabbits were inoculated with 0.13mg and 0.17mg of LMF per rabbit after the sixth and eleventh bleed respectively.

3.2.2 Identification of Cross-Reacting Antiserum by Binding to ¹²⁵I-Labelled LMF

3.2.2.1 Labelling of LMF with Iodine-125

A number of LMF preparations were labelled with I-125 using a method based on that of Hunter & Greenwood (1962) and Hunter (1973). Radioactive iodide, oxidised by chloramine T, substitutes onto the aromatic ring of tyrosine residues in the peptide. The reaction is stopped by sodium metabisulphite and excess iodide is removed from the labelled peptide by gel filtration.

A stock solution of 0.5 M phosphate buffer pH7.5 in distilled water was made by mixing 0.5M Na₂HPO₄ with 0.5M NaH₂PO₄ until pH7.5 was reached. This was diluted when necessary. A general assay diluent (GAD) which was used

throughout the immunological investigation was made up as follows. To 0.15M phosphate buffered saline, which consisted of 0.075M NaCl + 0.075M phosphate buffer pH7.5, bovine serum albumin was added to give a concentration of 0.1% (W/V). The following solutions were made on the day of use. 2g of chloramine T was made up to 50ml (4%, W/V) in 0.05M phosphate buffer pH7.5. 120mg of sodium metabisulphite was made up to 50ml (2.4mg/ml) in 0.05M phosphate buffer pH7.5. 0.5g of potassium iodide was made up to 50ml (1.0% W/V) in GAD. The peptide preparation to be labelled was the purest available at the time and was stored in 20 μ l aliquots of a 1mg/ml solution in distilled water at -18°C. The approximate activity of the iodide-125 used for labelling was 1000 μ Ci, though this decreased with storage time. A correction factor, depending on the day of use, was applied to calculate the activity of the iodide aliquot on the day of labelling.

On the day prior to iodination, 1g of Sephadex G-50 was weighed out and soaked overnight in GAD. The next day this was poured into a Whatman's 1 cm² x 15cm chromatography column and equilibrated with 25ml of GAD. 1.0ml of 4% BSA in 0.15M phosphate buffered saline was run through the column to saturate protein binding sites on the dextran and on the tube walls. The column was eluted with GAD until re-equilibrated.

The iodination of LMF preparations was carried out by mixing the following in a small reaction vessel; 5 μ l of 1mg/ml LMF; 25 μ l of 0.5M phosphate buffer pH 7.5;

10 μ l of 100 μ Ci/ μ l Iodide-125. To these was added 25 μ l of 4% (W/V) chloramine T and the vessel was shaken for 20 seconds. The reaction was stopped by the addition of 100 μ l of 2.4mg/ml sodium metabisulphite and the mixture diluted with 200 μ l of 1% (W/V) potassium iodide.

The reaction mixture was applied to the G-50 column, along with 2 x 200 μ l of 1.0% (W/V) KI used to wash out the reaction vessel. The column was eluted with GAD, 1ml fractions being collected. Operating pressure was maintained by keeping the buffer reservoir at a constant height above the column. The γ -radiation in each fraction was measured on a Mini-Assay γ -spectrometer. Elution continued until activity returned to near background levels, after the appearance of 2 peaks. This was usually after 18-20ml had eluted. The fraction with the highest activity in the first (breakthrough peak) was retained and the remaining ones discarded. The retained fraction was diluted to 10ml with GAD and was then known as stock label. For experimental use, 1ml of this was diluted with GAD to 1-2ng/ml of LMF, so that 100 μ l gave a reading of about 200 counts per second (c.p.s.) on a Wallac γ -spectrometer. This was the working label. Both this and the stock label were stored at 4°C.

The following were calculated:

$$\text{Yield of label on LMF} = \frac{\text{Counts in 1 st peak}}{\text{Total counts in all fractions}} \times 100\%$$

$$\begin{aligned} \text{Concentration of LMF in 1ml fraction before dilution } (\mu\text{g/ml}) \\ = \frac{\text{Count in Fraction}}{\text{Total counts in 1 st peak}} \times \frac{\text{Amount of LMF labelled}}{(\mu\text{g})} \end{aligned}$$

From this the LMF content of working label was derived. The specific activity of the labelled LMF was also calculated.

$$\begin{aligned} \text{Specific Activity of } ^{125}\text{I-labelled LMF } (\mu\text{Ci}/\mu\text{g}) \\ = \frac{\mu\text{Ci of } ^{125}\text{I used}}{\text{LMF used } (\mu\text{g})} \times \frac{\% \text{ yield of label}}{\text{on LMF}} \end{aligned}$$

The activity of the ^{125}I used was calculated by adjusting for the change in activity of 10 μl of ^{125}I between the day of use and an activity date named by the manufacturer. A table of correction factors was provided for this calculation.

3.2.2.2 Removal of Free ^{125}I and Damaged Labelled Peptide from ^{125}I -labelled LMF.

Polyacrylamide disc gel electrophoresis has been used in place of gel filtration to separate peptide bound to I-125 from free I-125 (Hunter, 1971). Although being a more tedious method, better separation can be obtained by electrophoresis and the method is frequently used in the preparation of iodinated human follicle-stimulating hormone.

a) By Electrophoresis of ^{125}I -labelled LMF. 50 μl of stock ^{125}I -labelled LMF was electrophoresed on a polyacrylamide disc gel (Section 2.2.1.3) which was immediately sliced into 2mm sections using a perspex die and a scalpel blade. The slices were counted for γ -radiation. Labelled material from

each peak of radioactivity was eluted from the gel slices at 4°C by soaking overnight in 1ml of GAD. Slices 11-13 (Figure 3.3) were combined for the first peak, while slices 21 and 25 represented the second and third peaks. The eluted radioactive material was diluted so that either 100µl would give 200 c.p.s. or to a volume (2ml) sufficient to establish an antiserum titration curve (Section 3.2.2.3). Serum from the seventh bleed of R199 was titrated against label from each peak and compared to a titration curve of non-electrophoresed label against the same antiserum.

b) Further Investigation of ^{125}I -labelled LMF after Electrophoresis. After electrophoresis of 50µl of stock ^{125}I -labelled LMF the gel was sliced as before. Alternate 2mm slices were placed in 2ml of GAD and labelled material allowed to elute overnight at 4°C. 100µl aliquots of these were dispensed in triplicate into polystyrene LP4 tubes either (i) alone, (ii) with 0.7ml of GAD or (iii) with 0.5ml of GAD plus 0.2ml of a 1:400 dilution of serum from the tenth bleed of R199. These were treated as totals, blanks and zero standards as in Section 3.2.2.3. The binding of the antiserum to labelled material eluted from the gel slices was found.

c) Preparation by Electrophoresis of ^{125}I -labelled LMF for use in an Assay. To improve the binding of label to antiserum in certain assays, the label was cleaned by electrophoresis on polyacrylamide disc gels prior to the assay. 4 x 50µl aliquots of label, from gel filtration without further dilution, were electrophoresed and the gels sliced

into 2mm sections as before. The slices were counted for γ -radiation on a Wallac γ -spectrometer. All the slices from the peak with lowest mobility were pooled and label eluted into 25ml of GAD overnight at 4°C. After dilution with GAD, so that 0.1ml gave a count of 200 c.p.s., the label was used in an assay as described in Section 3.2.2.4.

3.2.2.3. Assessment of Antiserum ; Antiserum Titration Curves

Sera from the immunized animals, were assessed for the presence of LMF binding antibodies by antiserum titration studies (Hurn & Landon, 1971). The percentage of ^{125}I -labelled LMF, binding to serial dilutions of the antisera, was determined by a method based on that of Hunter (1973). Antibody bound ^{125}I -labelled LMF was separated from free labelled hormone by precipitation with a second antiserum, which was specific for rabbit immunoglobulins.

The antisera were serially diluted with GAD in ratios of 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200 and 1:6400. 0.2ml aliquots of these were mixed with 0.5ml of GAD in polystyrene LP4 tubes and as they would contain no unlabelled LMF, they were known as zero standards. Blank LP4 tubes containing 0.7ml of GAD were prepared in triplicate. 0.1ml of a solution of ^{125}I -labelled LMF at the working concentration (1-2ng/ml; about 200 c.p.s. on γ -spectrometer) was added to the zero standards and blanks. A similar volume was dispensed to a further three tubes, which were stoppered and later counted as 'totals'. The tubes were mixed and incubated at 4°C for 48 hours.

Goat anti-rabbit immunoglobulin antiserum, the second antiserum, was added to the blanks and zero standards after the 2 day incubation. Immediately before the addition of the second antiserum, 0.1ml of a 1:400 dilution of normal rabbit serum was added to these tubes. 0.2ml of the second antiserum, at a 1:30 dilution and containing 0.01M EDTA, was then added. After mixing, the tubes were incubated at 4°C overnight.

The next day 2ml of GAD was added to all the tubes except the totals. The tubes were centrifuged at 2,500g for 20 minutes. The supernatants of the blanks and zero standards were decanted and, after draining for 30 minutes, any remaining liquid was aspirated. All the tubes, including the totals, were counted on a Wallac γ -spectrometer for 100s.

The percentage of ^{125}I -labelled LMF bound was calculated for the zero standards at each dilution of serum.

$$\text{Percentage bound} = \frac{\text{count in zero standard} - \text{mean count of blanks}}{\text{mean count of totals}} \times 100$$

The antiserum with the steepest negative slope, when the percentage of ^{125}I -labelled LMF bound to antiserum was plotted as ordinate against the antiserum dilution as abscissa, was the best for use in a radioimmunoassay (Berson & Yalow, 1964). The gradient of the antiserum titration curve reflected the avidity of binding of antiserum to labelled antigen.

3.2.2.4 Assessment of Antiserum ; Standard Curve and Specificity of Antiserum

A standard curve consisting of increasing proportions of unlabelled LMF in the presence of antiserum and ^{125}I -labelled LMF, was set up using the serum that showed the

highest percentage binding and steepest gradient in an anti-serum titration curve. In the same experiment increasing amounts of other pituitary peptides were incubated with the antiserum and ^{125}I -labelled LMF, to see if they caused any interference by cross-reaction. A Searle 'Analmatic' pipetting station was used to dispense the solutions, which were all made up in GAD. The totals, blanks and zero standards were in quadruplicate, whereas the standards and unknown samples were in triplicate.

Serum prepared from the tenth bleed of R199 at a dilution of 1:400 was used as the first antiserum. At this dilution, 20% of ^{125}I -labelled LMF was bound. The antiserum was not used at 1:200, which gave the maximum binding, in order to reduce any possible serum proteolytic effects. The standard curve consisted of serial dilutions of a 6.0 $\mu\text{g}/\text{ml}$ solution of the purest LMF preparation available (gmLMF_1) in GAD, giving concentrations of 5.9, 23, 94, 375, 1500 and 6000 ng/ml . These were diluted 1:10 and 1:5 with GAD while being dispensed to give a twelve-point standard curve ranging from 0.59 to 1200 ng/ml . A number of pituitary peptides and glycoproteins were dispensed at the final concentrations shown in Table 3.1. 0.5ml of the unknown samples and the standard dilutions of gmLMF_1 , were dispensed to LP4 tubes. Zero standards, consisting of 0.5ml of GAD were dispensed. 0.2ml of the first antiserum was added to all these tubes and thoroughly mixed. After 48 hours at 4 $^{\circ}\text{C}$, 0.1ml of working label was added to the tubes and also dispensed to blanks (0.7ml of GAD) and totals. The assay then proceeded as

Table 3.1 Concentrations of peptides and glycoproteins used in specificity test.

Peptide or glycoprotein	Species	Source of peptide or glycoprotein	Concentration (ng/ml)		
LPH	porcine	Dr. L. Graf	1000	166	83
ACTH	porcine	Sigma	1000	200	100
GH	porcine	[†] NIH-GH-P548	1220	144	122
PRL	ovine	NIH-P-S9	1190	238	119
LH	ovine	NIH-LH-S17	1025	203	103
FSH	ovine	NIH-FSH-S9	1000	200	100
TSH	ovine	NIH-TSH-S6	1080	216	108
LMF	porcine	Dr. O. Trygstad	1100	220	110
				42	21
				50	25
				60	30
				60	30
				50	25
				50	25
				51	25
				54	27

The peptides and glycoproteins, at the concentrations shown, were assayed as unknown samples for their inhibition of the binding of ¹²⁵I-labelled LMF to anti-LMF antiserum. Serum from bleed 10 of R199 at a dilution of 1:400 was used.

[†]National Institute of Health batch number.

described for the antiserum titration curves, with a further 2 day incubation at 4°C before addition of second antiserum, followed a day later by centrifugation and counting for γ -radiation.

After the tubes had been counted for γ -radiation, the proportion of labelled antigen bound to the antiserum in the presence of unlabelled antigen was calculated as a percentage of the amount of labelled antigen bound in the absence of unlabelled antigen (i.e. in the zero standard). This was known as Y and was found for each dilution of standard or unknown samples.

$$Y = \frac{\bar{B} - B1}{B0 - B1} \times 100 \text{ where } \bar{B} = \text{Count with unlabelled antigen (i.e. in standard or unknown sample)}$$

B1 = Mean count of blanks

B0 = Mean count of zero standards

The standard curve was plotted with either Y as a percentage or $\log Y$ as the ordinate and with the log of the concentration of LMF as the abscissa.

3.2.2.5. Assessment of Antiserum ; Nature of Non-Specific Cross-Reactions.

Three of the hormones, which had cross-reacted with the anti-LMF antiserum from R199 in the specificity test (Section 3.2.2.4), were examined to find the electrophoretic mobility of their cross-reacting fractions. The apparent LMF content of slices of polyacrylamide gels, on which the peptides had been electrophoresed, was assayed using an LMF standard curve between 12.5 and 1600ng/ml. The totals, blanks, zero standards and the standard curve were prepared as described in the previous section.

Two 0.1ml aliquots of 1.0mg/ml solutions of mLMFd, oLH, oFSH and LPH were electrophoresed on polyacrylamide gels at pH8.9 (section 2.2.1.3.). One gel with each sample was stained in amido black and destained as normal. The other gels were sliced into 2mm segments using a perspex die and soaked overnight at 4°C in 2ml of GAD. The resultant solutions were included in the assay as unknown samples at 1:10 and 1:5 dilutions. The apparent LMF content of each slice of gel was found by comparison to the standard curve.

Further investigation was made into antiserum from rabbit R199 by comparing ¹²⁵I-labelled LH to ¹²⁵I-labelled LMF in antiserum titration curves with the antiserum. The procedure described in section 3.2.2.3 was used for both labelled peptides except that serum from bleed 10 of R199 was present at dilutions of 1:100, 1:200 and 1:400.

LMF preparations were assayed for LH content in an LH radioimmunoassay (Carr & Land, 1975) which included a small standard curve of LH at 1.02, 4.10 and 10.24 ng/ml. Preparations of LMF from extraction XI and XII were assayed for LH content at a concentration of 0.02 mg/ml.

3.3 Results

3.3.1 Identification of Cross-Reacting Antiserum by binding to ¹²⁵I-labelled LMF

3.3.1.1 Labelling of LMF with Iodine - 125

Over a two year period LMF was labelled with Iodine - 125 eight times. Both G-50 and G-15 sephadex columns were used to separate labelled peptide from free iodide. No difference was observed in the elution volumes of the radio-

active peaks between the two sephadex grades. The breakthrough peak containing ^{125}I -labelled LMF had an elution volume of 5 or 6ml whereas free ^{125}I eluted in a volume of 12 or 13ml (Figure 3.2).

The yield of radioactivity bound to the peptide varied from 35% to 75% and the specific activities from 63 to 160 $\mu\text{Ci}/\mu\text{g}$. For the experiment illustrated in Figure 3.1, the yield was 50% and the specific activity was 71 $\mu\text{Ci}/\mu\text{g}$. Fraction 6 on dilution to 10ml became the stock label with a concentration of 125ng/ml. 1ml of the stock label, diluted to 50ml gave a working label of 2.5ng/ml, 0.1ml of which gave a count of 223 c.p.s. on the Wallac γ -spectrometer.

3.3.1.2 Removal of Free ^{125}I and damaged labelled peptide from ^{125}I -labelled LMF

a) By Electrophoresis of ^{125}I -labelled LMF. The pattern of γ -radiation in 2mm slices of the polyacrylamide gel on which ^{125}I -labelled LMF had been electrophoresed is shown in Figure 3.3. The peptide had been iodinated a week earlier. 22% of the total activity was in peak 1, 20% in peak 2 and 57% in peak 3. The antiserum titration curves of eluted label from the individual peaks with serum from the seventh bleed of R199, are shown in Figure 3.4. The radioactivity eluted from peaks 2 and 3 showed negligible binding to the antiserum. A maximum of 37% of the label from peak 1 was bound by the antiserum. This was an increase of 17% over the maximum binding found when non-electrophoresed label had been used. The gradient of the descending slope of the antibody titration curve was greater with ^{125}I -labelled material from peak 1 than with non-electrophoresed ^{125}I -labelled LMF.

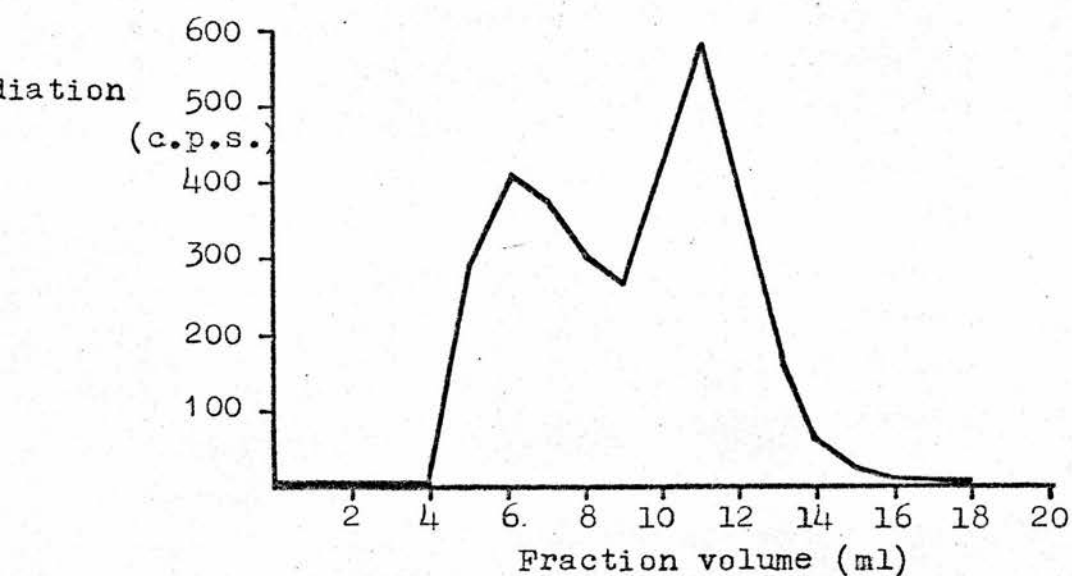


Figure 3.2 Separation of iodination reaction mixture by gel filtration

1ml fractions collected from the gel filtration of an iodination reaction mixture on a G-50 Sephadex column (1cm² x 15cm) were counted for γ -radiation.

