SERUM INHIBITORS OF INSULIN ACTION

A thesis presented to the University of London in part fulfilment of

the requirement for the degree of

DOCTOR OF PHILOSOPHY

by

NEELIMA AVASTHY BSc

MEDICAL LIBRARY,
ROYAL FREE HOSPITAL
HAMPSTEAD.

Department of Chemical Pathology and Human Metabolism,

Royal Free Hospital School of Medicine,

London

1990
This thesis is dedicated to the memory of my father

Dr. Suresh Kumar Avasthy
ACKNOWLEDGEMENTS

I should like to express my deepest gratitude to Dr. P. Dandona (Director Metabolic Unit), Dr. D.P. Mikhailidis (Senior Lecturer in Chemical Pathology) and Mr. J.Y. Jeremy (Senior Research Fellow) for their guidance and encouragement throughout this project.

To Professor D.N. Baron and all the members of the Department of Chemical Pathology & Human Metabolism, my thanks for much appreciated support and advice.

I should also like to thank Mr. A.M. Taylor (Institute of Child Health) who carried out the $[^{35}S]_i$-sulphate uptake by porcine cartilage work quoted in this work and Dr. M.A. Khokher (Research Assistant) who introduced me to the lipogenesis bioassay methodology.

Very special thanks go to Mr. Iain Haldane Taylor, Miss Lauren Ozin and all other friends, too numerous to mention individually, for their encouragement through the years.

Last but by no means least I would like to express my fondest love and thanks to my parents, Dr. & Mrs. S.K. Avasthy without whom none of this would have been possible.
This work describes an investigation into the presence of inhibitors of insulin and somatomedin C in sera of (i) normal, diabetic and obese humans and (ii) sera of normal and diabetic rats. The effect of whole sera and fractions prepared from sera was tested on (i) basal and insulin stimulated rat adipocyte lipogenesis and glucose oxidation and (ii) basal and serum somatomedin stimulated [35S] labelled sulphate uptake by porcine cartilage.

Fractions were prepared from human sera by using supernatants prepared after the precipitation of larger proteins with ammonium sulphate. Supernatants were further fractionated by ultrafiltration. Inhibitory activity in sera from normal subjects was found in 10-30kDa and 30-50kDa fractions. Sera from diabetic and obese patients had inhibitory activity in the fractions similar to those above and this activity was not significantly different to that found in normal subjects. However sera from obese non-insulin dependent diabetics showed significantly greater inhibitory activity than sera from non-obese non-insulin dependent diabetics.

A further small molecular weight inhibitor was identified by acid-ethanol extraction of human sera and during the course of these experiments it also became clear that glucose and insulin have to be excluded from inhibitory preparations and methodology was devised accordingly. No difference was found between the inhibitory activity of these fractions from sera of controls and non-insulin dependent diabetic
patients. However, fractions prepared from insulin dependent diabetics appear to have insulin inhibitory factors.

Studies to identify the site of action of the inhibitory fractions prepared by ammonium sulphate precipitation of human sera led to a series of experiments to determine a role for protein kinase C activation, calcium influx and calmodulin in basal and insulin stimulated lipogenesis. While verapamil, a calcium channel blocker, H7, a protein kinase C inhibitor and calmidazolium, a calmodulin inhibitor, inhibited basal and insulin stimulated lipogenesis, phorbol esters, known protein kinase C stimulators and calcium ionophore (A23187) stimulated basal and insulin induced lipogenesis. The serum inhibitory fractions (10-30kDa and 30-50kDa) from human sera inhibited not only insulin stimulated but also phorbol ester and A23187 stimulated lipogenesis. Thus the site of action of these inhibitors was distal to that of protein kinase C and calcium entry into the cell. These inhibitors did not affect the glucose transporter activity.

Sera from rats with streptozotocin induced diabetes had a marked inhibitory activity as far as \([^{35}S]\)-sulphate uptake by porcine cartilage is concerned. This inhibitory activity was exhibited in the \(<1kDa, 1-10kDa, 30-50kDa\) and \(>300kDa\) fractions. In the rat lipogenesis bioassay only the \(<1kDa\) fraction was found to be inhibitory.