Figure 3.3 Polyacrylamide disc gel electrophoresis of ^{125}I -labelled LMF

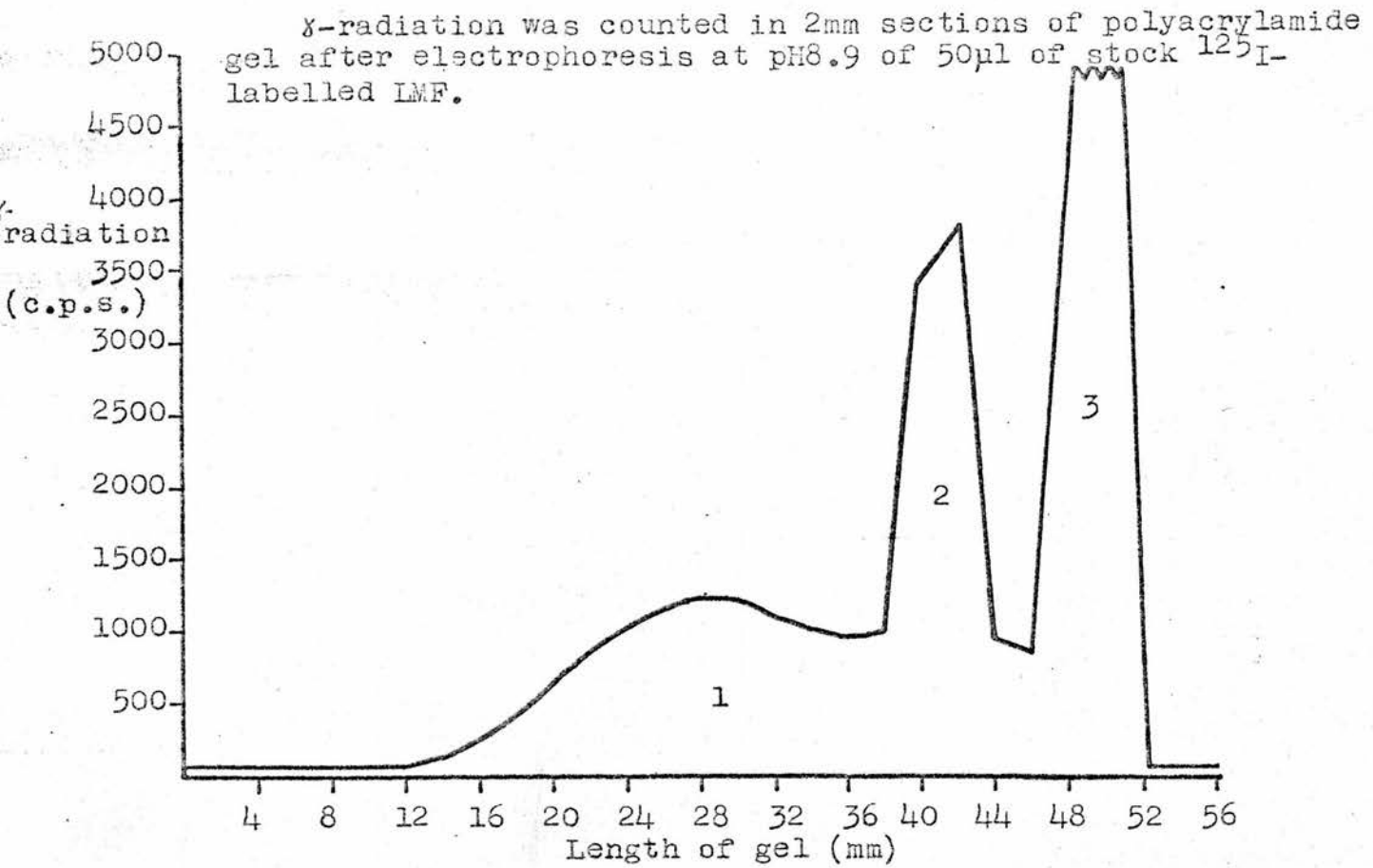
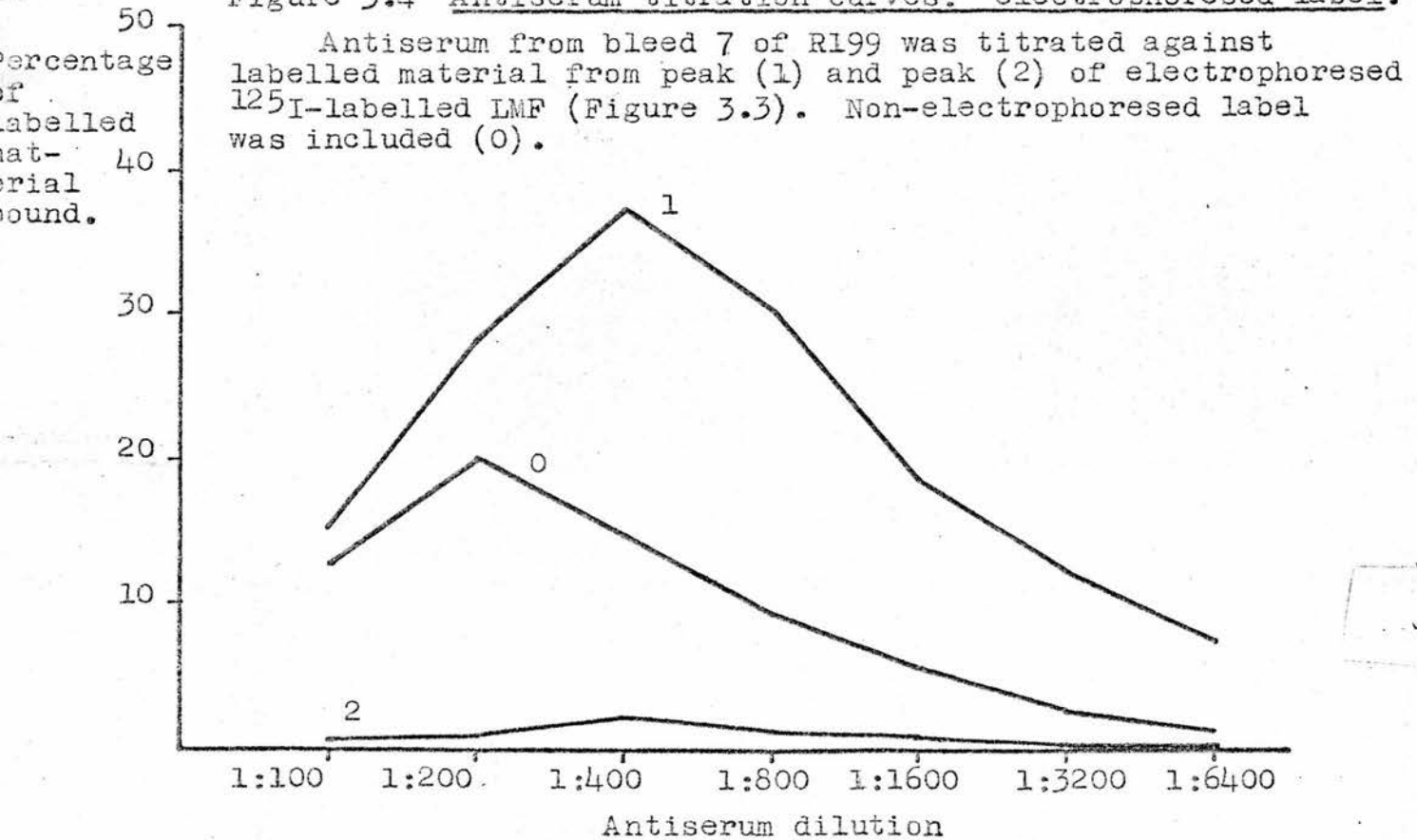


Figure 3.4 Antiserum titration curves: electrophoresed label.



b) Further Investigation of ^{125}I -labelled LMF after Electrophoresis.

The first peak in radioactivity coincided with the highest percentage of eluted label binding to the antiserum (Figure 3.5). Labelled material, eluted from the second and third peaks, exhibited negligible binding to the antiserum. The maximum percentage of eluted label bound by antiserum was 42.5%. The anti-LMF antiserum had only bound 22% of ^{125}I -labelled LMF before electrophoresis of the label.

The third peak, which had the highest radioactive count and the highest mobility, was associated with the bromophenol blue dye front. The second (middle) peak had the same mobility as a secondary band of bromophenol blue which separated from the leading dye front.

c) Time Course of Damage to ^{125}I -labelled LMF.

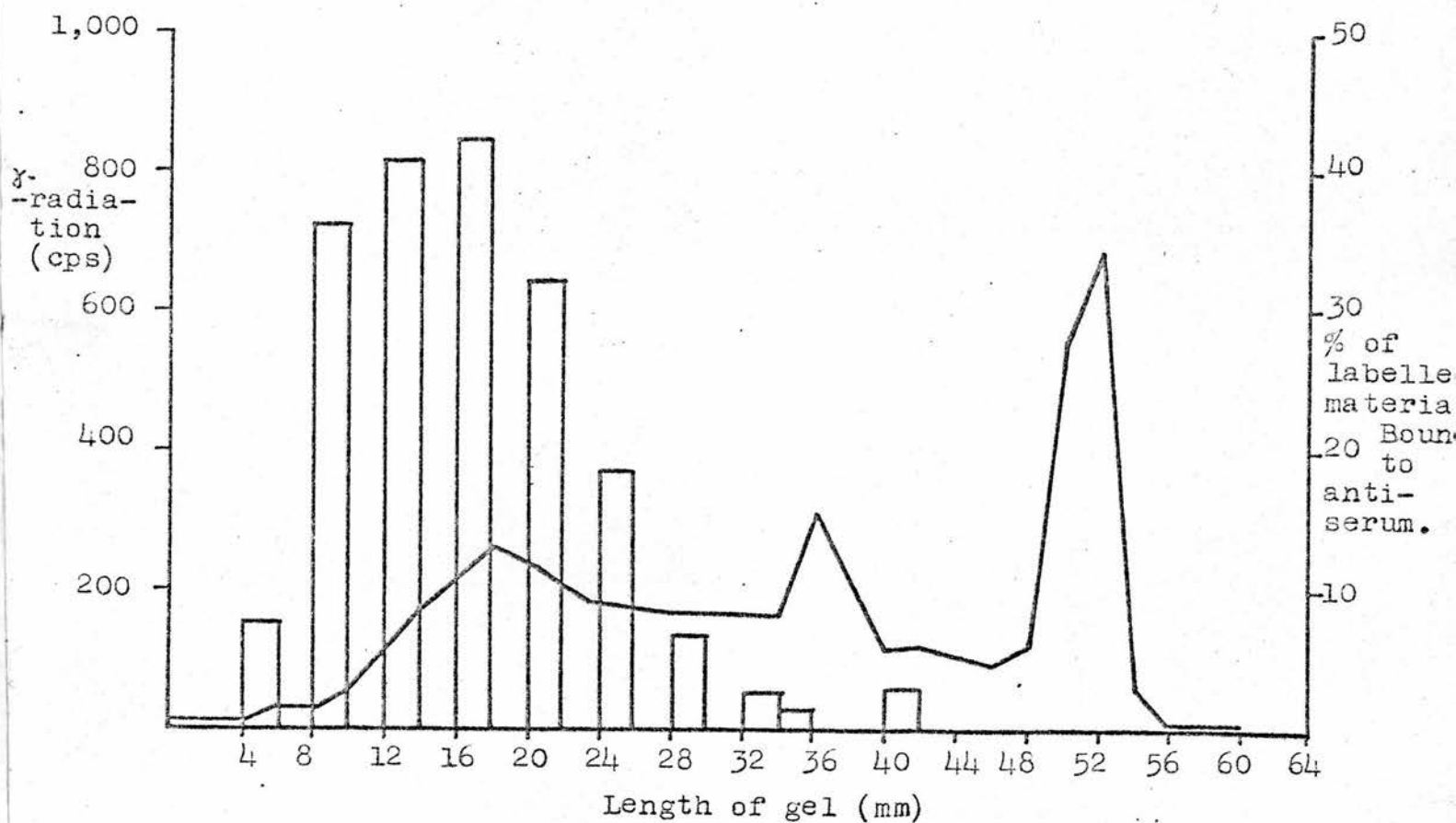
The preparation of ^{125}I -labelled LMF, which in section (a) on electrophoresis gave peaks containing 22%, 20% and 57% of the radioactivity respectively, was again electrophoresed 6 days later. In this case the proportions of radioactivity in peaks 1, 2 and 3 were 12%, 25% and 63%. Another preparation of label was electrophoresed on the same day as it was iodinated and gave 56%, 18% and 25% of the radioactivity in peaks, 1, 2 and 3 respectively.

d) Effect of Buffer on Electrophoresis of ^{125}I -labelled LMF.

The normal buffer (GAD) was replaced by the tris-glycine buffer pH8.9 used for electrophoresis (section 2.2.1.3) for gel filtration of the iodination reaction

Figure 3.5 Polyacrylamide gel after electrophoresis of
 ^{125}I -labelled LMF: binding of eluted labelled
material to antiserum.

The γ -radiation in 2mm slices of polyacrylamide gel after electrophoresis of ^{125}I -labelled LMF is shown by the line graph. The % binding of labelled material eluted from alternate slices to a 1:400 dilution of bleed 10 of R199 is represented by the vertical bars.



mixture of one preparation of ^{125}I -labelled LMF. 0.1% bovine serum albumin was included in the buffer. The elution of radioactive material from the Sephadex G-50 column was not affected by this change. The yield of ^{125}I attached to the LMF was 50% and the specific activity of the product was 85 $\mu\text{Ci}/\mu\text{g}$. The electrophoresis, immediately after gel filtration, of label in tris-glycine buffer on polyacrylamide gel is shown in Figure 3.5. There was no difference between this pattern of mobilities and with ^{125}I -labelled LMF in GAD buffer.

3.3.2 Assessment of Antisera for Radioimmunoassay

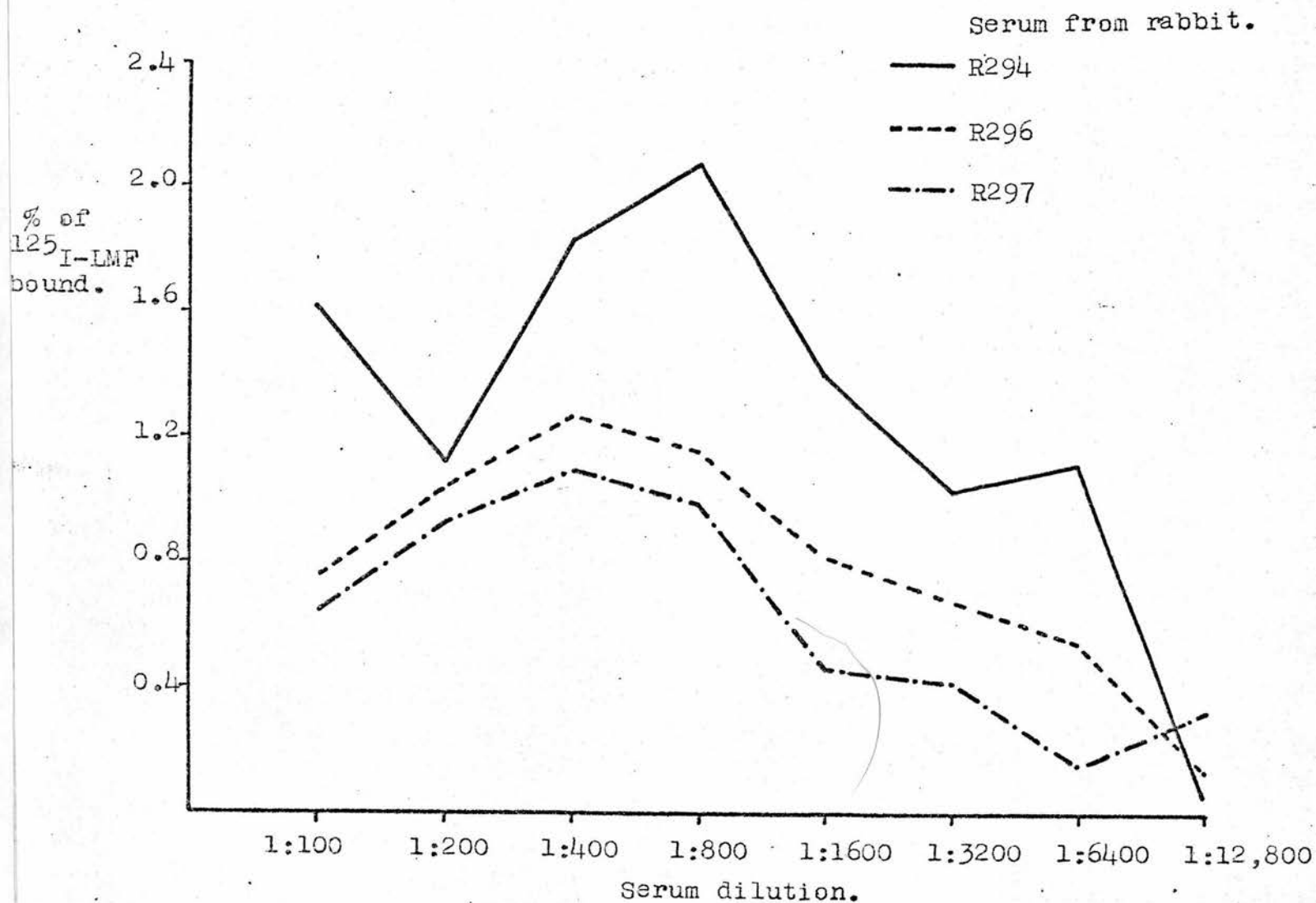
3.3.2.1 Antisera Raised against LMF Emulsified in Freund's Complete Adjuvant.

No sample of sera from rabbits R294, R296 or R297 bound more than 3% of ^{125}I -labelled LMF. The maximum was found with serum bled 4 weeks after the primary immunization. Antiserum titration curves for these sera are shown in Figure 3.6. The serum from R294 showed the maximum binding at a dilution of 1:800. Re-immunization did not increase the binding of serum from subsequent bleeds to ^{125}I -labelled LMF.

3.3.2.2 LMF Conjugated to Bovine Serum Albumin and Emulsified in Freund's Complete Adjuvant

a) Antiserum Titration Curves and Avidity of Binding. From the rabbits immunized with LMF-BSA conjugate, the serum from R199 consistently bound more ^{125}I -labelled LMF and had the steepest antiserum titration curve. Serum from the tenth bleed of all 3 rabbits (R180, R198, R199) gave the antiserum titration curves shown in Figure 3.7. At a dilution

Figure 3.6 Antiserum titration curves: Sera from rabbits R294, R296, R297.



Serum, prepared from blood taken 4 weeks after the rabbits were immunized with LMF in Freund's complete adjuvant was titrated against ^{125}I -labelled LMF.

of 1:400, serum from R199 bound 22% of the label. Sera from the other rabbits bound no more than 7% at any dilution.

The antiserum titration curves obtained with serum from different bleeds of rabbit R199 are compared in Figure 3.8. Though the results are not from one experiment, an increase in binding on re-immunization after the third and eighth bleeds could be seen. The ninth and tenth bleeds gave the maximum binding. Bleeds 1 and 2; 4, 5, 6 and 7; 8, 9 and 10, were assayed in three separate experiments while the third bleed was assayed alone.

The avidity of binding, an estimate of which is given by the steepness of the descending antiserum titration curve, increased up to bleeds 9 and 10. After the tenth bleed a further re-immunization increased neither the maximum binding of the serum to ^{125}I -labelled LMF, nor the avidity of its binding.

b) Standard Curve and Specificity of Antiserum. The standard curve of Y (as a percentage of zero standard) against the log of the dose of LMF (x) is shown in Figure 3.9. Below 10ng/ml unlabelled LMF did not inhibit the binding of the antiserum to ^{125}I -labelled LMF.

When the logit of Y was plotted against the log of the dose of LMF (x) a straight line could be drawn through the points (Figure 3.10). The logit of Y for pituitary hormones were also plotted against the log of their dose (x). pGH, oPRL and pACTH did not inhibit the binding. Trygstad's porcine LMF at 1,100ng/ml reduced the binding to

Figure 3.9 Standard Curve Inhibition of anti-LMF antiserum binding to ^{125}I -labelled LMF by unlabelled LMF.

Increasing concentrations of unlabelled LMF were incubated with serum from bleed 10 of R199 at 1:400 dilution and ^{125}I -labelled LMF. Inhibition of binding (Y) recorded as the amount of label bound as a percentage of the label bound in the absence of unlabelled LMF.

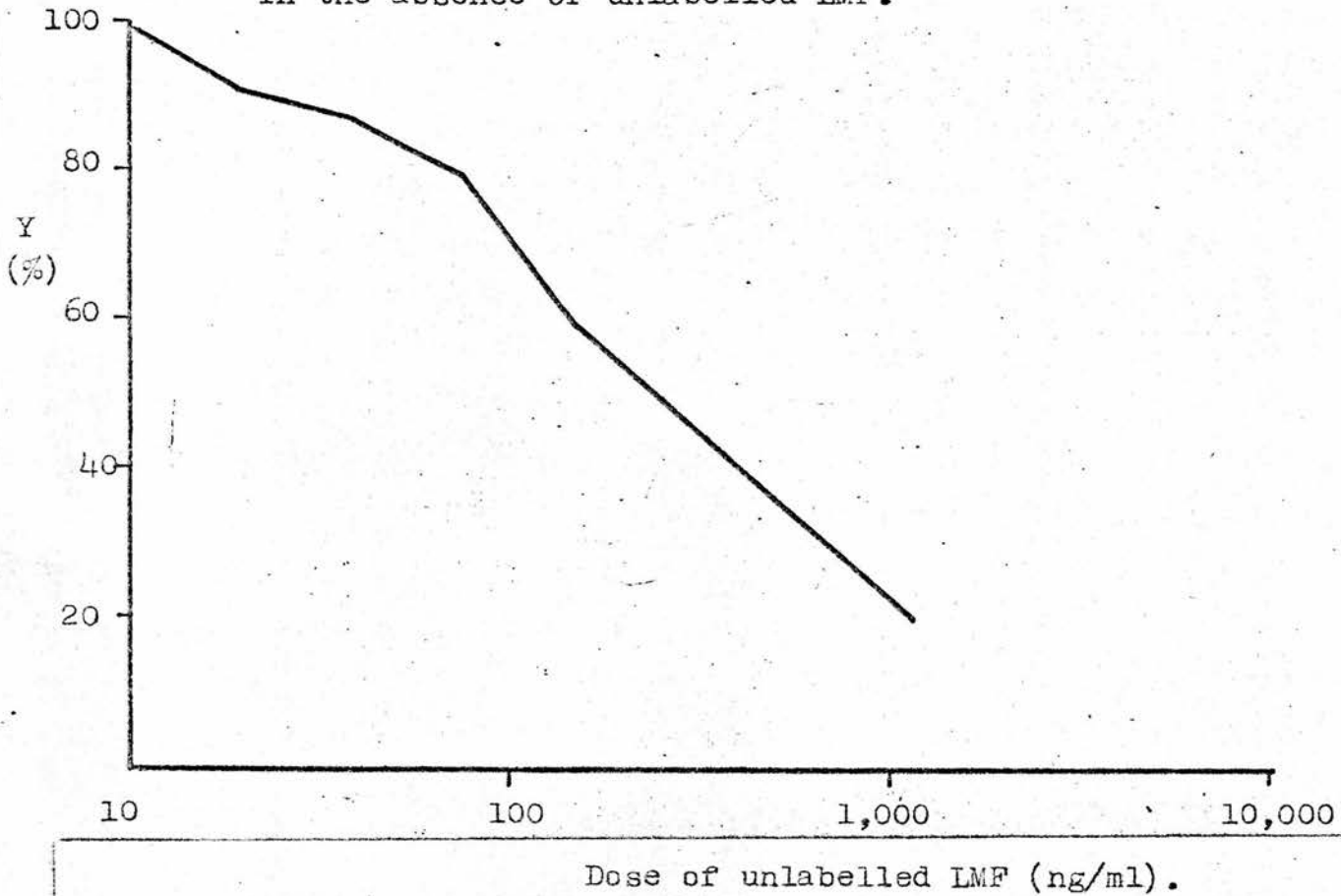
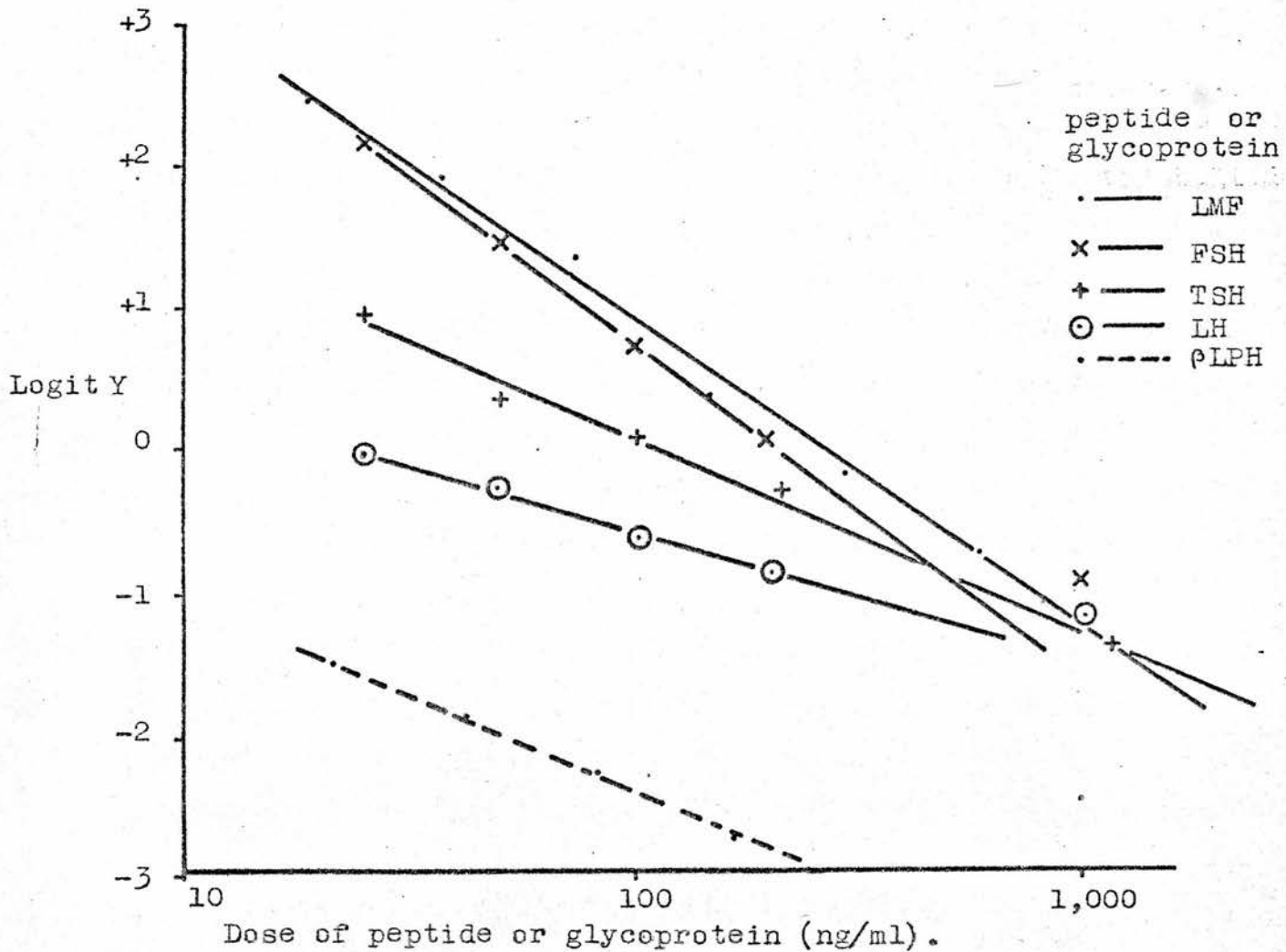


Figure 3.10 Specificity of antiserum from rabbit R199.

Increasing concentrations of pituitary peptides and glycoproteins were incubated with serum from bleed 10 of R199 at a dilution of 1:400 and ^{125}I -labelled LMF. Inhibition of binding recorded as Logit Y. Values for LMF are from the data used in Figure 3.9.

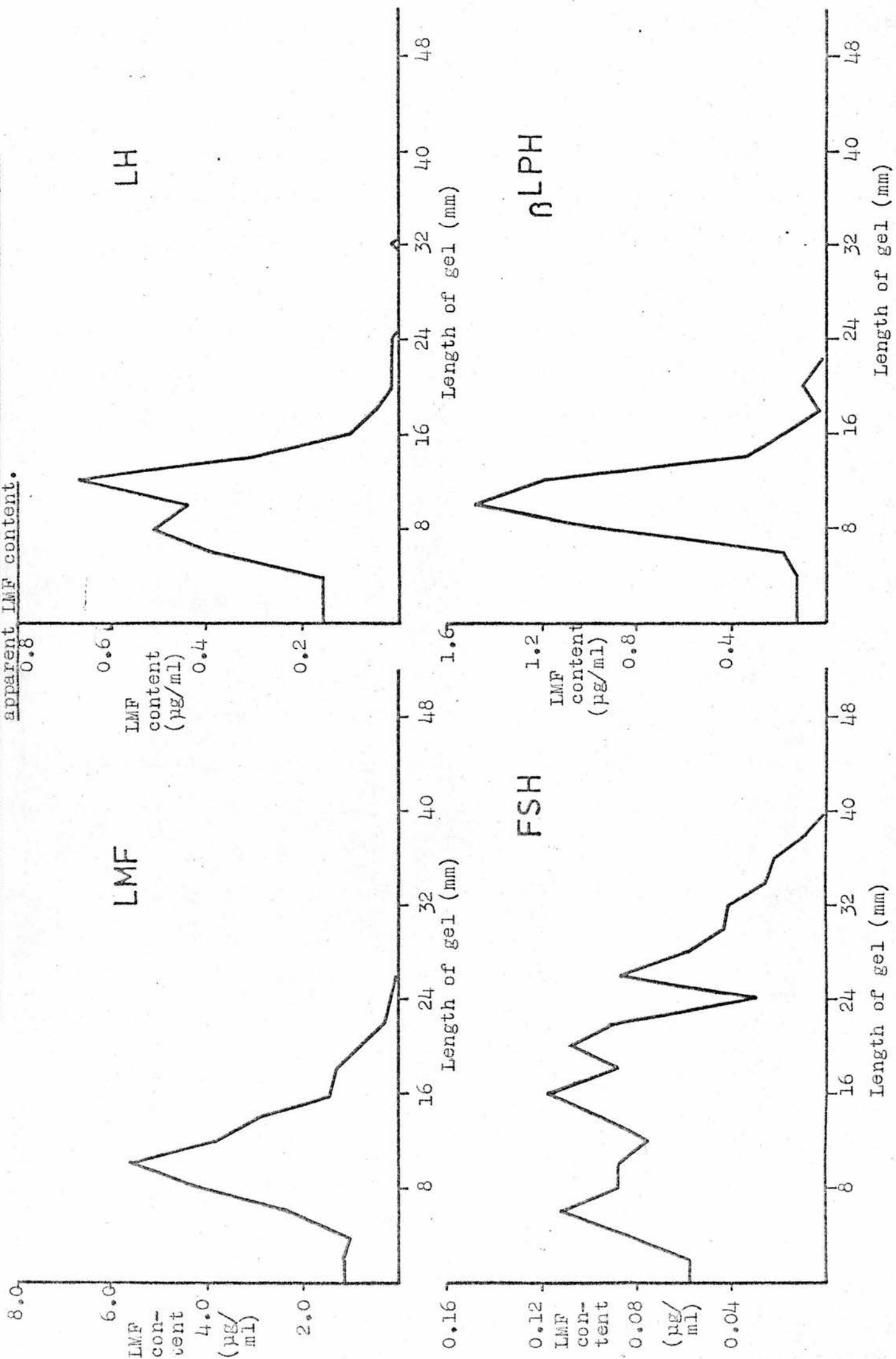
48% of the zero standards but at other concentrations was not effective. β LPH, oLH and oTSH showed a greater inhibition of binding than the LMF standards. oFSH was equivalent in inhibition to unlabelled LMF. The titration curves of the hormones were not parallel to the LMF standard curve, indicating that there was some difference in immunological reaction.

c) Nature of Non-Specific Cross-Reactions. After electrophoresis of mLMFd, the slice of polyacrylamide gel which, when eluted, gave the greatest cross-reaction with anti-LMF anti-serum (Figure 3.11) corresponded with the heavily stained protein band seen in plate 3.1. There was a similar peak of cross-reacting material in the eluates of the gel on which β LPH had been electrophoresed. This corresponded to the more basic (i.e. least mobile) of the two main stained protein bands on an identical gel. LH showed a double peak of cross-reacting material, neither of which had exactly the same mobility as the LMF peak. On the stained gel, a diffuse protein band covered both peaks. The stained FSH gel revealed the heterogeneity of the preparation. The main (acidic) protein band did not correspond in mobility to any of a number of irregular peaks of cross-reacting material eluted out of the sliced gel. These peaks were more basic in nature.

The assay detected 4 times more cross-reacting material in the LMF gel than in the β LPH gel, 10 times more in the LMF gel than in the LH gel and 50 times more than in the FSH gel.

Figure 3.11 100µg of LMF, β LPH, FSH and LH were electrophoresed at pH8.9, the polyacrylamide gels sliced and the segments soaked overnight in 2ml of GAD. The eluate from each slice was assayed for apparent LMF content in a radioimmunoassay using ^{125}I -labelled LMF, serum from bleed 10 of R199 at 1:400 dilution and an LMF standard curve. The apparent LMF content was recorded as its concentration in the slice eluates. Note the scale on the ordinate varies between diagrams.

Figure 3.11 Polyacrylamide gels of pituitary peptides, and glycoproteins assayed for apparent LMF content.



The antiserum used in the previous assay and prepared from the tenth bleed of rabbit R199, bound 4 times as much ^{125}I -labelled LH as ^{125}I -labelled LMF (Table 3.2). At dilutions of 1:200 and 1:400 the antiserum bound over 60% of ^{125}I -labelled LH while binding less than 20% of ^{125}I -labelled LMF.

Preparations from extractions XI and XII, assayed by an LH radioimmunoassay, contained little LH. The highest concentration (Table 3.3) of LH in preparations from both extractions was found in the acidic fraction, which was absorbed by DE 32 cellulose (dLMF₂). The fractions, which by polyacrylamide disc gel electrophoresis contained most LMF (mLMFd), had a lower concentration of LH. As the protein concentration was 20 $\mu\text{g}/\text{ml}$ and the estimated LH content of the mLMFd was 2.1 or 1.1 ng/ml , the maximum contamination of the latter by LH was estimated as 1:10,000.

3.3.2.3 LMF in Polyacrylamide Gel as the Immunogen

Sera from the rabbits which had been injected with homogenized polyacrylamide gel containing LMF, did not exhibit any binding to ^{125}I -labelled LMF. Re-immunization of the animals (R1298, R1299, R1300) after 4 weeks also failed to produce a response.

After immunizing the same animals after 12 weeks with LMF in Freund's Complete Adjuvant and not in polyacrylamide gel, a slight response was found in one rabbit. Serum from R1300 consistently bound to ^{125}I -labelled LMF but with a low percentage of the available label being bound (less than 10%). This serum, at a dilution of 1:400, was tested for specificity of cross-reaction in a

Table 3.2 The binding of ^{125}I -labelled LH to anti-LMF antiserum.

Serum Dilution	% of ^{125}I -labelled LH bound by serum	% of ^{125}I -labelled LMF bound by serum
1:100	8.4 \pm 2.3	1.9 \pm 0.3
1:200	69.4 \pm 1.2	17.6 \pm 0.3
1:400	60.3 \pm 0.2	14.6 \pm 1.1

Serum from bleed 10 of R199 was titrated, in triplicate, against both ^{125}I -labelled LH and ^{125}I -labelled LMF, as for the antiserum titration curves. Results are presented as mean \pm SEM and n=3.

Table 3.3 LH content of LMF preparations.

Extraction	LMF Preparation	^{125}I -labelled LH bound as percentage of zero standards (Y)	Apparent LH concentration (ng/ml) of peptide solution
XI	dLMF2	39.6 \pm 0.7	4.0
	mLMFa	64.9 \pm 1.6	1.7
	mLMFb	58.7 \pm 0.6	2.0
	mLMFc	45.8 \pm 0.8	3.2
	mLMFd	57.8 \pm 0.3	2.1
XII	dLMF2	41.2 \pm 0.5	3.7
	mLMFa	62.7 \pm 0.7	1.8
	mLMFc	55.8 \pm 0.6	2.3
	mLMFd	76.2 \pm 0.8	1.1

Preparations of extractions XI and XII at 20 $\mu\text{g/ml}$ were assayed in triplicate, for LH content. Their inhibition of binding of ^{125}I -labelled LH to anti-LH antiserum is recorded as mean \pm SEM (n=3) and from this the LH content was found by comparison to an LH standard curve.

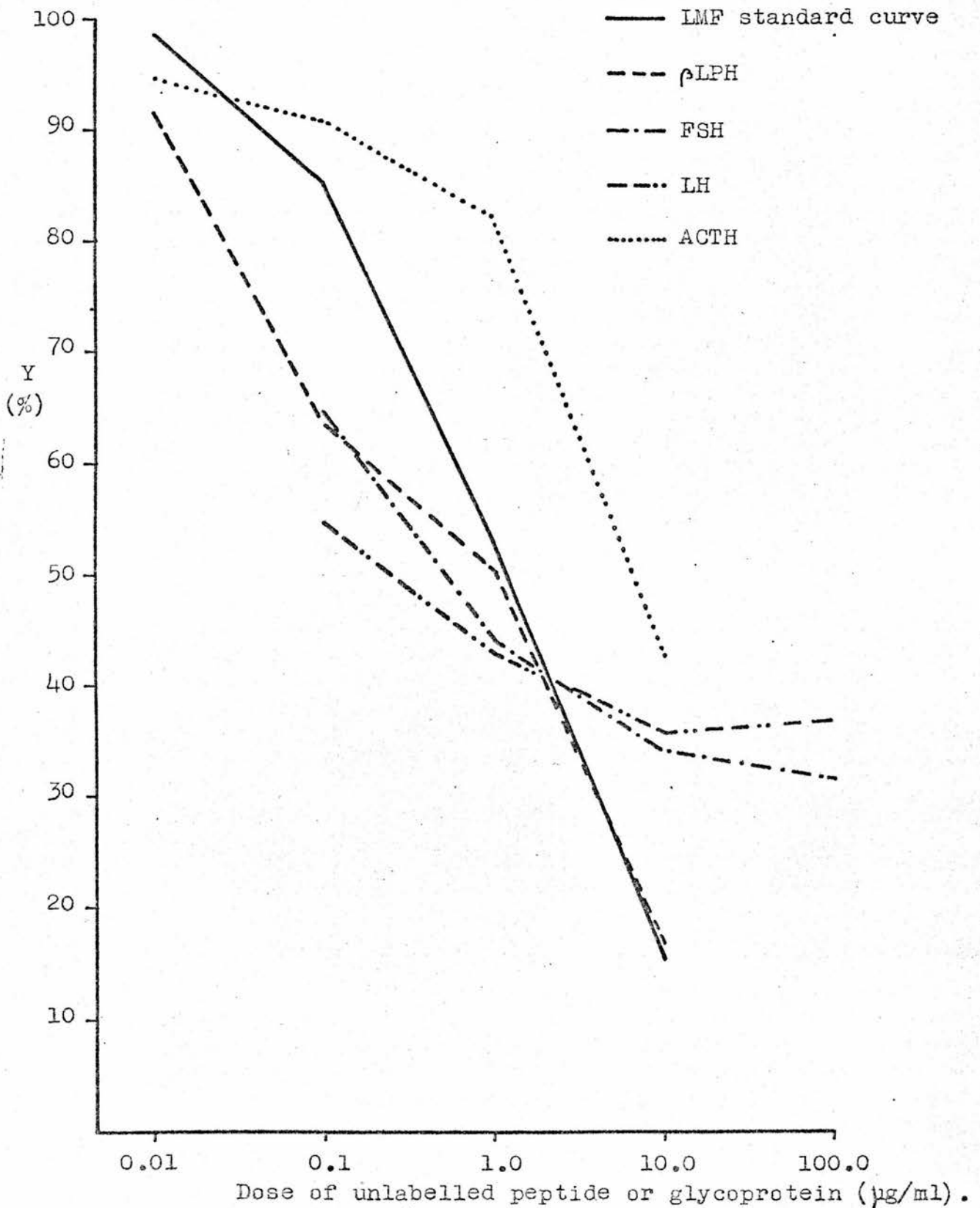
small assay (Figure 3.12). A 4 point standard curve of LMF from 0.01 to 10.0 μ g/ml was set up and compared to ACTH and LPH over the same range. oLH and oFSH were included at concentrations of 0.1 to 100 μ g/ml. The procedure was as described in section 3.2.2.4. LMF was equivalent to β LPH in this assay (Figure 3.12) and also parallel to ACTH which was, however, less potent. Both LH and FSH inhibited the binding of 125 I-labelled LMF to the antiserum but with less avidity than did unlabelled LMF or β LPH. This was reflected by the less steep slopes of the LH and FSH titration curves.

A further injection of LMF in Freund's Complete Adjuvant into R1300 increased neither the maximum binding of its serum to 125 I-labelled LMF, nor the avidity of its binding. LH remained a potent inhibitor of the antibody-antigen reaction.

3.4 Discussion

Labelling of LMF with Iodine - 125

Iodine - 125 was preferred to iodine - 131 as the isotope for labelling of LMF because of its longer half life and higher abundance (Hunter, 1971). Iodine - 125 therefore retains its radioactivity longer than iodine - 131, and in a preparation for labelling, has a higher percentage of the iodide as the radioactive isotope. 125 I is the most commonly used isotope at present for the labelling of peptides and proteins. Hunter (1971) advised that, for the iodination of a novel peptide, a trial with 125 I should be carried out, only changing to 131 I if any problems were encountered.

Figure 3.12 Specificity of serum from rabbit R1300.

A 1:400 dilution of Serum from R1300, which had been injected at first with LMF in polyacrylamide gel, then in FCA, was tested for specificity of inhibition of binding to ^{125}I -labelled LMF. Inhibition of binding (Y) recorded as a percentage.

The chloramine T oxidation method for the iodination of proteins was first introduced by Hunter and Greenwood (1962) for the labelling of human growth hormone with ^{131}I . The procedure, based on this method, proved to be effective for the iodination of LMF with ^{125}I , though considerable variations were found in the yield and specific activities of different ^{125}I -labelled LMF preparations. These variations possibly reflect differences which can occur between batches of the radioisotope (Landon, 1968).

During iodination some damage inevitably occurs to the peptide (Landon, 1968). Irradiation, from the presence of radioactive material, and oxidation by chloramine T, both contribute to this damage. The iodination procedure was designed to minimize the time the peptide was in contact with excess radioisotope and oxidising agent. These were removed by gel filtration, although the damaged products of iodination eluted from the sephadex column in the breakthrough peak, along with the undamaged ^{125}I -labelled peptide. The damaged products of iodination bind non-specifically to other proteins and especially to albumin (Landon, 1968), so even though they may be small the damaged products are not greatly retarded by Sephadex G-50. These damaged products may be labelled with ^{125}I and, if they are present in the label used for radioimmunoassay, lead to a loss of sensitivity and precision in the assay (Greenwood, 1967). In an extreme case, with a highly damaged labelled peptide, the gradient of the standard curve would be zero.

When maximum sensitivity and precision were required, for example, when the specificity of antiserum was assayed, labelled damaged peptide was removed by electrophoresis. The plot of the γ -radiation, detected in slices of polyacrylamide gel following the electrophoresis of ^{125}I -labelled LMF (Figures 3.3 & 3.5) resembled the chromatophoresis of ^{131}I -labelled ACTH described by Landon (1968) although on the latter, ^{131}I -labelled ACTH remained at the origin. The resemblance was seen in the presence of a large peak of high mobility closely followed by a second, smaller peak of radioactivity. On polyacrylamide gel electrophoresis the former peak moved with the bromophenol blue dye at the anion migration front and consisted of free ^{125}I ions, which were thereby removed from ^{125}I -labelled LMF. The second peak of radioactivity consisted of damaged ^{125}I -labelled peptide bound to the albumin present in the diluent. Albumin also binds to bromophenol blue and the correspondence of this second peak of radioactivity with a secondary band of blue dye confirmed that the damaged peptides were associated with albumin. These two peaks contained little labelled material capable of binding to anti-LMF antiserum (Figure 3.5). The low, wide and least mobile peak contained all the immunologically reactive ^{125}I -labelled material. Presumably this was ^{125}I -labelled LMF. The increase in maximum binding and avidity of binding of antiserum to ^{125}I -labelled LMF (Figure 3.4) following electrophoresis revealed the benefit of removing free ^{125}I and damaged labelled peptides from the working label before an assay.

Damage to ^{125}I -labelled LMF did not only occur at iodination. Immediately after iodination 56% of the radioactivity was associated with the immunologically active peak on electrophoresis. Another preparation of ^{125}I -labelled LMF, electrophoresed a week after iodination showed 22% of the radioactivity in this peak and after a further week only 18%. Thus on storing there was a progressive decrease in the proportion of immunologically active, ^{125}I -labelled LMF in the preparation. The free ^{125}I and the damaged labelled peptide peaks both increased over the same period.

The electrophoretic mobility of the peak of ^{125}I -labelled LMF did not correspond to that of unlabelled LMF stained with amido black. The latter had a mobility of approximately 0.28 while the former had a variable but higher mobility of between 0.33 and 0.57. The introduction of negatively charged iodine into the peptide molecule was a likely cause of the higher mobility. The different mobilities were probably due to differences in specific activities of the preparations. However, there was the possibility that the product of iodination was not ^{125}I -labelled LMF but labelled peptide with a normally higher mobility which migrated to its usual position on electrophoresis. If this were the case, when unlabelled LMF was electrophoresed, the gel sliced and the segment eluates assayed for inhibition of the binding of anti-LMF antiserum to the labelled peptide, the greatest inhibition should also have exhibited a higher mobility. This did not happen, and material eluted from the normal, migratory position of LMF

was the most potent inhibitor of the antibody-antigen reaction (Figure 3.11). Electrophoresis was therefore able to separate ^{125}I -labelled LMF from any unlabelled peptide as well as from damaged labelled peptide and free ^{125}I .

The peak of radioactivity which contained immunologically active ^{125}I -labelled LMF was low and broad. At first, it was thought that the width of the peak could be due to extraneous ions, present during electrophoresis, coming from the buffer in which the labelled peptide had been prepared and stored. However, the same wide peak was found on electrophoresis of an aliquot of the labelled peptide in the tris-glycine buffer used for electrophoresis. Possibly the wide peak was another result of the introduction of ^{125}I into the LMF molecule.

Production of Antiserum

The production of antisera, suitable for radioimmunoassay, against a specific immunogen has been called more of an art than a science, with chance playing an important part (Hurn & Landon, 1971). When faced with the problem of raising antisera against a novel antigen, the recommended course of action is to employ a simple technique. If this fails the method can be altered until success is achieved. Factors which can affect the production of a specific antigen have been reviewed by Chase (1967) and Hurn & Landon (1971). They include immunogenicity of antigen, the immunized species, dose of immunogen, route of administration, use of adjuvant and timing of injections and bleedings.

The rabbit was chosen as the animal to be immunized with LMF. They are the most widely used species for antibody production and are relatively easy to handle. In a previous attempt to produce anti-LMF antiserum, Norman & Turter (1968) raised an antiserum in rabbits, which cross-reacted with growth hormone instead of LMF. The report of at least some response to LMF was an encouragement to use the same species.

Only three rabbits were immunized with each method of administration, as there was not a plentiful supply of LMF. A compromise was reached between immunizing a large number of animals with a small dose of LMF or less rabbits with more immunogen. In the first attempt to raise anti-LMF antiserum, 250 μ g of LMF, emulsified in Freund's Complete Adjuvant, was injected into each rabbit. 100 μ g per animal of immunogen can stimulate the immune response (Hurn & Landon, 1971), but a larger dose of LMF than this was administered in the attempt to produce antiserum specific for this peptide. The dose of LMF was increased to 330 μ g per animal in the subsequent booster injections. None of the rabbits so treated produced serum which bound more than 3% of 125 I-labelled LMF. It was concluded that in its native state LMF could not easily stimulate the production in rabbits of antibodies suitable for immunoassay.

LMF Conjugated to Bovine Serum Albumin as an Immunogen

Before a further 3 rabbits were immunized, LMF was conjugated to bovine serum albumin (BSA) by the 'carbodiimide'

method first described by Goodfriend et al. (1964) and reviewed by Lekhite & Sehon (1967). A possible reaction pathway was illustrated in Figure 3.1. In this case, LMF was the hapten and BSA was the protein. Not all the bonds formed are protein-hapten linkages. Other products can be protein-protein or hapten-hapten conjugates, but these are not likely to interfere with the immune response and could augment it.

The scarcity of LMF was a problem in using the conjugation reaction. Goodfriend et al. (1964) conjugated 20mg of bradykinin to albumin and seldom has less than 5mg of hapten been conjugated to a carrier protein (Lekhite & Sehon, 1967). For the small amount of LMF conjugated to BSA a lengthy reaction time was employed. The success of coupling bradykinin to albumin had been assessed by amino acid analysis of reactants and products. It was estimated that 12 molecules of the hapten had been bound to 1 molecule of albumin (Goodfriend et al., 1964). As LMF was in short supply, the successful completion of conjugation was gauged by the success of the complex stimulating the production of anti-LMF antiserum.

Assessment of Antiserum

At first the conjugation of LMF to BSA did appear to increase the immunogenicity of the LMF. One of the immunized rabbits (R199) responded to the treatment and serum, prepared from the ninth and tenth bleed of this animal, bound over 30% of ^{125}I -labelled LMF. Further injection of conjugate in FCA did not increase the binding.

Serum from bleeds 9 and 10 of R199 also had the steepest descending slope of the antiserum titration curves (Figure 3.8), which was originally thought to indicate a high avidity of binding to ^{125}I -labelled antigen (Berson & Yalow, 1964). Although the steepness of this part of the curve is now considered to be a poor guide to avidity and sensitivity (Hurn & Landon, 1971), the serum from these bleeds was the most eligible, of the sera produced, for use in a radioimmunoassay. However, the finding that serum from R199 also cross-reacted with a number of other pituitary hormones (Figure 3.10) indicated that it was not suitable for a radioimmunoassay.

For the binding of ^{125}I -labelled LMF to the antiserum to be inhibited by non-porcine glycoprotein hormones such as ovine LH, TSH and FSH the antiserum must have had a wide spectrum of cross-reaction. That porcine ρ LPH should have inhibited the binding and have been the most potent inhibitor, was less surprising, as this peptide as well as being from the same species, possesses similar biological properties to LMF (Gilardeau & Chrétien, 1970). There could be structural resemblances between LMF and ρ LPH. However, it seems likely that the ρ LPH preparation, which was only 90% pure (Graf, personal communication) was contaminated with a peptide resembling LMF. The immunologically active peptides in both LMF and ρ LPH had the same electrophoretic mobility (Figure 3.11) and this corresponded to the stained peptide band of mLMFd in plate 3.1. The slower subsidiary

band of ρ LPH had this mobility on staining and it was possible that this either was LMF or bore a close resemblance to it. The main peptide band of ρ LPH was not immunologically active.

Nature of Non-Specific Cross-Reactions

The cross-reaction between the glycoprotein pituitary hormones and ^{125}I -labelled LMF could be due to contamination of the LMF preparation, conjugated to albumin, with one or more of the hormones. Another possibility is that LMF has a similar antigenic determinant to the glycoprotein hormones, which have a common α -subunit (Pierce, 1971; Maguin-Rogister *et al.*, 1976).

In the first case, contamination of LMF, or even the bovine serum albumin, with one of the more immunogenic glycoprotein hormones could have stimulated the production of antibodies. Antibodies produced against the common α -subunit of the glycoprotein hormones would cross-react not only with the particular hormone present in the emulsion injected, but also with the other glycoprotein hormones. However, for antibodies produced against a contaminant of LMF to interfere in a radioimmunoassay for LMF, the peptide preparation used for labelling with ^{125}I must also have contained the contaminant. The preparation used as label in the majority of assays (gmLMF₁) appeared to be homogeneous on electrophoresis at pH 8.9 in polyacrylamide gel (Plate 2.4). A similar preparation (mLMFd) to the one used as hapten (mLMF-VIID) showed, on electrophoresis, one main band of LMF mobility with some contamination (Plates 2.1,

2.5 and 3.1). On electrophoresis in SDS polyacrylamide gels, this preparation showed (Plate 2.2) a large molecular weight contamination which could well have been a glycoprotein hormone. Possibly this contamination stimulated the production of antiserum and was present in sufficient amount in the ^{125}I -labelled peptide to cause the variety of cross-reactions seen in Figure 3.10. The contaminant was probably not LH, as various LMF preparations showed only slight inhibition of the binding of anti-LH antiserum to ^{125}I -labelled LH in an LH radioimmunoassay (Table 3.3). The proportion of LH in the LMF preparation appeared to be in the order of 1:10,000.

On electrophoresis, the LH cross-reactivity with anti-LMF antiserum appeared as a twin peak, the trough of which corresponded to LMF's mobility (Figure 3.11). Apparently the immunological activity of the unlabelled LH and LMF did not occur in identical fractions. The immunological activity of FSH which had irregular mobilities after electrophoresis and which did not correspond to the main protein band was probably due to contamination of the FSH preparation.

The inhibition of the binding of anti-LMF antiserum to ^{125}I -labelled LMF by the glycoprotein hormones could be explained if LMF had a similar structure to their common subunit. In this case antibodies raised against some of the antigenic determinants of LMF would also bind to the α -subunit of the glycoproteins. This would result in the cross-reactions seen in Figure 3.10. The cross-reaction of β LPH,

which had a higher potency than any of the glycoproteins, could still be explained by assuming that LMF was the minor band seen on electrophoresis of β LPH. This would still have a stronger cross-reaction with anti-LMF than the glycoproteins, which would only contain similar sections to LMF and not the whole molecule. However, a much larger inhibition of the binding of anti-LH antiserum to ^{125}I -labelled LH by LMF than that found might be expected if this were the case, especially as LH was the most potent of the glycoproteins to inhibit the binding of ^{125}I -labelled LMF. Alternatively little inhibition was seen in the LH radioimmunoassay because the anti-LH antiserum did not bind to the antigenic determinants which were common to the α -subunit and to LMF. The anti-LH antiserum could have been specific for the β -subunit of LH.

A third possible reason for the production of antiserum, which could bind to LH, by the immunization of rabbits with LMF-BSA conjugate could be that glycoprotein contaminants of LMF were preferentially bound to the albumin during conjugation. Thus the main immune response would produce antibodies against the glycoprotein contaminants rather than against LMF. This would be supported by the finding that antiserum from R199 could bind 4 times as much ^{125}I -labelled LH than ^{125}I -labelled LMF (Table 3.2).

It was apparent that the antiserum raised against LMF-BSA conjugate was not suitable for a radioimmunoassay of LMF due to its cross-reaction with other pituitary hormones. A third method was attempted to stimulate the production of anti-LMF antiserum.

LMF in Polyacrylamide Gel as Immunogen

Purification of LMF by polyacrylamide disc gel electrophoresis, before immunization of rabbits with the homogenized gel containing the peptide, was intended to remove any possible contamination by glycoprotein hormones. After injection into the animal the immunogen slowly diffuses out of the polyacrylamide gel and stimulates the immune response. The method has been successfully used by Weintraub & Raymond (1963) and Green *et al.* (1974). Unfortunately LMF in polyacrylamide gel was no more effective in the production of antibodies than LMF in Freund's Complete Adjuvant.

Further immunization of the rabbits with non-electrophoresed LMF in Freund's Complete Adjuvant did produce antibodies, in one rabbit (R1300), that would bind a small amount of ^{125}I -labelled LMF. This serum also cross-reacted with β LPH, ACTH and the glycoprotein hormones LH and FSH (Figure 3.12). The slope of the titration curves with the last two hormones were less steep than the slope of the LMF standard curve. The titration curve of β LPH closely resembled that of LMF while ACTH gave a parallel curve displaced from the LMF standard curve. This indicated that there was a similarity of cross-reaction between LMF, β LPH and ACTH. The glycoproteins were only partially cross-reacting and therefore a lower gradient in their curves was found (Greenwood, 1967).

Neither increased binding or improved specificity resulted from a further immunization of rabbit R1300 so LMF had proved again to be a poor stimulator of antibody pro-

duction. There are a number of possible alterations which would have been made to the procedure for antibody production if time had allowed. These are mentioned in the general discussion (Chapter 5) which includes a section on possible future work.

3.5 Summary

- 1) LMF was successfully labelled with ^{125}I on a number of occasions. The ^{125}I -labelled LMF was purified for some assays by polyacrylamide disc gel electrophoresis, which removed free ^{125}I , damaged labelled peptide and unlabelled peptide from the labelled antigen.
- 2) The immunization of 3 rabbits with LMF emulsified in Freund's Complete Adjuvant failed to produce antiserum for use in a radioimmunoassay.
- 3) The immunization of 3 rabbits with LMF conjugated to BSA and emulsified in Freund's Complete Adjuvant produced anti-LMF antiserum which also cross-reacted with other pituitary peptides and glycoproteins. Due to this cross-reaction the antiserum was not suitable for use in a radioimmunoassay.
- 4) The immunization of 3 rabbits with LMF in homogenized polyacrylamide gel after electrophoresis of LMF failed to produce anti-LMF antiserum. A later injection of LMF in Freund's Complete Adjuvant did stimulate one of these rabbits to produce antiserum which was again not suitable for use in a radioimmunoassay due to non-specific cross-reactions.

CHAPTER 4The Effect of Serum on LMF Induced Lipolysis4.1 Introduction

Trygstad & Foss (1967) reported that human serum could inhibit the adipokinetic activity of human LMF in vivo in rabbits. Serum from one group of obese patients inhibited LMF more than did serum from normal patients. Serum from children with lipodystrophy had a negligible effect. Later they partially purified the inhibitory factor from human serum and concluded that it was a proteolytic enzyme associated with albumin (Trygstad & Foss, 1972).

The situation in swine was investigated (Trygstad et al., 1972; Standal et al., 1973) using samples from pigs which had been bred with growth rate and leanness as selection criteria. Over ten generations of a breeding experiment (Vangen, 1974a), 3 lines of Norwegian Landrace pigs had evolved. The HP-line had pigs with low backfat and high growth rate. The LP-line had animals with high backfat and low growth rate. The control line (C-line) had not been subjected to selection. The lines also differed in a number of non-selected traits (Table 4.1). Serum, taken at slaughter from these 3 lines of pigs caused differing inhibition in vivo of the lipolytic action of human LMF in rabbits. Serum from the HP-line was least inhibitory while serum from the LP-line caused most inhibition. Subcutaneous fat pads which had also been taken from the animals at slaughter, showed a variable release of free fatty acids on incubation in vitro with porcine LMF.

Table 4.1. Pigs have been selected over 10 generations for high growth rate and low backfat thickness (HP-line) or low growth rate and high backfat thickness (LP-line). The control animals (C-line) were produced by crossing the other two lines in the early stages of the experiment and were then maintained with no selection. A number of reports have been published describing these animals.

References:

1. Vangen (1974a); 2. Vangen (1974b); 3. Vangen (1972);
4. Bakke & Standal (1975); 5. Standal et al (1973);
6. Bakke (1975); 7. Lund-Larsen & Bakke (1975);
8. N. Standal, personal communication.

Table 4.1

Differences between lines of pigs bred using criteria based on growth rate and backfat thickness.

Trait or Biochemical Parameter Measured	Year of pigs tested	Ref.	HP-line	Control line	LP-line
a. Selected Traits					
Rate of Growth (g/day)	1967 - 1971	1	515	504	484
Thickness of Backfat (mm)	1966 - 1971	2	22.5	28.3	37.4
b. Non-selected Traits					
Feed conversion ratio (F.U./kg gain)	1966 - 1971	1	3.07	3.24	3.30
Pre-weaning weight at 21 days (kg)	1966 - 1971	2	5.9	5.6	5.4
Mortality: Number alive at 6 weeks as percent of total born	1966 - 1970	3	70.7	-	75.8
Intramuscular fat content (%)	1971	4	1.21	1.01	1.47
c. Biochemical Parameter					
FFA released <u>in vitro</u> from fat pads by 1 μ g of porcine LMF (μ mol/100 mg/3hrs)	1969	5	1.16	0.83	0.40
Serum FFA in rabbits 90 min. after injection with 0.1mg LMF+3ml pig serum (meq/l)	1969	5	5.03	3.35	1.29
Pig serum FFA after 25 hr fast (meq/100ml)	1971 - 1972	6	121.08	47.41	30.96
Pig serum Glucose after 25hr fast (mg/100ml)	1971 - 1972	6	62.36	73.06	80.09
Pig serum Triiodo-thyronine (nmol/Litre)		8	1.49	2.30	1.15
Pig serum Growth Hormone (ng/ml)	1973	7	23	20	14

Lipolysis was greatest with the fat of the lean HP-line animals and least from the fat of the LP-line pigs. Fat from the control line animals gave an intermediate release of free fatty acids.

The objective here was to develop a method, based on these observations, which could be used to predict the future growth rate and leanness of an animal. Measurement of serum inhibition of LMF induced lipolysis would be more convenient than an assay of induced lipolysis in excised adipose tissue from individual animals. The serum samples would, have to be taken (with the minimum of stress) from a young animal, not at slaughter, for a prediction of future growth to be of value. Serum from the pigs in the breeding experiment (Vangen, 1974a) described above provided suitable material for use in establishing a method to predict the growth of an animal.

Initially an attempt was made to quantify the reported proteolytic nature of the serum inhibition of LMF (Trygstad & Foss, 1972). Incubation of LMF with the partially purified inhibitory factor for an hour, prior to electrophoresis, resulted in the stained band of LMF completely disappearing. The reduction in precipitation of ^{125}I -labelled LMF by trichloroacetic acid following incubation with serum was used to measure the degradation of LMF.

To investigate the effect of serum on the biological action of LMF, incubation in vitro was preferred to an in vivo method. The latter would have involved a large number of

rabbits and a large amount of lipolytic peptide to survey a reasonable number of individual pigs. Rabbit adipose tissue was used in the in vitro assay, as Trygstad (1968b) had reported that human LMF did not stimulate lipolysis in fat from normal mice or rats. By using an isolated fat cell suspension based on the rat fat cell suspension of Rodbell (1964), instead of fat pad slices, the accuracy of the results was improved in that standard errors were reduced. The results within an assay were more consistent though inter-assay variation was increased (Schleyer et al., 1971). Pooled serum collected from animals at slaughter was first used to establish standard conditions. Then sera from a number of animals in the HP, LP and C-lines were examined for any variation in their effect on LMF induced lipolysis.

4.2 Methods

4.2.1 Degradation of ^{125}I -labelled LMF

A sample of Trygstad's porcine LMF was labelled with iodine-125 as described in Section 3.2.2.1. Serum, from each line of pigs in the breeding experiment (Standal et al., 1972; Vangen, 1974a) was pooled according to line. 1ml of serum from 20 animals in each line was mixed.

0.1ml of LMF working label was incubated, in quadruplicate, at 37°C with 0.5ml of the pooled serum from each of the 3 lines of pigs. After a trial experiment, when incubation time varied from 0 to 2 hours, the serum and labelled peptide mixtures were incubated for 4 days. To duplicate samples, 0.5ml of 15% (W/V) trichloroacetic acid (TCA) was added prior to the 4 day incubation as controls.

4 x 0.1ml aliquots of working label were incubated under identical conditions.

After 4 days 0.5ml of 15% (W/V) TCA was added to the incubation tubes, which were mixed and, together with the controls, were centrifuged at 2,500g for 10 minutes. The supernatants were discarded and the precipitates washed twice with 1.0ml of 7.5% (W/V) TCA. The remaining precipitates were counted for 50s in a Nuclear Enterprise γ -spectrometer. The percentage degradation of label over the 4 days was calculated from the difference in precipitated γ -radiation between the incubated samples and the corresponding controls.

4.2.2 In vitro Assay of Lipolytic Activity

The in vitro assay of lipolytic activity described here, but also used in chapter 2, consisted of measuring the effect of the agent under test on the rate of glycerol production from isolated rabbit fat cells. Rabbit fat cells were used in preference to slices of fat pads because trial experiments with fat pads had led to inconsistent and inaccurate results. Standard errors with fat pads were large.

Rodbell (1964) was the first to introduce a method for the isolation of rat fat cells, for the investigation of fat cell metabolism, by digestion of adipose connective tissue by collagenase. There have been few reports of the isolation of rabbit fat cells, but Vezinhet (1973) did use them for the investigation of growth hormone action. The method described here is based on Rodbell's original procedure and on that of Langslow & Hales (1969) who isolated chicken fat cells.

4.2.2.1 Preparation of Buffer

The buffer used throughout the assay was Krebs Ringer bicarbonate (KRB) buffer pH7.4 (Cohen, 1964), which contained half the prescribed calcium content (Rodbell, 1964). Stock solutions at 5 times the required strength were made up in distilled water and stored at 4°C in a cold room, where they remained stable for months. These were, as W/V percentages, NaCl 4.5%, KCl 5.75%, CaCl₂ 3.05%, MgSO₄ · 7H₂O 19.1% and KH₂PO₄ 10.55%. At working strength these were all 0.154M except CaCl₂ which was 0.055M. The concentration of the latter was tested by titration against 0.1M AgNO₃. 5.0ml of 0.055M CaCl₂, with 2 drops of 10% KCrO₄ as indicator, reacted with 5.50ml of AgNO₃ before the end point, when a brown precipitate appeared (Long et al., 1961).

A stock mixture, which was usable for a week if stored at 4°C, was made with the following proportions of stock solutions. NaCl: 100, KCl: 4, Ca Cl₂: 3, MgSO₄ · 7H₂O: 1, KH₂PO₄: 1. This was diluted 5 times with distilled water. A fresh preparation of 1.30% NaHCO₃ (0.154M) was made on the day of use and gassed for 1 hour with CO₂. 16.0ml of this was diluted to 100ml with the stock mixture and gassed for 20 minutes with 95% O₂: 5% CO₂. When necessary bovine serum albumin at 4.0% (W/V) was added to the buffer and stirred under the same gas phase for 20 minutes (KRB-albumin). Finally the buffer was equilibrated at 37°C.

4.2.2.2 Isolation of Rabbit Fat Cells

Male or female rabbits of various strains (New Zealand White, Lop etc.) approximately six months old, weighing

3-4 kg and fed ad libitum were killed by cervical dislocation. Sufficient perirenal fat (6g) was excised as soon as possible after death and placed in KRB buffer at 37°C. A segment of fat was chopped up on a plastic petri dish. Plastic containers were used throughout the assay as glass disrupts the fat cells. 1-2g of the minced fat was weighed into a 25ml plastic capped vial which contained 5.0ml of a 0.4-1.5mg/ml solution of collagenase in KRB-albumin buffer. The vial was shaken vigorously at 37°C for 30-60 minutes on a metabolic shaker, occasionally being shaken by hand. Undigested fat was removed with forceps and the fat cell suspension transferred to a plastic centrifuge tube. Centrifugation at 250g for 1 minute caused the isolated fat cells to form a surface layer. The sediment and infranatant were aspirated by pasteur pipette without disturbing the fat cell layer. The cells were washed 3 times with 5.0ml of KRB-albumin buffer and finally resuspended in the same buffer. The dry weight of fat cells in 1ml of suspension was determined. This was the difference between the mean dry weight of 4 x 1.0ml aliquots of KRB-albumin buffer and the mean dry weight of 4 x 1.0ml aliquots of the fat cell suspension.

4.2.2.3. Assay Conditions

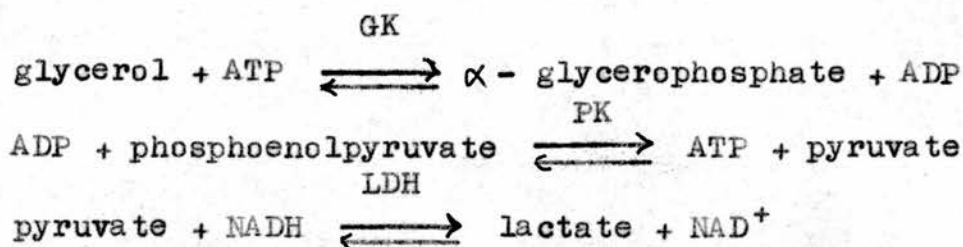
In all in vitro assays the various treatments whether standards, samples or blanks were performed in quadruplicate. Lipolytic agents were dispensed, usually made up in KRB buffer in 0.1ml aliquots to 25ml plastic capped vials. The basal rate of lipolysis was found in blank vials which con-

tained 0.1ml of KRB-albumin buffer. 1.0ml of fat cell suspension was dispensed to each vial by a 1.0ml automatic pipette fitted with a plastic tip with a wide nozzle. The fat cell suspension was constantly shaken to provide an even dispersion of the adipocytes. To further reduce error, which could be caused by uneven distribution of the adipocytes, the suspension was dispensed in a specific order. The first vials of each set of 4 were dispensed in order followed by the second vial of each set in the reverse order. The third and fourth vials of each set were dispensed in a similar order to the first and second vials respectively.

The vials were gassed with 95% O₂ : 5% CO₂, sealed and placed in a shaking water bath at 37°C. After a 2 hour incubation, the individual fat cell suspensions were deproteinized by pouring into 1.0ml of 10% W/V TCA in 7ml centrifuge tubes. After centrifugation at 2,500g for 10 minutes the infranatants were transferred to 10ml test tubes. Care was taken not to disturb a surface layer of fat cell debris. The tubes were stored at 4°C overnight unless the samples were assayed for glycerol immediately. Prior to the glycerol assay TCA was removed from the samples by extraction with diethyl ether. 5ml of diethyl ether was mixed thoroughly with the samples and the ether layer was then aspirated. The samples were extracted a further 2 times with the solvent. Residual ether evaporated on incubation of the tubes at 40°C for 1 hour.

4.2.2.4 Glycerol Assay

The enzymatic glycerol assay, first reported by Garland and Randle (1962) was based on the following reactions:



where GK = glycerol kinase, PK = pyruvate kinase and LDH = lactate dehydrogenase. The oxidation of NADH was followed spectrophotometrically by the reduction in its absorbance at 340nm.

500ml of a double strength glycerol assay medium (GAM) was prepared in distilled water as follows. 18.4g of triethanolamine hydrochloride, 1.48g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.148g of KCl were dissolved in distilled water and the pH adjusted to pH7.6 with 50% (W/V) KOH. 144.2mg of phosphoenolpyruvate tricyclohexylammonium salt and 100mg of NADH were added and the solution made up to 500ml. This was stored at -18°C in 60ml aliquots. For an assay of 48 ether extracted samples, 60ml of GAM was thawed, to it were added 225mg of ATP disodium salt, 300 μl of 1.0mg/ml LDH, 30 μl of 10.0mg/ml PK and distilled water up to 120ml. The final concentration of the medium was: triethanolamine hydrochloride 0.1M; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 5mM; KCl 2mM; phosphoenolpyruvate tricyclohexylammonium salt 0.54mM; NADH 0.14mM; ATP 3mM; LDH 2.5 $\mu\text{g}/\text{ml}$ and PK 2.5 $\mu\text{g}/\text{ml}$. For each assay, 50 μl of 1.0mg/ml of glycerol kinase (GK) was diluted to 550 μl with distilled water.

Glycerol was assayed by mixing 0.5ml of sample with 2.5ml of GAM in a 1cm path length cuvette. The absorbance at 340nm was taken and was usually between 0.4 and 0.6. 10 μl of diluted GK was added and after thorough mixing left

at room temperature for 20 minutes. The decrease in absorbance was found. The concentration of glycerol in the sample was calculated as follows:

$$\begin{aligned} \text{Concentration of glycerol} &= \frac{\text{Change in absorbance at } 340\text{nm} \times \text{Reaction volume in Cuvette}}{\text{Molar Extinction Coefficient of NADH at } 340\text{nm} \times \text{Sample Volume in Cuvette}} \\ &= \frac{E_{340} \times 3.0}{6.22 \times 0.5} \mu \text{ mol/ml} \quad (\text{Molar Extinction Coefficient of NADH at } 340\text{nm} = 6.22 \times 10^3 \text{ Litre/mol/cm}) \end{aligned}$$

From this the glycerol production rate or rate of lipolysis was calculated:

$$\begin{aligned} \text{Glycerol production rate} &= \frac{\text{Glycerol Concentration} \times \text{Dilution by TCA} \times \text{Incubation Volume}}{\text{Dry weight of 1ml of fat cell} \times \text{Time of incubation suspension}} \\ &= \frac{E_{340} \times 3.0 \times 1.909 \times 1.1}{6.22 \times 0.5 \times w \times 2} \mu \text{ mol/gfat/hour} \end{aligned}$$

where E_{340} = change in absorbance at 340nm

w = dry weight of fat cells in 1ml of fat cell suspension

It is assumed that for every mole of glycerol a mole of NADH is oxidised. The mean and standard error of the estimate of mean value (SEM) were calculated for each treatment which were tested in quadruplicate (Section 4.2.2.6).

The validity of the glycerol assay was assessed by estimating the glycerol content of various dilutions of a glycerol standard solution (1.0mM). The whole in vitro assay was assessed by incubation of ACTH at various concentrations in the assay and plotting a dose-response relationship.

4.2.2.5 Optimum Collagenase Concentration

The activity of different batches of collagenase were found to vary and optimum conditions for each new batch had to be found (Fain et al., 1973).

1g of minced rabbit adipose tissue was digested at 37°C in 5.0ml of KRB-albumin buffer, which contained collagenase at concentrations of 0.5, 1.0, 2.0 and 3.0mg/ml. After an hour on the metabolic shaker, the isolated fat cells were collected by centrifuging at 250g for 1 minute and washed 3 times with 5.0ml of KRB-albumin buffer. Each fat cell preparation was suspended in 20ml of buffer and undigested fat removed with forceps. 1ml aliquots were dispensed into vials containing 0, 1.0µg or 10.0µg of ACTH in KRB buffer. After a 2 hour incubation, the glycerol production rate in the vials was found as before, using the mean dry weights, which had been found for 1ml of each of the fat cell suspensions. The concentration of collagenase, which gave the most responsive fat cell suspension to ACTH, was used in subsequent preparations of isolated fat cells with that batch of collagenase.

4.2.2.6 Statistical Methods

The following statistical treatments were used in the analysis of the results of the in vitro assay. Most calculations were performed on a Sumlock Computcorp 344 Statistician calculating machine.

Mean (\bar{x}) = $\frac{\sum x}{n}$ where x = an observation n = the number of observations

$$\text{Standard Deviation (SD)} = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n - 1}}$$

Standard Error of the estimate of Mean value (SEM) = $\frac{SD}{\sqrt{n}}$

The results for glycerol production rates are presented as means \pm SEM.

Student's t Test for Independent Means

$$\bar{x} - \bar{y}$$

$$t_{\text{ind}} = \frac{\bar{x} - \bar{y}}{\sqrt{\left[\frac{(n_x - 1) SD_x^2 + (n_y - 1) SD_y^2}{n_x + n_y - 2} \right] \times \left[\frac{1}{n_x} + \frac{1}{n_y} \right]}}$$

where

x = observations of treatment x y = observations of treatment y

n_x = number of x observations n_y = number of y observations

SD_x = Standard deviation of \bar{x} SD_y = Standard deviation of \bar{y}

\bar{x} = mean of x \bar{y} = mean of y

Linear Regression

$$\text{Coefficient of correlation (r)} = \frac{\sum xy - \frac{\sum x \sum y}{n}}{\sqrt{\left(\sum x^2 - \frac{(\sum x)^2}{n} \right) \left(\sum y^2 - \frac{(\sum y)^2}{n} \right)}}$$

$$\text{The slope of the regression (m)} = \frac{\sum xy - \frac{\sum x \sum y}{n}}{\sum x^2 - \frac{(\sum x)^2}{n}}$$

The intercept (b) of the regression on the y axis = $\frac{\sum y}{n} - m \frac{\sum x}{n}$

Probabilities

The probability (P) of the 2 independent means of the 't' test being significantly different was found using a table

of the distribution of 't' from Fisher & Yates (1963). The degree of freedom was $(n_x + n_y - 2)$.

The probability (P) of the linear regression being significantly different from zero was found from a table of the correlation coefficient (Fisher & Yates, 1963) where the degree of freedom was $n-1$.

In presenting the results the different levels of significance are represented as follows: $P < 0.001$ by ''', $0.01 > P > 0.001$ by '''', $0.02 > P > 0.01$ by '''', $0.05 > P > 0.02$ by '' and $P > 0.05$ by N.S. indicating a non-significant result.

4.2.3 Application of the In Vitro Assay : Investigation of the Serum Effect on LMF Induced Lipolysis

The in vitro assay was used initially in the isolation of LMF and to establish the lipolytic activities of the purer LMF preparations. These applications were described in chapter 2. The assay was used here to investigate the effect of porcine serum on the lipolysis induced in rabbit adipocytes by porcine LMF.

4.2.3.1 A Standard Procedure

At first a standard method was devised using pooled serum, taken at slaughter, from 4 randomly chosen pigs. 1 litre of serum was collected after centrifugation of coagulated blood at 6000g for 20 minutes. The serum was known as 'normal serum' and stored at -18°C .

The basic incubation procedure was to dispense 0.2ml of serum or 0.2ml of KRB-albumin buffer to the 25ml plastic capped vials prior to addition of lipolytic agent and fat cell suspension, prepared as previously described. The

incubation then proceeded normally except that the calculation of glycerol production rate was adjusted to allow for a total incubate volume of 1.3ml. This procedure was used for a number of short experiments, the designs of which are described in conjunction with the results they produced. The LMF used in these and subsequent experiments came from different extractions, but all the preparations were equivalent in purity to mLMFd from extraction XI. They had only one major band of electrophoretic mobility at pH8.9.

4.2.3.2 Sera from Individual Animals

After the establishment of a standard procedure, sera from individual animals in the three lines of pigs, described by Vangen (1974a), were assessed for effect on LMF induced lipolysis. Sera from 54 animals were assayed and as only 9 sera could be measured at once the results had to be adjusted to allow for inter-assay variation.

15ml of blood was taken from the anterior vena cava of 60 Norwegian Landrace pigs of either sex. The pigs had been fasted overnight for 14-16 hours and were between 100 and 170 days old. The animals were restrained during bleeding. After centrifugation the sera were aspirated and stored at -18°C . 20 animals from each line were sampled, consisting of 5 groups of 4 siblings each. The pigs were from the tenth generation of the selection experiment.

In each of the 6 assays carried out, 3 serum samples were included from each of the 3 lines of pigs. In a particular assay, no more than one serum sample came from any one family of pigs. The glycerol production rates when

0.2ml of these sera was incubated with 1.0 μ g of mLMFd and 1.0ml of fat cell suspension were found as previously described. The basal rate of glycerol production and that caused by LMF alone were found for each assay.

To adjust the results of the 6 assays to allow for inter-assay variation, the overall mean (\bar{x}) rate of lipolysis (glycerol production rate) was calculated. The difference between this mean and the mean for a particular assay (\bar{x}_i where i = the number of the assay) gave a correction factor for that assay.

Adjusted rate of lipolysis = experimental rate of lipolysis + ($\bar{x} - \bar{x}_i$).

The probability of a correlation between the rate of lipolysis (x) in the presence of pig serum, after adjustment for inter-assay variation, and either the growth rate (y) or backfat thickness (y) of the original animal was found by calculation of the linear regression (section 4.2.2.6).

4.2.3.3 Dialysed Serum from Individual Animals

The effect of serum after dialysis against KRB buffer from individual pigs on LMF induced lipolysis was examined. 3 sera samples, from animals with full growth and fatness data, were selected from each of the lines of pigs. 1.0ml of these sera were placed in separate dialysis bags identified by plastic tape fixed round a loop of dialysis tubing above the closing knot. The tubing had been washed twice in an excess of distilled water to remove the glycerol used in the storage of the dry tubing. The sera were dialysed overnight against 800ml of freshly prepared KRB buffer. The

KRB-albumin buffer for the in vitro assay was dialysed overnight against 1000ml of the same KRB buffer.

The resultant rates of lipolysis when the dialysed sera were assayed for effect on LMF induced lipolysis were examined for correlation with growth rate and backfat thickness. These correlations were compared to the effect of the same sera on lipolysis before they had been dialysed.

4.3 Results

4.3.1 Degradation of ^{125}I -labelled LMF

After a 4 day incubation with serum, ^{125}I -labelled LMF exhibited less than 10% degradation (table 4.2). Label with serum from the control line of pigs showed 8.3% degradation. With serum from the fat, slow growing LP-line the degradation was 8.0% while with serum from the lean, fast growing HP-line 7.1% of the label was degraded. 4% (W/V) albumin in 0.01M phosphate buffered saline, incubated as a control, caused the most reduction in recovery of γ -radiation, with 21% of the label being degraded.

In the earlier trial, over 0-2 hours, no decrease in precipitated radioactivity was found, either with serum or with albumin containing buffer.

4.3.2 In Vitro Assay of Lipolytic Activity

4.3.2.1 Validation of Glycerol Assay

A 1.09mM stock solution of glycerol in distilled water was diluted to give 0.218mM and 0.436mM solutions. 4 x 0.5ml aliquots of both of these were assayed for glycerol content with the results shown in table 4.3. The experimentally derived glycerol concentrations were $0.205 \pm 0.002\text{mM}$ and $0.432 \pm 0.001\text{mM}$ respectively.

Table 4.2 The Proteolytic action of Serum on ^{125}I -labelled IMF.

Sample	Incubation Time (days at 37°C)	Counts per 50 sec Mean \pm SEM (n=4)	% loss of Radioactivity
0.1ml of label	4 days	32,000 \pm 709	
0.5ml of 0.01M Phosphate Buffered Saline pH7.5 + 4% Albumin	0	25,862 \pm 229	21%
	4	20,329 \pm 121	
0.5ml of pooled serum From HP-line pigs	0	26,918 \pm 337	7.1%
	4	25,018 \pm 61	
0.5ml of pooled serum From control line pigs	0	27,395 \pm 398	8.3%
	4	25,108 \pm 202	
0.5ml of pooled serum From LP-line pigs	0	27,363 \pm 119	8.0%
	4	25,178 \pm 244	

Degradation of ^{125}I -labelled IMF by serum from 3 lines of pigs was estimated by % loss of radioactivity after precipitation of protein by trichloroacetic acid.

The glycerol content of standards was assayed before and after addition of TCA and extraction 3 times with diethyl ether. The 1.09mM stock glycerol standard was diluted to 0.109mM, 0.218mM and 0.327mM. To 4 x 1ml aliquots of each, 1ml of 10% (W/V) TCA was added and thoroughly mixed. After 3 extractions with 5.0ml of diethyl ether, the samples were assayed for glycerol content along with duplicate samples which had received neither TCA nor ether extraction. The calculation of glycerol content was adjusted to allow for the 2 fold dilution resulting from the addition of TCA to the extracted samples. At each glycerol concentration, the latter had been concentrated by their treatment (Table 4.4). The mean increase in concentration was by a factor of 1.14.

4.3.2.2. ACTH Dose-Response Curve

The dose-response curve resulting from the incubation of ACTH with 1.0ml of the fat cell suspension at 0.05, 0.2, 0.5, 2.0 and 5.0 μ g per 1.1ml is shown in figure 4.1. Above 0.2 μ g per 1.1ml, ACTH produced a significant rise in the rate of glycerol production. With 0.05 μ g of ACTH, the glycerol production rate did increase, but not significantly.

Typical isolated rabbit fat cells from a suspension are shown in plate 4.1. (a&b), where the adipocytes are magnified approximately 500x and stained in methylene blue.

4.3.2.3 Optimum Collagenase Concentration

The most responsive fat cell suspension to both 1.0 and 10.0 μ g of ACTH was prepared with 2.0mg/ml of collagenase (figure 4.2). The lipolytic response of the suspensions rose with increasing collagenase concentration up to 2.0mg/ml,

Table 4.3 Validation of Glycerol Assay Method

Calculated Glycerol concentration (mM)	E_{340}	Glycerol concentration (mM)	Mean Glycerol conc. \pm SEM (mM)
0.218	0.220 0.218 0.210 0.214	0.212 0.206 0.203 0.206	0.205 \pm 0.002
0.436	0.448 0.446 0.450 0.446	0.432 0.430 0.434 0.430	0.432 \pm 0.001

Glycerol diluted from 1.09mM gave the samples assayed. Reduction in absorbance at 340nm was measured after addition of 0.9 μ g of glycerokinase to 0.5ml of sample mixed with 2.5ml GAM in a 1cm path length cuvette.

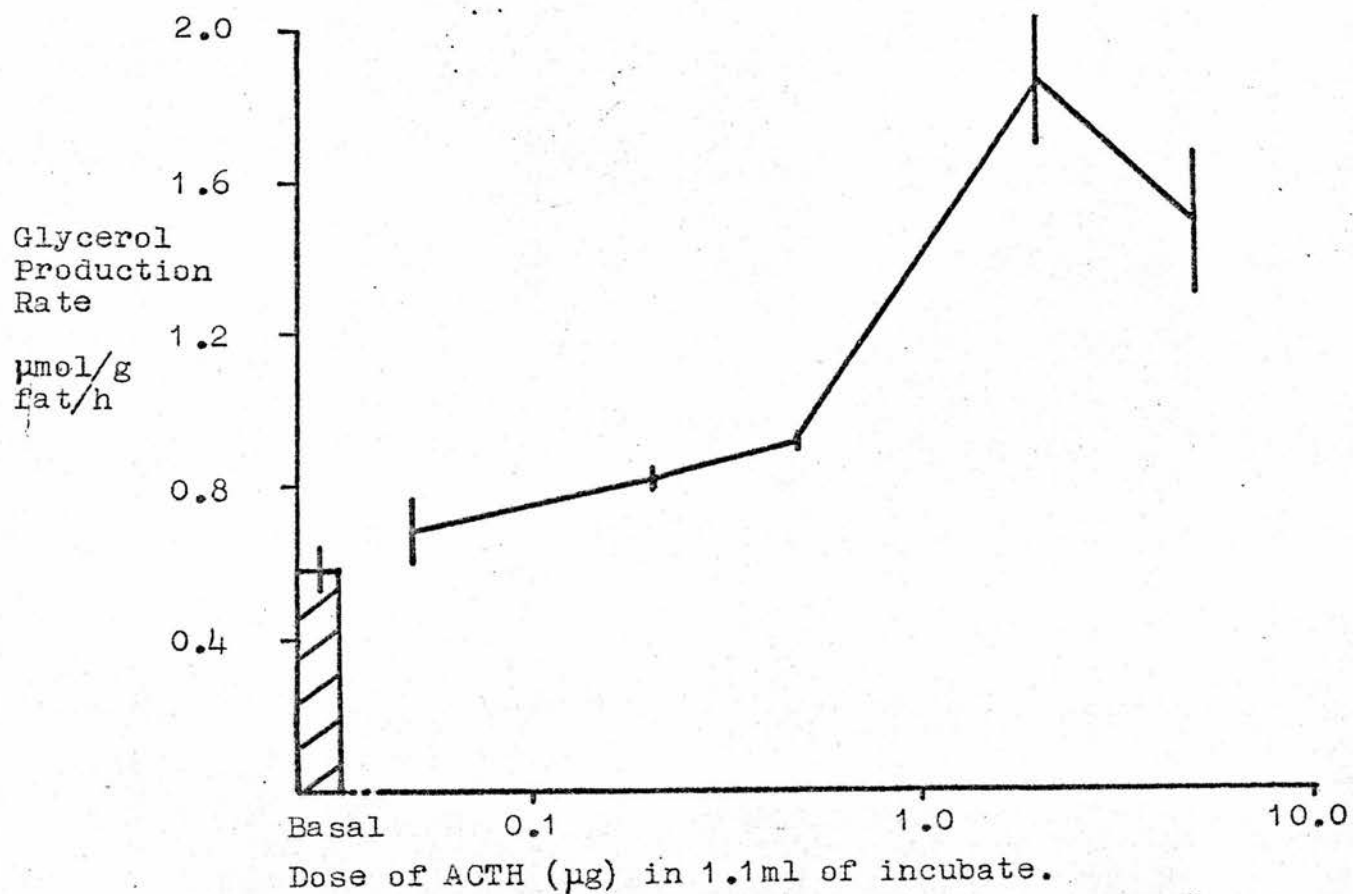
Table 4.4 Effect of Extraction on Glycerol Concentration

Glycerol Concentration before extraction (mM)	Glycerol Concentration after extraction (mM)	Correction factor	Mean Correction factor
0.113 \pm 0.003	0.123 \pm 0.01	1.09	1.14
0.210 \pm 0.003	0.254 \pm 0.001	1.21	
0.315 \pm 0.005	0.356 \pm 0.004	1.13	

Identical glycerol standards were assayed (in quadruplicate) before and after addition of an equal volume of 10% (W/V) TCA and extraction 3 times with 5 volumes of diethyl ether. The original concentrations of the extracted samples were calculated by their dilution from the stock solution. The correction factor =

$$= \frac{\text{glycerol concentration after extraction}}{\text{glycerol concentration before extraction}}$$

The results are presented as mean \pm SEM (n=4).

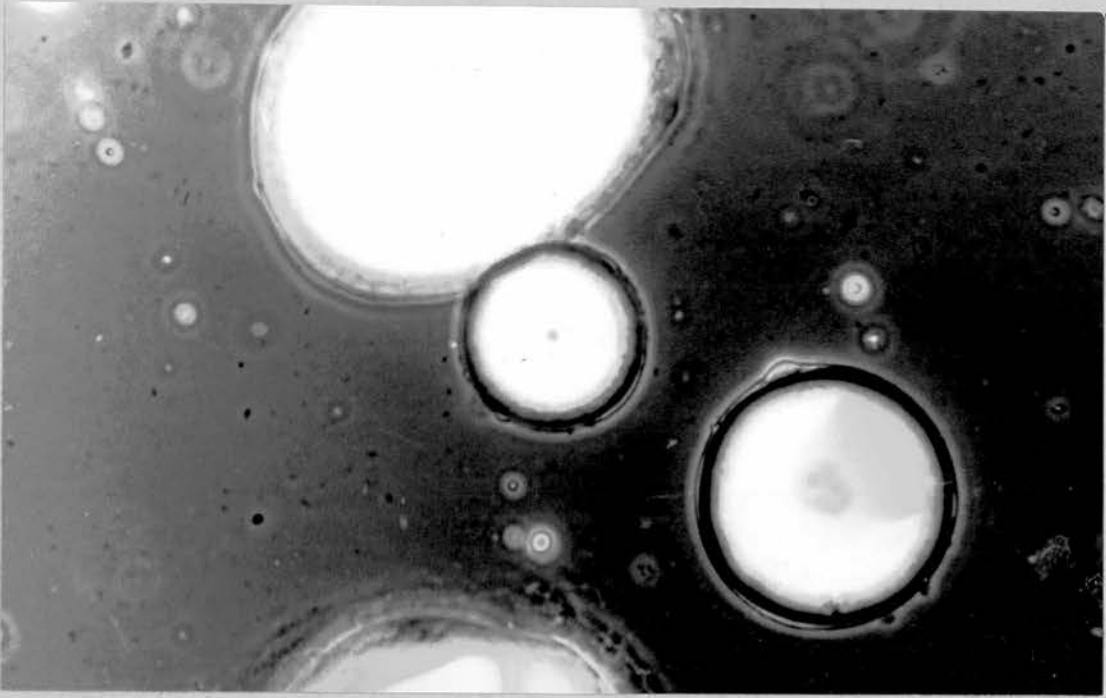
Figure 4.1 Dose-Response Curve of ACTH in Fat Cell Assay

Each point represents mean \pm SEM of quadruplicate samples. Basal lipolysis is included.

Plate 4.1

Rabbit fat cells isolated by collagenase digestion were examined under a microscope at approximately 500x magnification and stained in methylene blue dye. Free fat droplets could be seen in (a).

Plate 4.1 Isolated rabbit fat cells.

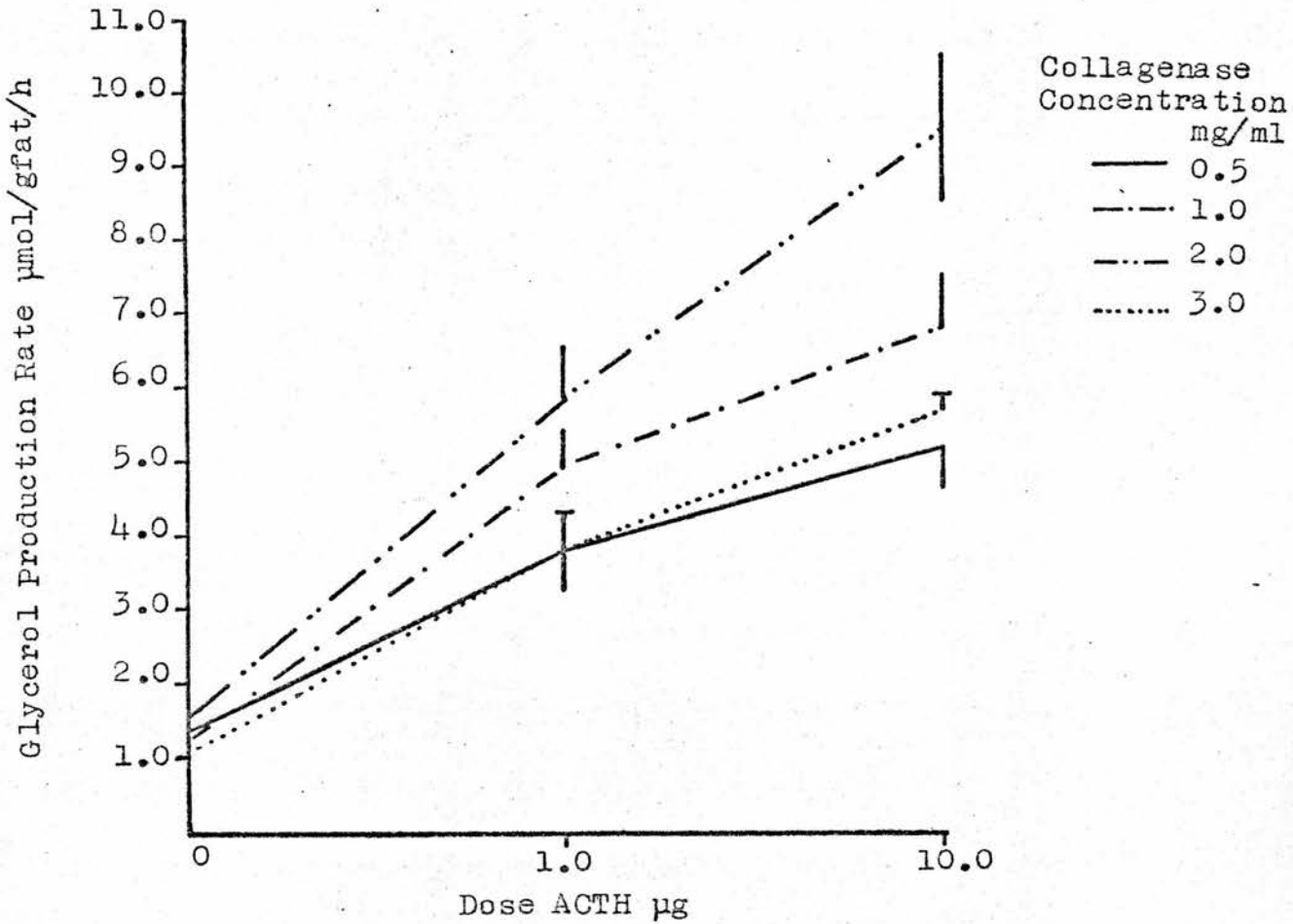


(a)



(b)

Figure 4.2

Optimum Collagenase Concentration

Rabbit Fat Cells isolated at various concentrations of collagenase were assayed for their response to ACTH. Each point represents the mean \pm SEM of quadruplicate samples.

then fell with a low rate of lipolysis in the suspension prepared with 3.0mg/ml collagenase. This batch of collagenase was used at a concentration of 1.5mg/ml for the subsequent isolation of rabbit adipocytes.

Considerable difference was found between batches of collagenase. With some batches, the incubation time, as well as the enzyme concentration, had to be reduced to prevent disruption of the fat cells. The optimum conditions, for a number of collagenase batches, shown in Table 4.5, varied by a factor of 5.

4.3.3 Application of the In Vitro Assay

4.3.3.1 Investigation of Serum Effect on LMF Induced Lipolysis

a) Serum Effect on Various LMF preparations and on Basal Lipolysis

The effect of serum on the lipolysis induced by 2.0 μ g of fractions from extraction XIII (similar to extraction VIII) is shown in Table 4.6. Inhibition of lipolysis by serum was found with acidic protein (mLMF-XIII₁), which was not absorbed by a CM cellulose column (Section 2.3.2.3). Serum had a potentiating effect on the remaining LMF fractions and on the lipolysis induced by both β LPH and ACTH. Of the LMF fractions, mLMF-XIII₃ was both the most active alone and when potentiated by serum. This fraction resembled mLMF₃ in containing most LMF, as seen by electrophoresis.

In a subsequent assay, the lipolytic activity of this peptide was again potentiated by the addition of serum (Table 4.7). This assay also showed that serum did not affect basal lipolysis, a result confirmed in a further assay (included in Table 4.7).

Table 4.5 Optimum Conditions for Batches of Collagenase

Collagenase Batch No.	Concentration mg/ml	Incubation Time (min)
743411/1 Nov 1975	1.5	50-60
7365412/1 Oct 1976	0.5	40-50
1445313/1 Nov 1976	0.4	40-50
1515313/1 Jan 1977	0.3	30-40

The best conditions were found by trial and error, with each new batch of collagenase, for the digestion of rabbit adipose tissue.

Table 4.6 The Effect of Serum on Induced Lipolysis

Peptide	Amount (μ g)	Glycerol Production Rate (μ mol/gfat/h)	
		Serum Absent	Serum Present
mLMFXIII-1	2.0	2.35 ± 0.36	1.38 ± 0.11 '
mLMFXIII-3	2.0	2.61 ± 0.72	9.40 ± 0.82 '''
mLMFXIII-4	2.0	1.98 ± 0.08	6.50 ± 0.60 '''
ACTH	2.0	4.59 ± 1.56	10.18 ± 0.58 '''
β LPH	2.0	3.83 ± 0.17	10.51 ± 1.12 '''
None	-	0.57 ± 0.06	-

0.2ml of normal serum or 0.2ml of KRB-albumin added to quadruplicate protein samples (in 0.1ml) incubated for 2 hours with 1ml of rabbit fat cell suspension.

† mean \pm SEM, n=4 ' 't'-test for significant difference between incubates with or without serum ($0.05 > P > 0.02$; ''' $0.01 > P > 0.001$).

Table 4.7 The Effect of Serum on Basal Lipolysis

Assay No.	Peptide	Amount (μg)	Glycerol Production Rate ($\mu\text{mol/gfat/h}$)	
			Serum Absent	Serum Present
50	mLMFXIII-3	2.0	$^{+}7.80 \pm 0.94$	$13.73 \pm 0.28''''$
	None	-	1.29 ± 0.17	$1.00 \pm 0.11 \text{N.S.}$
51	None	-	3.07 ± 0.38	$2.97 \pm 0.19 \text{N.S.}$

0.2ml of normal serum or 0.2ml of KRB-albumin was added to quadruplicate protein samples (in 0.1ml) incubated for 2 hours with 1ml of rabbit fat cell suspension.

$^{+}$ mean \pm SEM, n=4 't'-test for significant difference between incubates with or without serum (''''' $P < 0.001$; N.S. $P > 0.05$).

Table 4.8 The Effect of Dialysed Serum on LMF Induced Lipolysis

Added to Incubates	Glycerol Production Rate ($\mu\text{mol/gfat/h}$)
KRB albumin	$^{+}1.57 \pm 0.02$
normal serum	2.54 ± 0.52
dialysed serum	$2.40 \pm 0.13 \text{N.S.}$

Quadruplicate 0.2ml aliquots of serum or buffer were added to 1ml of rabbit fat cell suspension plus 0.5 μg of LMF(mLMFd) in 0.1ml of KRB buffer and incubated for 2 hours.

$^{+}$ mean \pm SEM, n=4 N.S. 't' test for significant difference between addition of normal or dialysed serum.

(N.S. $P > 0.05$)

b) Dialysed Serum and Excess Albumin

The possibility that the potentiating action of serum on LMF induced lipolysis was a result of more effective buffering in the presence of serum was investigated. 20ml of 'normal' serum was dialysed against 1000ml of KRB buffer at 4°C overnight and the effect of 0.2ml of this on LMF induced lipolysis was compared to the effects of the same volume of both 'normal' serum and KRB-albumin buffer (Table 4.8). Dialysis of serum did not alter its potentiating action.

In another experiment, the effect of dialysed serum was again compared to that of normal serum, but on ACTH, as well as, LMF induced lipolysis. This assay also examined the possibility that lipolysis was limited by the fatty acid-free albumin concentration. Addition of serum with extra albumin and more vacant fatty acid binding sites might be potentiating lipolysis by removing free fatty acids from the medium, thereby allowing more to be released.

This was investigated by the addition of KRB buffer containing 8% (W/V) albumin to the incubates, instead of either serum or normal buffer.

10ml of serum and 10ml of KRB buffer plus 8% (W/V) albumin were dialysed overnight at 4°C against 800ml of KRB buffer. 110ml of KRB-albumin buffer was dialysed against 1000ml of the same buffer. The effects of 0.2ml aliquots of the dialysed serum and KRB buffer plus 8% (W/V) albumin on the lipolysis induced by 1.0µg of LMF and 1.0µg of ACTH, were compared to the lipolysis in the presence of 0.2ml aliquots of normal serum and normal KRB-albumin buffer.

The results confirmed that dialysis of serum did not significantly affect its potentiation of LMF induced lipolysis (Table 4.9). A similar situation occurred with ACTH induced lipolysis. Excess albumin in an assay incubate did not significantly increase the lipolysis stimulated by either LMF or ACTH. In this experiment ACTH induced more lipolysis under all conditions than did a similar amount of LMF.

4.3.3.2 Effect of EDTA on LMF induced Lipolysis

In two assays, fat cell suspensions were incubated with LMF, β LPH and ACTH in the presence of EDTA (ethylenediamine tetra-acetic acid), which chelates both Ca^{++} and Mg^{++} ions. Ca^{++} ions were necessary for the lipolytic activity of both ACTH and β LPH (Lis et al., 1972; Lopez et al., 1959).

In (a), 0.2ml of 0.1M EDTA, added in the place of serum reduced the lipolysis caused by 1.0 μ g of both LMF and β LPH to the basal rate (Table 4.10). The concentration of EDTA in the incubation medium was 15mM. In (b), EDTA at a final concentration of 0.75mM (0.2ml of 0.005M EDTA), inhibited the lipolysis induced by 1.0 μ g of LMF and ACTH. Apparently Ca^{++} , or possibly Mg^{++} , ions were necessary for the stimulation of lipolysis by LMF as well as for stimulation by ACTH and β LPH.

4.3.3.3 Sera From Individual Animals

a) Animal Data Of the 60 pigs bled, complete data for growth rate and backfat thickness was available for 48 (Table 4.11). Some animals were retained as breeding stock and carcass information, including backfat thickness was not

Table 4.9 The Effects of Excess Albumin and Dialysed Serum on Induced Lipolysis

Peptide	Added to Incubates (0.2ml)			
	KRB+ 4% albumin	KRB+ 8% albumin	normal serum	dialysed serum
mLMFd (1.0µg)	$^{+}1.76 \pm 0.08$	1.92 ± 0.16 N.S.	5.06 ± 0.30	4.21 ± 0.76 N.S.
ACTH (1.0µg)	3.76 ± 0.33	3.50 ± 0.57 N.S.	7.69 ± 0.18	7.12 ± 0.20 N.S.

Quadruplicate 0.2ml aliquots of buffer plus albumin or serum added to 1ml of rabbit fat cell suspension plus 1µg of lipolytic peptide and incubated for 2 hours. Basal lipolysis rate was $0.84 \pm 0.12 \mu\text{mol/gfat/h}$.

(⁺ glycerol production rate ($\mu\text{mol/gfat/h}$); mean \pm SEM, n=4)

N.S. 't' test for significant difference between KRB+4%(W/V) albumin and KRB+8%(W/V) albumin or between 'normal' and dialysed serum (N.S. P>0.05).

Table 4.10 Effect of EDTA on Induced Lipolysis

Expt.	Treatment	No Addition	15mMEDTA
a	Basal	$^{+}0.46 \pm 0.03$	-
	1.0 μ g mLMFd	6.07 ± 0.33	0.51 ± 0.03
	1.0 μ g β LPH	3.65 ± 0.36	0.49 ± 0.03
b	Treatment	No Addition	0.75mMEDTA
	Basal	0.84 ± 0.12	-
	1.0 μ g mLMFd	1.76 ± 0.08	0.70 ± 0.11
	1.0 μ g ACTH	3.76 ± 0.33	1.00 ± 0.13

mLMFd, β LPH or ACTH in 0.1ml of KRB buffer were incubated in quadruplicate with 1ml of rabbit fat cell suspension and 0.2ml of EDTA which gave a final concentration of 15mM or 0.75mM.

$^{+}$ glycerol production rate (μ mol/gfat/h); mean \pm SEM, n=4

Table 4.11

Serum from 54 pigs was assayed for effect on LMF induced lipolysis. 6 assays which each had 3 serum samples from the 3 lines of pigs were carried out. 0.2ml of each serum sample, in quadruplicate, was incubated for 2 hours with 1ml of rabbit fat cell suspension plus 1.0 μ g of mLMF α in 0.1ml of buffer. The observed glycerol production rate was adjusted to allow for inter-assay variation.

Adjusted Lipolysis (glycerol production rate) = Observed Lipolysis + (Overall Mean-Assay Mean)

The overall mean of the 54 incubates was 5.7 \pm 0.30 μ mol/gfat/h (mean \pm SEM).

The growth rate and backfat thickness were provided by Dr N. Standal of the Institute of Animal Genetics and Breeding, Agricultural University of Norway, Aas-WLH, Norway.

Animal Number	Line	Growth Rate (g/day)	Backfat Thickness (mm)	Observed Lipolysis ($\mu\text{mol/gfat/h}$)	Adjusted Lipolysis ($\mu\text{mol/gfat/h}$)	Assay Mean \pm SEM ($\mu\text{mol/gfat/h}$)	Correction Factor
0702	HP	536	18.7	6.38	6.57		
1508	C	588	26.3	4.14	3.93		
0207	LP	532	37.6	4.53	4.32		
9302	C	545	25.0	6.08	5.87	5.95 \pm 0.34	-0.21
0303	LP	523	42.6	6.11	5.90		
1206	HP	674	24.0	6.37	6.16		
0803	LP	558	44.3	6.49	6.28		
1704	HP	590	-	7.50	7.29		
1403	C	688	28.6	5.93	5.72		
9402	C	512	26.0	5.37	6.19		
0707	HP	653	27.3	6.86	7.69		
0904	LP	572	42.6	5.46	6.28		
1703	HP	584	17.0	1.35	2.17	4.92 \pm 0.51	+0.82
2305	LP	484	36.0	4.55	5.37		
1401	C	632	27.0	5.85	6.67		
0208	LP	526	43.0	4.34	5.16		
0605	C	640	-	4.87	5.69		
2709	HP	582	22.3	5.63	6.45		
2306	LP	598	37.0	10.04	8.41		
1411	C	431	-	7.10	5.47		
2708	HP	596	19.0	5.23	3.60		
0602	C	552	28.3	6.23	4.60	7.37 \pm 0.57	-1.63
0106	HP	630	25.0	7.85	6.22		
0202	LP	524	-	7.70	6.07		
0711	HP	584	-	8.88	7.25		
0309	LP	547	44.0	8.54	6.91		
9406	C	594	34.0	4.78	3.15		

Animal Number	Line	Growth Rate (g/day)	Backfat Thickness (mm)	Observed Lipolysis ($\mu\text{mol/gfat/h}$)	Adjusted Lipolysis ($\mu\text{mol/gfat/h}$)	Assay Mean \pm SEM ($\mu\text{mol/gfat/h}$)	Correction Factor
1701	HP	630	24.0	8.50	7.88		
0901	LP	526	44.6	8.61	7.99		
9314	C	528	25.6	3.56	2.94		
0313	LP	578	43.0	5.87	5.25		
1504	C	580	26.6	3.04	2.42	6.36 \pm 0.67	-0.62
0103	HP	666	20.3	7.30	6.68		
1404	C	700	31.0	8.16	7.54		
1210	HP	653	21.3	6.44	5.82		
2310	LP	553	-	5.72	5.10		
0610	C	585	-	6.08	4.16		
0206	LP	524	-	8.06	6.14		
1208	HP	600	24.3	7.73	5.81		
0802	LP	489	-	7.81	5.89	7.66 \pm 0.26	-1.92
9408	C	549	27.3	8.60	6.68		
0708	HP	630	23.0	7.09	5.17		
0902	LP	578	49.0	8.60	6.68		
1502	C	632	23.3	7.21	5.29		
2707	HP	625	11.3	7.84	5.92		
0903	LP	-	-	2.81	6.34		
0609	C	618	30.6	1.54	5.07		
0108	HP	657	-	2.38	5.91		
9410	C	578	32.6	1.55	5.08	2.21 \pm 0.16	+3.53
2701	HP	600	-	2.23	5.76		
0806	LP	517	39.0	1.79	5.32		
1207	HP	541	17.6	2.63	6.16		
2304	LP	606	32.3	2.86	6.39		
9312	C	545	27.6	2.15	5.68		

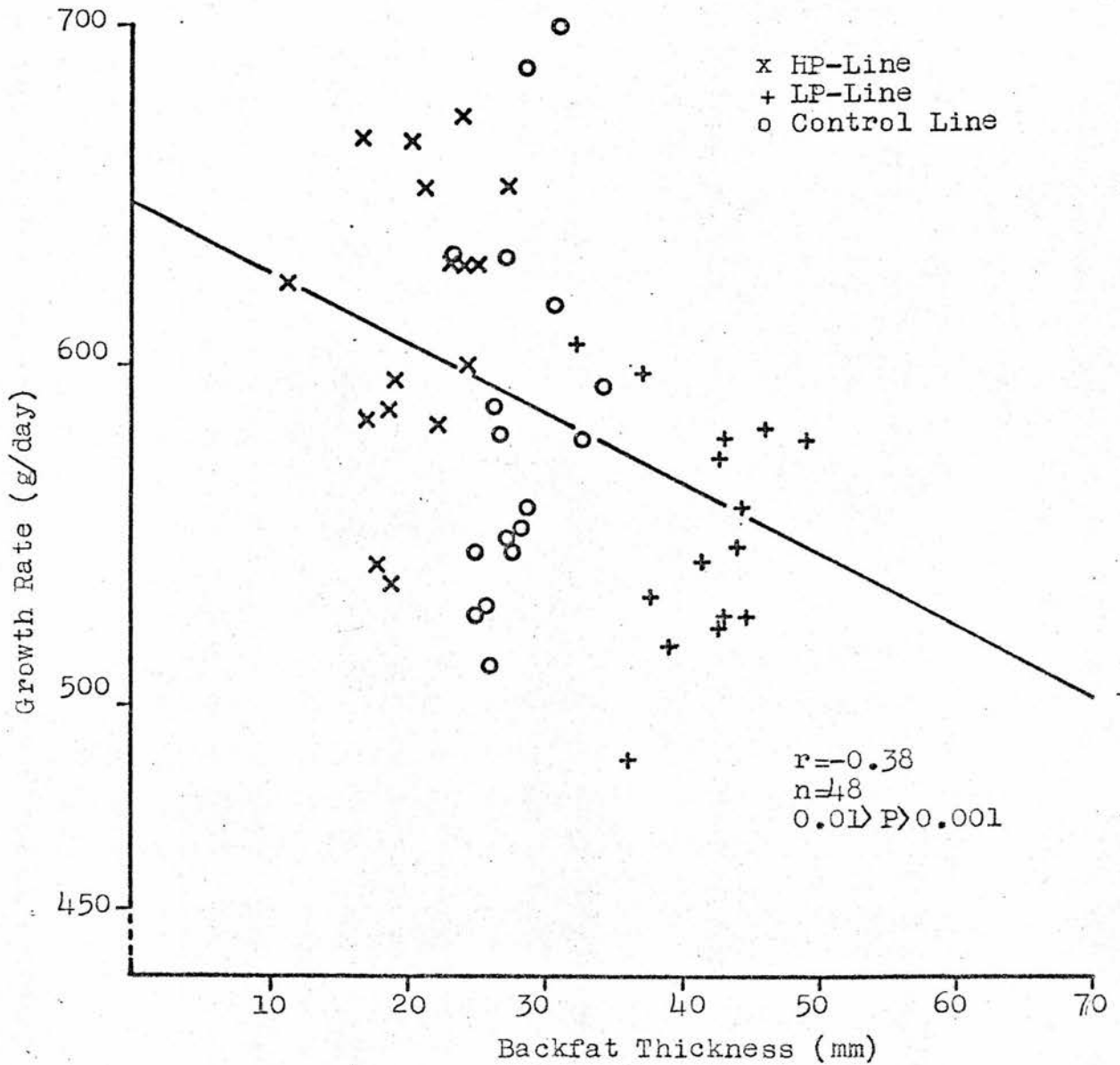
Table 4.11 Data from Individual Pigs and the Effect of their Serum on IMF Induced Lipolysis

obtained for these animals. One pig died soon after bleeding and no data was available for this animal. The pigs of the three lines could be divided into their groups by examination of the data (Figure 4.3). Growth rate (Y) correlated to be backfat thickness (X) with a coefficient of correlation (r) of -0.38 which, for $n=48$, gave $0.01 > P > 0.001$. There was a significant correlation between growth rate and backfat thickness in these pigs. The mean (\pm SEM) growth rates were 543 ± 35 , 579 ± 62 and 614 ± 40 g/day for the LP-line, control line and HP-lines respectively. The HP and LP lines differed significantly from the control line ($0.05 > P > 0.02$). Means (\pm SEM) of the backfat thickness for the three lines of pigs were 41.5 ± 4.3 , 27.8 ± 2.8 and 20.6 ± 4.6 mm in the same order. The two selected lines were significantly different ($P > 0.001$) from the control line in backfat thickness.

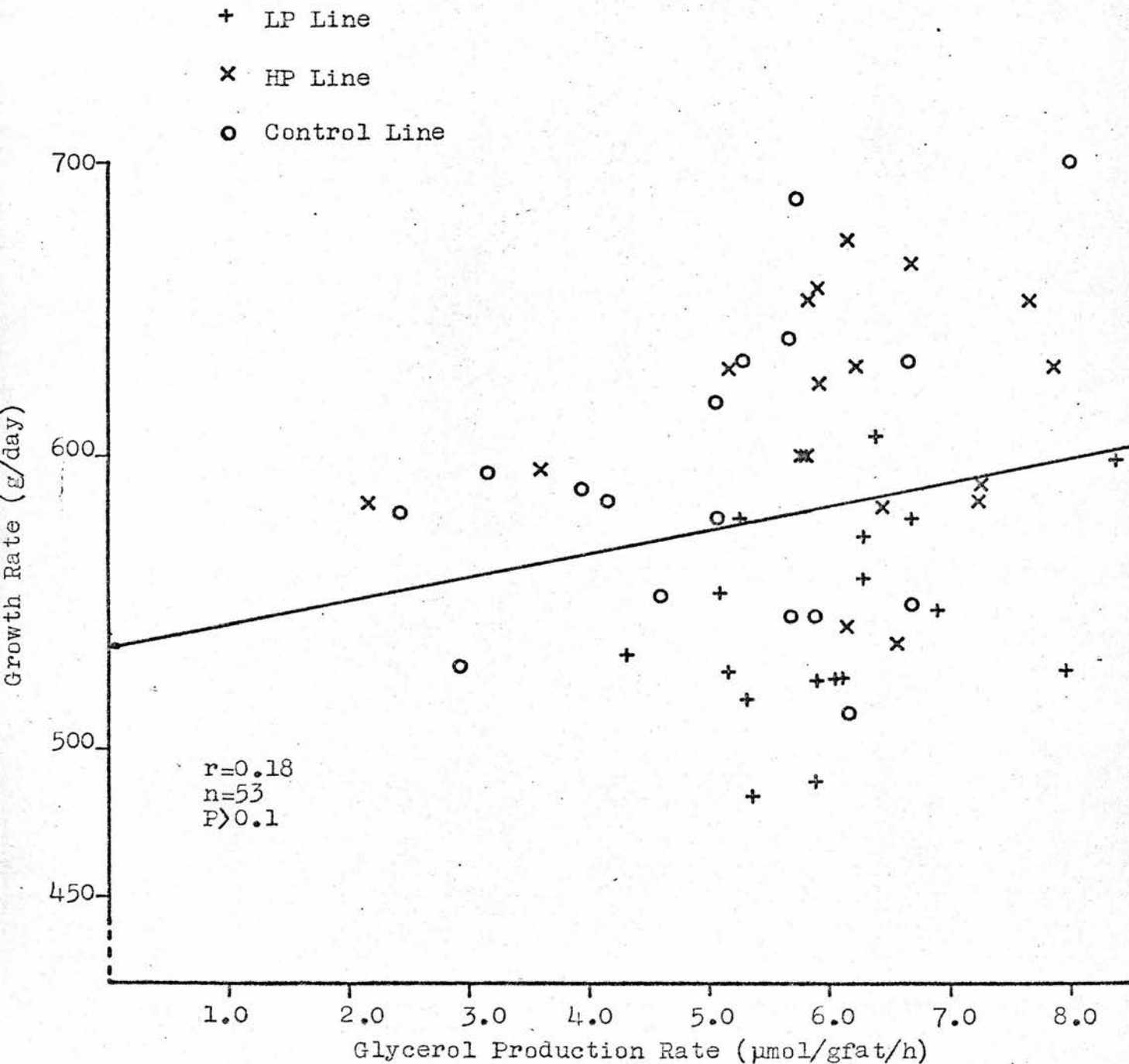
b) Effect of Sera on LMF induced Lipolysis. A wide range of glycerol production rates was found when the sera were incubated in vitro with LMF and fat cell suspension (Table 4.11 Figures 4.4 & 4.5). The overall mean glycerol production rate was $5.74 \mu\text{mol/gfat/h}$ with the means of the 6 assays varying from 2.21 to $7.66 \mu\text{mol/gfat/h}$.

The coefficient of correlation (r) for lipolysis (X) against growth rate (Y) was 0.18. For $n=53$, $P > 0.1$ indicating that there was no significant correlation between these parameters. Similarly lipolysis (X) was not significantly correlated to backfat thickness ($r=0.16$, $n=42$, $P > 0.1$).

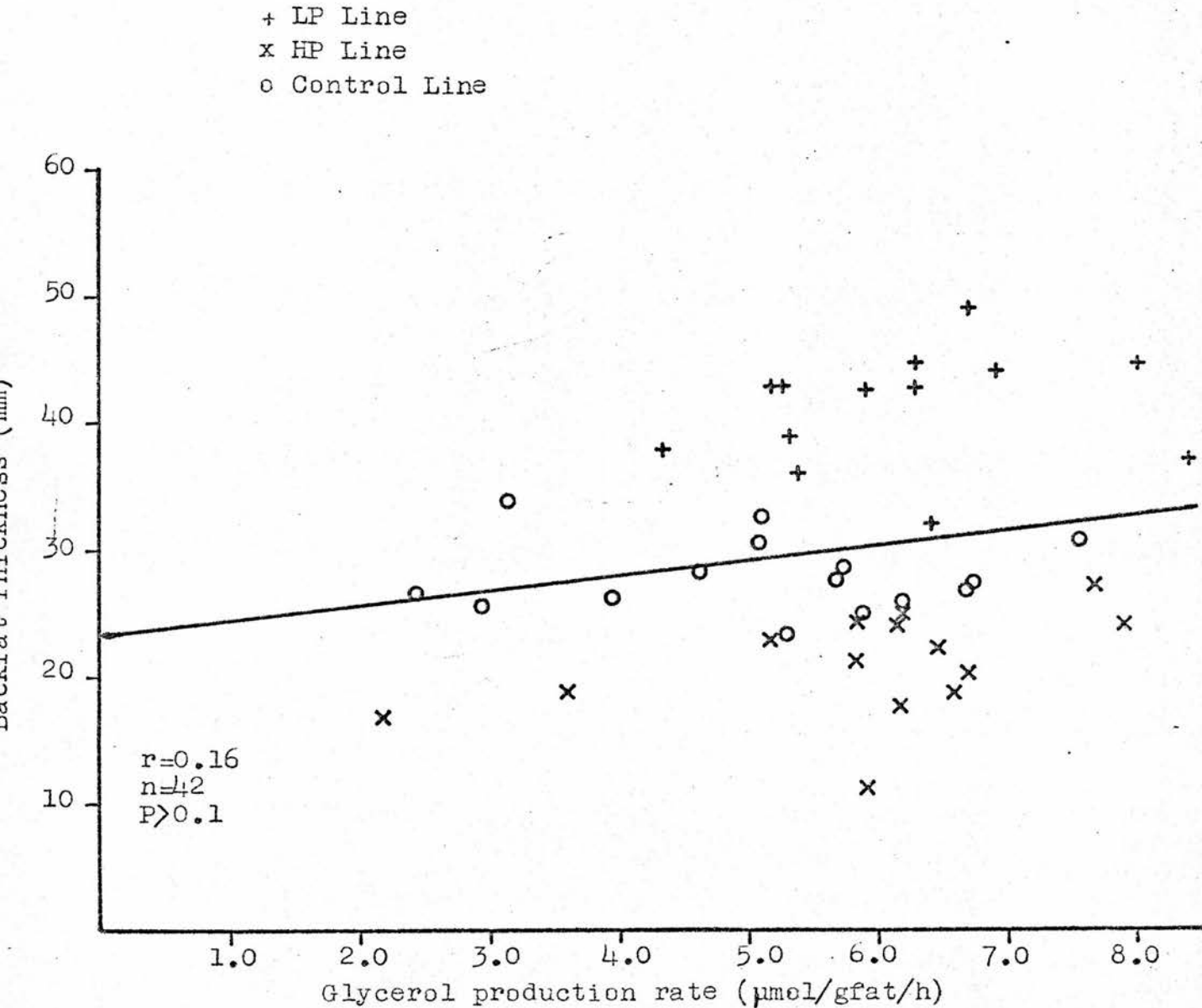
Figure 4.3 Correlation of Growth Rate To Backfat Thickness of Pigs



Data provided by N. Standal on 60 pigs bled for the investigation of serum effect on LMF induced Lipolysis. There were 20 pigs from each line. Complete data was only available for 48 animals.

Figure 4.4 Relation of Growth rate to Lipolysis

Adjusted lipolysis was found after incubation of 0.2ml of pig's serum with 1.0 μg of LMF (in 0.1ml) plus 1ml of fat cell suspension (in quadruplicate). Plotted against growth rate of the individual pigs, no significant correlation was found.

Figure 4.5 Relation of Backfat Thickness to Lipolysis

The adjusted lipolysis was found after incubation of 0.2ml of pigs serum with 1.0 μg of LMF (in 0.1ml) plus 1ml of fat cell suspension (in quadruplicate) plotted against backfat thickness of the individual pigs. No significant correlation was found.

The means (\pm SEM) of the lipolysis in the presence of serum from the 3 lines, after adjustment for inter-assay variation, were 6.12 ± 0.24 , 5.14 ± 0.32 and 6.02 ± 0.32 $\mu\text{mol/gfat/hour}$ for the LP-line, control line and HP-line respectively. These were not significantly different.

4.3.3.4 Dialysed Sera from Individual Animals

When dialysed sera were included in one assay, instead of 'normal' sera, a change was found in the comparative rates of lipolysis (Figure 6a&b). The correlation coefficient for growth rate (Y) against lipolysis (X) in figure 6a was 0.56 for dialysed sera, compared to -0.18 when the data was taken for the results of same sera incubated before dialysis. However there was still not a significant correlation, although for the former $0.1 > P > 0.05$ while for the latter $P > 0.1$.

For backfat thickness (Y) against lipolysis (X) in the presence of the dialysed sera $r = -0.41$ while with the same sera before dialysis the coefficient of correlation (r) had been 0.17. As for both of these $P > 0.1$, neither was a significant correlation.

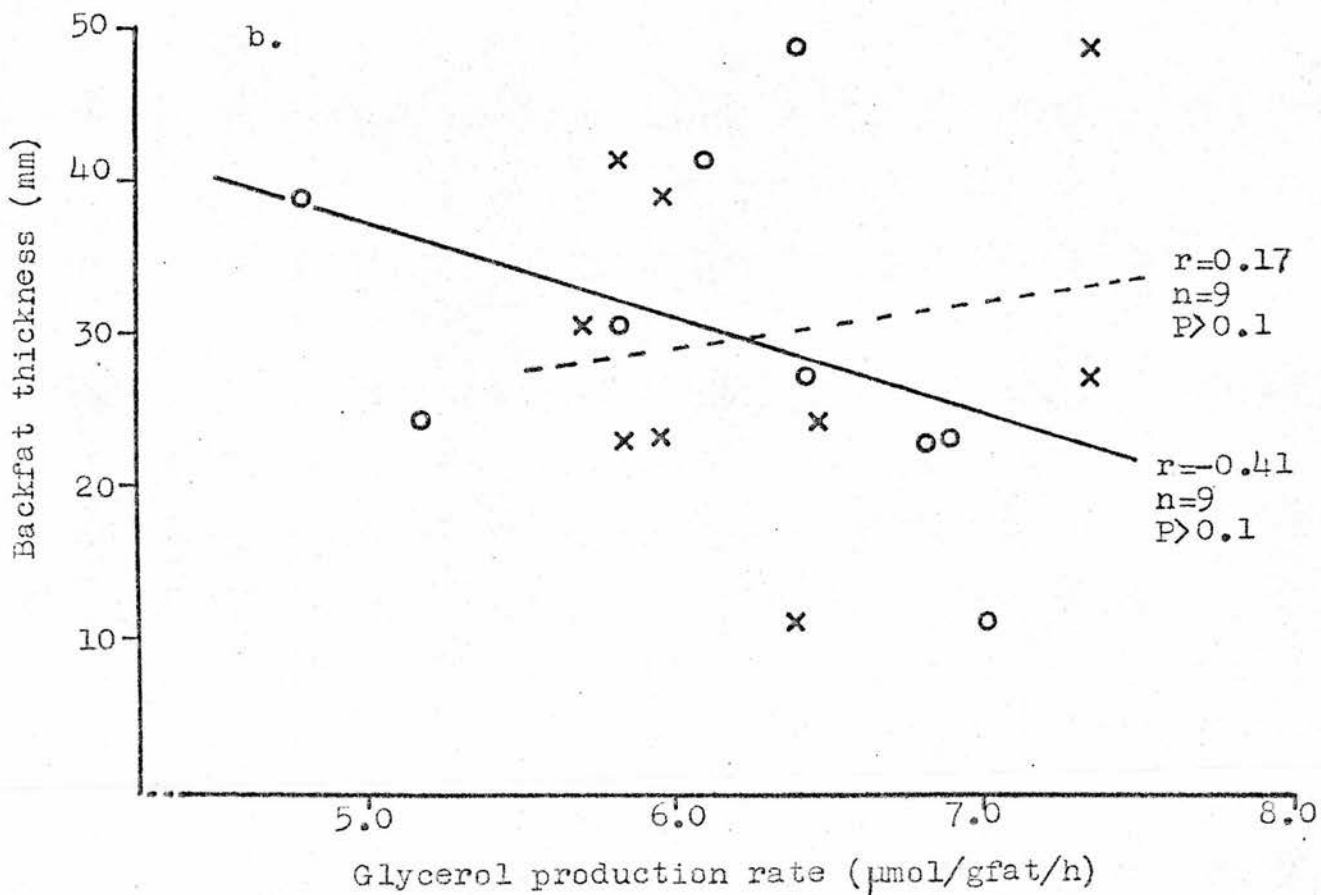
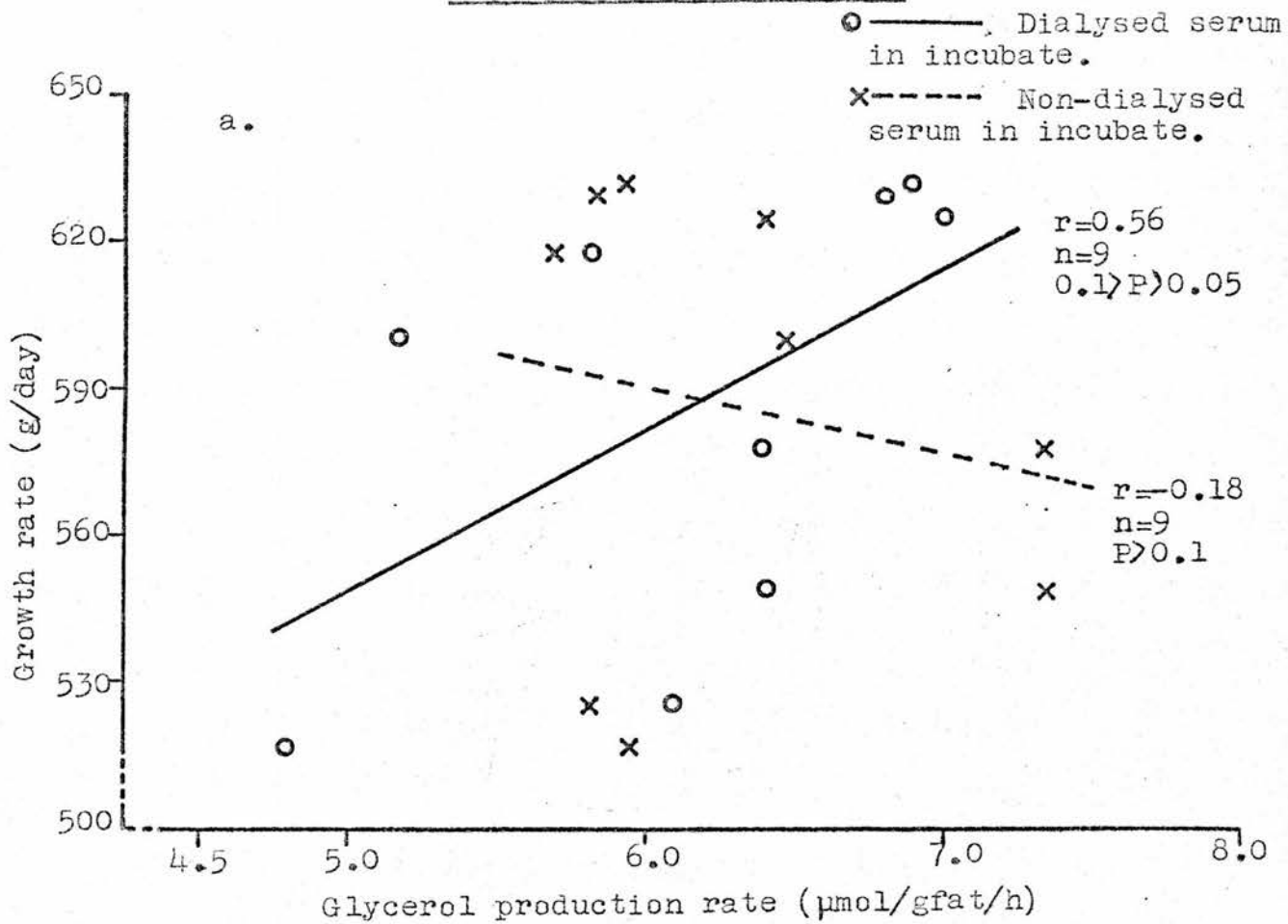
4.4 Discussion

Degradation of ^{125}I -Labelled LMF

The postulation by Trygstad & Foss (1972) that serum inhibits the lipolytic action of LMF by hydrolysis of the peptide was not confirmed by incubation of ^{125}I -labelled LMF with serum for up to 4 days. A reduction in TCA precipitable radioactivity of less than 10% was found with serum from all three lines of pigs, whereas the radioactivity precipitated by TCA, when 4% (W/V) albumin was the only protein

Figure 4.6a: The relation of 9 pigs' growth rates to the lipolysis produced when 0.2ml of their serum, either dialysed or non-dialysed, was incubated with 1.0µg of LMF + 1ml of fat cell suspension. Each point is mean of four samples. The linear regression with both types of serum is shown.

Figure 4.6b: The relation of 9 pigs' backfat thickness to the lipolysis produced when 0.2ml of its serum, either dialysed or non-dialysed, was incubated with 1.0µg of LMF + 1.0ml of fat cell suspension. Each point is the mean of four samples. The linear regression with both types of serum is shown.

Figure 4.6 Dialysis of serum and Effect on relation of Lipolysis to Animal Data

present, was reduced by 21%. Possibly the products of hydrolysis of ^{125}I -labelled LMF bind non-specifically to other proteins. This occurs with damaged labelled hormones (Landon, 1968). LMF could have been degraded in all the incubates, but less δ -radiation was precipitated in those samples with less protein capable of binding the hydrolysed products. With only 4% (W/V) of protein in the control (phosphate buffered saline + albumin), less radioactivity would be precipitated than when serum, with approximately 7% (W/V) protein, was present. This method would be impractical for a quantitative assessment of the proteolysis of LMF by serum, as the final result would depend on the amount and type of protein present in the sample. Another possibility was that serum did not degrade the LMF, as predicted by Trygstad & Foss (1972) but that its lipolytic action was inhibited by serum by another mechanism. To investigate this, a method to assess the action of serum on the biological activity of LMF was required.

In Vitro Assay of Lipolytic Activity

The in vitro assay for lipolysis adapted by the inclusion of serum, provided a method to measure the effect of serum on the biological activity of LMF. The assay was shown to be responsive to increasing doses of ACTH (figure 4.1) although the minimum effective dose at 0.2 μg was slightly higher than that reported by Rudman (1963) of 0.1 μg for the ACTH in rabbit adipose tissue. The validity of the glycerol assay was confirmed by the accuracy in measurement of standard glycerol (Table 4.3). Small

differences could be expected between the calculated glycerol concentration and the observed value due to dilution errors. The apparent increase in glycerol concentration following TCA treatment and ether extraction is due to removal of a small amount of water in the ether phase (Garland & Randle, 1962). The concentration factor of 1.14 (Table 4.4) agrees well with their figure of 1.15. As the assay results were used mainly for intra-assay comparisons rather than to find exact activity of a preparation, and all the results were treated the same, this correction factor was not included in the calculations of glycerol production rate.

The quantity of fat cells in a suspension was measured by dry weight, the simplest of the available methods, as this was adequate for intra-assay comparisons. The assays for effect of individual sera on LMF induced lipolysis were the only ones where comparisons were made between results of different assays. The adjustment made to the results of these 6 identical assays enabled the results to be directly compared.

The difference in collagenase activity between batches of the enzyme means that the optimum conditions for the collagenase incubation had to be found for each fresh batch by trial and error (Fain et al. 1973). Highly purified collagenase is not active, it seems that activity depends on the presence of proteolytic enzyme contaminants (Kono, 1969). The collagenase assessed in Figure 4.2, was used at a concentration of 1.5mg/ml not 2.0mg/ml which produced the most responsive

fat cell suspension. This was in case fat from other rabbits was more easily disrupted by the higher collagenase concentration and also to conserve the supply of the enzyme. The activity of isolated adipocytes was reduced by excess collagenase or a long incubation time by disruption of the cells. On centrifugation of such a preparation a free fat layer appeared and the fat cell layer reduced in size. The glycerol production rate from these suspensions was always low, though significant increases could be measured.

Even under identical conditions of preparation and incubation, fat cell suspensions varied in sensitivity. This is illustrated by the difference between the means of the 6 assays for effect of sera from individual pigs on LMF lipolysis Table 4.11. Here the mean glycerol production rates varied from 2.21 to 7.66 $\mu\text{mol/gfat/h}$. Another example of the variation is the 2 assays of the lipolytic activity of gmLMR, (Figure 2.9 a & b) described in Chapter 2. Here the maximum rates of glycerol release were 3.29 and 15.28 $\mu\text{mol/gfat/h}$. The source of the rabbit fat was the only difference between the assays in both examples. Such variation made it impossible to directly compare the results of different assays unless an adjustment, based on the assay means, was made as described in section 4.2.3.2. Schleyer *et al.* (1971) found that by incubating sliced rat fat pads, rather than isolated rat fat cells, in an in vitro assay for lipolysis, inter-assay comparisons could be more easily made. Isolated fat cells were used here in vitro, rather than sliced fat pads, because the accuracy of the latter was found to be much less. The standard errors of the estimated means (SEM) were usually $> 10\%$ of the mean value when sliced fat pads were used.

Investigation of Serum Effect on LMF Induced Lipolysis

The inclusion of 0.2ml of serum in an in vitro assay with LMF fractions, β LPH and ACTH consistently potentiated the activity of the lipolytic agent (Tables 4.6, 4.7, 4.9). Induced lipolysis was only inhibited in one case, with the acidic protein which eluted from a CM cellulose column while LMF was absorbed (Section 2.3.2.3). Serum had no effect on basal lipolysis (Table 4.7), indicating that any endogenous lipolytic agents, such as catecholamines, were at too low a concentration to activate lipolysis. Curtis-Prior (1973) and Curtis-Prior & Hanley (1973) found that rat serum stimulated lipolysis from rat fat cells of the same animal. As propranolol a β -adrenergic blocking drug, inhibited this lipolysis, catecholamines in the circulation could have caused his findings. However, as catecholamines are inactive in rabbit tissue (Rudman, 1963), it was unlikely that they played a significant role in the potentiation of lipolysis. The hormones (growth hormone, glucocorticoids and thyroid hormones), which usually act synergically with stimulators of adenylyl cyclase, were unlikely to be potentiating lipolysis here, as they require a lag period of over 2 hours (Fain, 1973).

The potentiation of the lipolytic action of LMF and other peptides by porcine serum did not agree with the findings of Trygstad & Foss (1972) or Standal et al. (1973). In vivo in rabbits, both human and porcine LMF had been inhibited by serum and in vitro, human serum had inhibited human LMF induced lipolysis from human adipose tissue pieces.

Possibly inhibition required some factor present in the connective tissue which was digested by incubation with collagenase. This would explain the lack of inhibition found when isolated fat cells were used.

Standal (personal communication) has been unable to confirm his results with samples taken from pigs under normal conditions and not at slaughter. Possibly the extreme stress endured by the pigs at slaughter helped to exaggerate the difference between lines of the inhibitory effects of serum and adipose tissue. The inhibitory action of serum on lipolysis found by Trygstad & Foss (1972) could be related to the other antagonists of lipolysis stimulators mentioned in the introduction (Section 1.3.3.3). The lipolytic peptide inactivating system of Rudman et al. (1964a,b) was absent from rabbit adipose tissue, though present in other organs of the animal. If Trygstad's inhibitory factor depended on the existence of such a system, serum inhibition of LMF in vivo in rabbits, but not in vitro in rabbit fat cells, could be explained. In vivo, the peptide could be inactivated by non-adipose tissues while in vitro no inactivation takes place due to absence of the antagonist.

The potentiation of lipolysis by serum has been reported (Benuzzi-Badoni et al., 1968). Rat and guinea pig serum were found to potentiate ACTH induced lipolysis from rat adipocytes. The effect of pig serum on rabbit adipocytes stimulated by LMF is probably of the same origin as these findings, even though experimental results in one species can not be applied to another species (Rudman, 1963).

The possibility that serum potentiation was caused indirectly was investigated. The potentiation was not caused by more efficient buffering in the presence of serum. The dialysis of serum against KRB buffer should have brought the ionic composition of serum to resemble the KRB buffer. As this dialysed serum still enhanced lipolysis (Table 4.8) stimulated by LMF it was unlikely that the potentiation was a result of better buffering. On the other hand, as the inclusion in the in vitro assay of KRB + 8% (W/V) albumin with LMF and ACTH did not potentiate lipolysis (Table 4.9), it can be concluded that the 4% (W/V) albumin in other incubations was not at a limiting concentration. The potentiating effect of serum on LMF induced lipolysis would appear to be an inherent characteristic and not an artefact of the assay conditions.

Correlation of the potentiation of LMF induced lipolysis by individual serum, to traits of the animals might have indicated a physiological role for such potentiation in the regulation of lipid metabolism. A similar method might have been used for selection of such traits. However when 54 individual sera were examined neither growth rate nor backfat thickness correlated to potentiated lipolysis (Figures 4.4, 4.5). The animals, which were bled, showed significant differences between the three lines in both growth rate and backfat thickness, which correlated to one another (Figure 4.3). The breeding experiment provided a suitable model for the examination of factors involved in genetic differences between fat and lean animals.

There is the possibility that significant correlations would have been found under a different regime. For instance, increasing the serum content of the incubates, fasting the animals longer before bleeding, increasing the samples per assay to reduce inter-assay variation, might all help in producing a significant result. The stress endured by the animals during bleeding might have influenced the final results and a less stressful means of obtaining serum might improve the experimental findings.

Dialysis of 9 of the previously assayed serum samples was designed to provide identical ionic environments in the incubates so that any differences would be due entirely to larger molecular weight species. The linear regression of lipolysis (X) against growth rate (Y) for these 9 samples happened to be negative. When the dialysed serum was assayed the regression became positive, steeper and was nearer significance (Figure 4.6a). The latter would be the more expected gradient, with a high growth rate corresponding to a high lipolysis, which was the situation found in the larger experiment with all 54 non-dialysed samples. The slope of the linear regression of backfat thickness (Y) against lipolysis (X) changed from positive to negative following dialysis of sera. This would correspond to serum from animals with high backfat having a low potentiating effect on lipolysis which differed from the findings of the original experiment with non-dialysed serum. Even though the linear regressions were affected by dialysis, none of the results proved to be significant. Possibly by assay of

more dialysed samples, a significant result could have been achieved but the serum sample volumes were by then too low for further studies to be undertaken.

Further areas for study for early selection of animals for leanness and growth rate will be dealt with in the following general discussion.

4.5 Summary

- 1) The degradation of ^{125}I -labelled LMF on incubation with serum could not be quantified and was therefore not suitable for the estimation of the serum inhibition of the lipolytic activity of LMF.
- 2) An assay for lipolytic activity in vitro was developed and used to investigate the effect of serum on LMF induced lipolysis.
- 3) Serum was found to potentiate the lipolysis stimulated in vitro by LMF. This effect was not due to different ionic composition of serum or to serum containing extra albumin for the binding of released free fatty acids.
- 4) The potentiation of LMF induced lipolysis caused by the sera from individual pigs was not significantly correlated to either the pigs' growth rate or backfat thickness.
- 5) Dialysis of sera prior to investigation of any such correlation did not alter the non-significance of the results.

CHAPTER 5DiscussionConclusions From Experimental Chapters

The lipid mobilizing factor, isolated from deep frozen porcine pituitaries, stimulated the release of glycerol from rabbit adipocytes in vitro. On polyacrylamide disc gel electrophoresis at pH 8.9 the LMF preparation had an identical mobility to that of Trygstad's porcine LMF. These two peptides also had a similar mobility on sodium dodecyl sulphate polyacrylamide gel electrophoresis, though a higher molecular weight contaminant was present in the sample (mLMFd). LMF alone did not stimulate the production of specific antibodies in immunized rabbits. A non-specific antiserum, which cross-reacted with other pituitary hormone, was raised in one rabbit against an LMF-BSA conjugate. Immunization of rabbits with LMF after electrophoresis on polyacrylamide gels did not stimulate the immune response. The lipolytic action of LMF, in vitro, was potentiated by replacement of 15% of the incubation medium by porcine serum. Non-dialysible material in the serum, but not albumin, was responsible for the potentiation of LMF induced lipolysis. The potentiating effect of serum from individual pigs did not correlate to either the growth rate or backfat thickness of the respective animal. There seemed to be little hope of using such a method for the selection of lean, fast growing animals.

Nature of LMF

Indications to the nature of the LMF isolated here, can be gained from examination of various experimental results.

The ability of LMF to stimulate lipolysis in rabbit adipocytes within 2 hours, indicated that the cAMP mediated lipolytic cascade (Figure 1.1) ^{could be} ~~was~~ involved. The mechanism could be similar to that of β LPH and ACTH, both of which required Ca^{++} ions in the medium for their lipolytic action to be seen (Lopez et al., 1959; Lis et al., 1972). EDTA (at 15 and 0.75mM), which chelates Ca^{++} and Mg^{++} ions, inhibited the lipolytic action of LMF and showed that these ions are necessary for LMF to stimulate lipolysis.

Comparison of the chromatographic isolation procedures and electrophoresis gels of LMF with γ LPH and β LPH indicated that LMF was more basic than these lipotropins. All three porcine peptides were absorbed on to a column of CM cellulose equilibrated with 0.01M ammonium acetate, although the buffer was at pH 4.6 for the lipotropins (Chrétien, 1973), and pH 5.5 for LMF. Judging from the elution diagram (Graf et al., 1969), δ LPH was desorbed before the buffer gradient reached either pH 5.5 or 0.05M, conditions under which LMF is still absorbed by the cellulose (Figures 2.8, 2.10). The interpretation of the elution of β LPH (Gilardeau & Chrétien, 1970) with respect to LMF is more difficult. The gradients of buffer pH and concentration were not shown for the elution diagram of β LPH so the conditions at which it desorbed were harder to estimate. However β LPH was eluted before the buffer pH and concentration rose to pH 6.7 and 0.1M, conditions which would also desorb LMF (Figures 2.8, 2.10). Thus there is some similarity in the ionic nature of β LPH and LMF. On electrophoresis at pH 8.9 (Plate 3.1), LMF was

less mobile and therefore more basic than the main peptide band of β LPH but had the same mobility as the minor band. Presumably the main peptide band was β LPH, so LMF was more basic than this peptide as well as being more basic than γ LPH.

The third member of the lipotropin group of peptides, which have similar peptide sequences, β MSH, was desorbed from a CM cellulose column under similar condition to LMF. Lande et al. (1965) in their investigation of various peptides from porcine pituitaries, found that a fraction (fraction D) which resembled β MSH, eluted from a CM cellulose column when the buffer concentration rose from 0.027M to 0.068M. Although they used a succinate-acetic acid buffer and not ammonium acetate, a similar rise in buffer concentration would desorb LMF from CM cellulose. It is possible that the isolated LMF and the minor band of peptide seen on electrophoresis of β LPH are both β MSH. The high mobility of LMF on SDS polyacrylamide electrophoresis (Plate 2.2), which indicates a low molecular weight, supports this theory, as β MSH is also a small molecule. That LMF and the peptide present in the minor stained band of LPH on electrophoresis are similar was confirmed by the finding that this band was the one which inhibited the binding of ^{125}I -labelled LMF to anti-LMF antiserum (Figure 3.11). It is possible therefore, that the LMF, isolated as described in Chapter 2, resembles the lipotropins and may even be identical to β MSH.

The Relation of the Purification of LMF to the Production of Antiserum

One possible reason, discussed in Chapter 3, for the production of non-specific antiserum to LMF, was that the

preparation of LMF (mLMF-VIId) conjugated to bovine serum albumin was contaminated with a glycoprotein hormone. Electrophoresis on SDS polyacrylamide showed that a similar LMF preparation (mLMFd) contained a high molecular weight contamination which could have been a glycoprotein. The main difference in purification of these two LMF preparations was that mLMF-VIId had been rechromatographed on a CM32 cellulose column. The procedure from residue 4 being G-15 \longrightarrow DE32 \longrightarrow CM32 \longrightarrow CM32. Possibly, gel filtration could have removed contamination of LMF with a larger molecule. However mLMFd, eluted from a G-50 sephadex column with an apparent molecular weight of between 14,500 and 20,000 (Figures 2.13), due either to the association between LMF and the large contaminant or to the aggregation of LMF molecules. Therefore, further gel filtration to separate mLMFd from the contaminant might not work.

Possibly all the binding of ^{125}I -labelled LMF to the antiserum from rabbit R199 was a result of the binding of ^{125}I -labelled contamination of LMF to antibodies specific for the contaminant. This would not explain why the most potent inhibition of the binding was found to be by β LPH. Unless it was similarly contaminated with glycoproteins, β LPH would only inhibit a real interaction between ^{125}I -labelled LMF and the antiserum. The fact that it was β LPH which was the most potent inhibitor of this antibody antigen reaction would indicate that LMF more closely resembled a peptide present in the β LPH preparation than the glycoproteins.

Although it was unlikely that all the precipitated γ -radiation was due to ^{125}I -labelled contaminant binding to specific antibodies, a proportion might have been precipitated in this way.

The results here suggested that LMF was not immunogenic in rabbits. This confirmed Norman and Turtter's (1968) finding that immunization of rabbits with human LMF did not produce anti-LMF antiserum. Possibly the rabbit has a lipid mobilizing factor, similar in structure to both the porcine and human peptides, so that the rabbit's immune system could not distinguish injected LMF from the native peptide. The high lipolytic activity of LMF in rabbit tissue would support this theory. The same does not apply to ACTH or β LPH as these are both able to stimulate the production of antibodies in rabbits. The small size of LMF seen by electrophoresis on SDS polyacrylamide gels, and the relatively low doses available for injection probably contributed to the lack of response.

Some antibodies were produced against LMF when it was conjugated to BSA but a greater response was found in the production of anti-glycoprotein antibodies. The 70% binding of ^{125}I -labelled LH to the supposedly anti-LMF antiserum (Table 3.2) showed that more antibodies had been produced that would react with LH than would react with LMF. Such an antiserum was not of practical use in a radioimmunoassay for LMF.

Is There a Physiological Role for LMF and its Potentiation by Serum?

The question as to whether LMF has a definite role to play in the lipid metabolism of the body remains unanswered.

Both the attempts to develop a radioimmunoassay for measurement of the peptide in serum and to develop a method capable of giving an indication of an animals potential growth were unsuccessful. However, the ability of porcine serum to potentiate LMF induced lipolysis may reflect the presence of an LMF regulating system in serum. If such a system exists and is shown to be specific for LMF then a major advance would be made in assigning to LMF a physiological role.

The nature of the factor in serum responsible for the enhancement of LMF stimulated lipolysis has not been fully investigated but it is non-dialysible. Small molecules including the catecholamines, and ions are therefore not the cause of the potentiation. The hormones, which are known to potentiate adrenalinestimulated lipolysis, growth hormone, thyroid hormones and glucocorticoids, all have a lag period of over 2 hours, before their effects are seen (Fain,1973). As the potentiation of LMF induced lipolysis occurred within a 2 hour incubation period, these hormones were probably not responsible. It is unlikely that other known lipolytic agents in the serum were the cause of the observed potentiation as they would have stimulated lipolysis when serum was incubated with the fat cell suspension in the absence of LMF.

Future Areas for Investigation

A physiologically significant role for LMF may still be confirmed by radioimmunoassay if a specific antibody could be produced. The animal species immunized is probably of importance in the raising of anti-LMF antiserum. Antisera

against insulin and parathyroid hormone could only be produced in guinea-pigs and not in rabbits (Hurn and Landon, 1971). Perhaps the situation is similar with LMF and changing the species immunized might help the production of antiserum specific for LMF.

To ensure the specificity of any antiserum raised against LMF, the large molecular weight contaminant should be removed prior to immunization. Gel filtration under different conditions to minimise the aggregation of LMF to itself or contaminants might be useful (Chapter 2). If this still failed to purify the peptide a different procedure such as preparative electrophoresis on SDS polyacrylamide gels could be attempted.

Full characterization of LMF must await the preparation of a homogeneous sample of sufficient quantity. An N-terminus analysis has been carried out on mLMFd, the preparation which had a high molecular weight contamination. Glutamic acid appeared to be the major N-terminal amino acid (Dunn MJ, personal communication). α LPH and β LPH have glutamate as their N-terminal residue, emphasising the similarity between LMF and the lipotropins. A second N-terminal amino acid was evident but not enough material could be spared for further investigation. Amino acid analysis of LMF and determination of its sequence would help establish the relation of LMF to the lipolytic peptides and other hormones. Up to 10mg of homogeneous material would be required for amino acid sequence analysis and this amount of purified peptide has not been available.

The relationship of LMF to other lipolytic peptides could be investigated by examination of fat cell membrane hormone receptors. Rat fat cell membrane preparations have been made that bind ^{125}I -labelled hormones, such as glucagon (Desbuquois & Laudat, 1974) and insulin (Cuatrecasas, 1971). These were bound by specific hormone receptors. For instance, bound labelled glucagon could not be replaced by other hormones but only by unlabelled glucagon. If a fat cell membrane preparation was made, capable of binding ^{125}I -labelled LMF then the specificity of the membrane receptors could be examined. The effect of serum on the binding could also be investigated. If LMF was bound specifically by the membrane preparation, the latter could replace antiserum in a saturation analysis (Ekins, 1970). LMF would then be measured by a radioreceptor assay in the same way as Lefkowitz et al. (1970) estimated ACTH, though they used adrenal rather than fat cell membranes.

The potentiating effect of serum on LMF induced lipolysis, could be further investigated by fractionation of the serum, for instance on DEAE ion-exchange cellulose (Sober & Petersen, 1958). The fraction, with which the potentiating effect was associated might give an indication of the relation of the potentiation to other serum effects on lipolysis (Trygstad & Foss, 1967, 1972; Benuzzi-Badoni, 1968; Curtis-Prior & Hanley, 1973). The serum effect on lipolysis from adipose tissue of different species and on other lipolytic agents should be investigated in vivo as well

as in vitro. Perhaps only the lipotropins, ACTH and LMF are potentiated and then, only with a rabbit fat cell suspension. Possibly alterations in the assay conditions could have produced more exaggerated and significant results when the serum potentiation was correlated to growth rate and backfat thickness of individual pigs. Thus the inclusion of a greater volume of serum, a change in the dose of LMF and an alteration of incubation time might be worth attempting to produce a significant correlation. Incubating more samples in a single assay would help reduce inter-assay variation and possibly increase the chance of a significant finding.

Measurement of alternative biochemical parameters, as selection markers for potential growth in pigs or other animals, is worthy of investigation. In the pigs described by Vangen (1974a), serum growth hormone concentration varied between the lines (Lund-Larsen & Bakke, 1975). There was a greater concentration of growth hormone in serum from animals of the high growth, low fat (HP) line than in serum from low growth rate and high fat (LP) animals. The growth hormone content of serum from control line pigs was between the other two lines. Serum content of somatomedin, the growth hormone intermediate, varied in the same way. However measurement of serum growth hormone concentration in pigs selected only for their backfat thickness, did not reveal such differences (Althen & Gerrits, 1976). They found that high fat pigs had a lower serum content of growth hormone than controls at both weaning and slaughter but that low fat pigs could not be distinguished from the controls. Serum growth hormone

concentration could not be used to directly select for low fat pigs but might be used to select against high fat animals.

Free fatty acid concentration in serum has also been examined in the three lines of pigs described by Vangen (1974a). Bakke (1975) found that after a 25 hour fast, the FFA concentration varied between the lines in serum from 140-160 day old pigs. Younger animals fasted for a shorter time showed more variation in results and no significant differences between the lines. A 25 hour fast is probably necessary to reveal any differences and this may be deleterious to young animals. Instead of starving the animals, the FFA concentration of their serum could be measured after injection of a lipolytic agent, such as LMF. The response after a certain period could be examined for any correlation with the animals growth and leanness data.

If the serum content of any of the other lipolytic peptides and hormones were shown to correlate with growth rate or backfat thickness of domestic animals then measurement of these would also be useful as selection criteria.

There are therefore, a number of possible directions further research into LMF could take. Some of these depend on the isolation of a homogeneous preparation. With such a preparation, the relation of LMF to other peptides could be established. A specific antiserum for a radioimmunoassay might be produced but probably in a different species to the rabbit. The potentiation of LMF induced lipolysis could be investigated for its mechanism and relevance to growing animals. Biochemical parameters unconnected with LMF could

be examined for any relation to beneficial traits both in selected and non selected animals. Hopefully a method, involving these techniques, might eventually be developed to provide an early estimate of a young animals potential growth and thereby increase the efficiency of animal production.

APPENDIX 1Nomenclature of LMF preparations.

- crude LMF: salt free fractions following gel filtration on Sephadex G-15 of residue 4.
- dLMF₁ or 2: fractions from DEAE cellulose ion-exchange chromatography of crude LMF.
- mLMF_{1,2,etc.}: fractions of extraction VIII from CM cellulose ion-exchange chromatography of crude LMF.
- gmLMF₁ or 2: fractions from gel filtration following CM cellulose ion-exchange chromatography.
- mLMFa,b,etc.: fractions of extraction XI from CM cellulose ion-exchange following DEAE ion-exchange chromatography.
- mLMF-VIIId (and similar): fractions from none of the extractions described in detail (V, VIII or XI) but in this case from extraction VII. The Roman numerals show which extraction the fraction comes from. The first and last letters or figures indicate the most closely resembling of the preparations described in detail, here mLMFd.

APPENDIX 2Material

All chemicals, which were of Analar grade, were supplied by BDH Chemicals Ltd., Poole, Dorset, apart from the following.

The Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, supplied bovine serum albumin, ECDI, porcine ACTH (100I.U./mg), TRIZMA BASE, glucagon, bovine insulin (24I.U./mg), lysozyme (25,000u/mg), chymotrypsinogen (30u/mg), carbonic anhydrase (2,500u/mg), dithioerythritol, and triethanolamine hydrochloride.

The Boehringer Corporation (London) Ltd., Bilton House, Ealing, London, supplied phosphoenolpyruvate hydrochloride, NADH, ATP disodium salt, collagenase (0.15u/mg), lactate dehydrogenase (550u/mg), pyruvate kinase (200u/mg) and glycerol kinase (85u/mg).

The ovine hormones PRL, LH, FSH and TSH and porcine GH were supplied by the National Institute of Health, Bethesda, Maryland U.S.A. (see table 3.1 for batch numbers).

Bordetella pertussis vaccine was provided by Wellcome Reagents Ltd., Beckenham, Kent. Freund's Complete Adjuvant was supplied by Gibco Bio-Cult Ltd., Paisley, Renfrewshire.

Sephadex of various grades was supplied by Pharmacia (G.B.) Ltd., 75 Uxbridge Rd., London. Ion-exchange celluloses were supplied by Whatman Biochemicals Ltd., Maidstone, Kent.

Oxygen, nitrogen and 95% O₂:5% CO₂ were supplied by BOC Ltd., Seafield Road, Edinburgh.

Tween 80 was supplied by Koch-Light Ltd., Colnbrook, Buckinghamshire.

Iodide-125 was supplied by the Radiochemical Centre, Amersham, Buckinghamshire.

Goat anti-rabbit immunoglobulin antiserum was provided by Mr. W.R. Carr, of the Department of Physiological Genetics, ABRO. Normal rabbit serum was supplied by Mr. D. McVitie of the Moredun Institute, Edinburgh who also provided rabbits for experimental use. Pig serum was collected at the slaughterhouse of the City of Edinburgh District Council and from pigs of the Institute of Animal Genetics and Breeding, Aas-NLH, Norway.

Porcine pituitaries were supplied by Lawson of Dyce, Dyce, Aberdeenshire.

Appendix 3 Equipment

The following specialised equipment was used.

LKB Instruments Ltd., South Croydon, Surrey supplied the fraction collector (Ultrorac 7000), the gradient maker (Ultrograd 11300) and the peristaltic pump (Varioperpex 12000).

A Searle Instruments, Harrow, Essex 'Analmatic' preparation unit and centrifuge (CRU-5000) were used.

Both a PYE Scientific Instruments Ltd., Cambridge (Unicam SP800A) and a Cecil Instruments Ltd., Cambridge (CE272) spectrophotometer were used.

Both a Wallac DECEM-GTL (LKB Instruments Ltd.) and a Mini-Assay (Mini Instruments Ltd., Burnham-on-Crouch, Essex) γ -spectrometer were used.

Electrophoresis was carried out in a Shandon kit (Shandon Southern Instruments Ltd., Camberley, Surrey) using a Shandon Vokam SAE2761 power pack.

MSE High Speed 18 and desk-top centrifuges were both employed (MSE Ltd., Crawley, Sussex). The freeze-drier was supplied by Edwards High Vacuum Ltd., Crawley, Sussex, (Model 30.P 2/797).

Polystyrene LP4 tubes were supplied by Luckham Ltd., Burgess Hill, Sussex.

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