It is concluded that normal human sera have insulin and somatomedin inhibitory activity and that these inhibitory fractions do not demonstrate increased activity in either type I or type II diabetes. However, obese patients with type II diabetes have a significantly greater insulin inhibitory activity in their sera. Sera from rats also have somatomedin inhibitory fractions. The inhibitory activity of these
fractions increases following induction of diabetes with streptozotocin; this increase in inhibitory activity can be prevented by treating diabetic rats with insulin. The role of the inhibitors in human serum in the pathogenesis of insulin resistance requires further investigation.
# LIST OF CONTENTS

<table>
<thead>
<tr>
<th>CONTENTS</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dedication</td>
<td>2</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>3</td>
</tr>
<tr>
<td>Abstract</td>
<td>4</td>
</tr>
<tr>
<td>List of Contents</td>
<td>7</td>
</tr>
<tr>
<td>List of Tables</td>
<td>8</td>
</tr>
<tr>
<td>List of Figures</td>
<td>14</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>20</td>
</tr>
<tr>
<td>CHAPTER 1 - INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>AIMS OF THE STUDY</td>
<td>50</td>
</tr>
<tr>
<td>TECHNIQUES USED</td>
<td>51</td>
</tr>
<tr>
<td>CHAPTER 2 - MATERIALS AND METHODS</td>
<td>54</td>
</tr>
<tr>
<td>CHAPTER 3 - RESULTS OF INSULIN INHIBITORY ACTIVITY FOUND IN</td>
<td></td>
</tr>
<tr>
<td>SUPERNATANTS OBTAINED AFTER AMMONIUM SULPHATE</td>
<td>93</td>
</tr>
<tr>
<td>CHAPTER 4 - RESULTS OF INVESTIGATION INTO THE POST-RECEPTOR</td>
<td></td>
</tr>
<tr>
<td>MECHANISMS MEDIATING INSULIN STIMULATED</td>
<td></td>
</tr>
<tr>
<td>LIPOGENESIS IN RAT ADIPOCYTES AND THE EFFECT OF</td>
<td></td>
</tr>
<tr>
<td>SERUM INSULIN INHIBITORY FRACTIONS PREPARED BY</td>
<td></td>
</tr>
<tr>
<td>AMMONIUM SULPHATE PRECIPITATION ON THESE PATHWAYS</td>
<td>145</td>
</tr>
<tr>
<td>CHAPTER 5 - RESULTS OF INSULIN INHIBITORY ACTIVITY FOUND IN</td>
<td></td>
</tr>
<tr>
<td>SUPERNATANTS OF ACID ETHANOL PRECIPITATED SERUM</td>
<td></td>
</tr>
<tr>
<td>CHAPTER 6 - DISCUSSION</td>
<td>220</td>
</tr>
<tr>
<td>FUTURE WORK</td>
<td>257</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>258</td>
</tr>
<tr>
<td>PUBLICATIONS OF AUTHOR</td>
<td>309</td>
</tr>
</tbody>
</table>
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>3:1</td>
<td>97</td>
</tr>
<tr>
<td>3:2</td>
<td>98</td>
</tr>
<tr>
<td>3:3</td>
<td>99</td>
</tr>
<tr>
<td>3:4</td>
<td>100</td>
</tr>
<tr>
<td>3:5a</td>
<td>102</td>
</tr>
<tr>
<td>3:5b</td>
<td>102</td>
</tr>
</tbody>
</table>

3:1 Effect of unfractionated inhibitor obtained from supernatants of ammonium sulphate precipitated control sera on basal and insulin-stimulated rat adipocyte lipogenesis.

3:2 Effect of unfractionated inhibitor obtained from supernatants of ammonium sulphate precipitated control sera on IgG-stimulated rat adipocyte lipogenesis.

3:3 Effect of unfractionated inhibitor obtained from supernatants of ammonium sulphate precipitated control sera on NSILA-s stimulated rat adipocyte lipogenesis.

3:4 Effect of unfractionated inhibitor obtained from supernatants of ammonium sulphate precipitated control sera on NSILA-p stimulated rat adipocyte lipogenesis.

3:5a Effect of ethanol on glucose oxidation in rat adipocytes.

3:5b Effect of ethanol on lipogenesis in rat adipocytes.
3:6 Effect of heat treatment (37°, 60° & 80°C), on the action of inhibitors obtained from supernatants of ammonium sulphate precipitated control sera on basal and insulin-stimulated rat adipocyte lipogenesis.  

3:7 Effect of trypsin on basal and insulin-stimulated rat adipocyte lipogenesis.  

3:8 Effect of trypsinisation and soya bean trypsin inhibitor on the action of inhibitors obtained from supernatants of ammonium sulphate precipitated control sera on rat adipocyte lipogenesis.  

3:9 Effect of immobilized trypsin on the action of serum inhibitors (from control subjects) on basal and insulin stimulated rat adipocyte lipogenesis.  

3:10a Effect of insulin and unfractionated serum inhibitor on the incorporation of [3H]-glucose into triglycerides, fatty acids and glycerol in rat adipocytes. (Incubation media contained 0.1mM unlabelled glucose.)  

3:10b Effect of insulin and unfractionated serum inhibitor on the incorporation of [14C]-glucose into triglycerides, fatty acids and glycerol in rat adipocytes. (Incubation media contained 0.1mM unlabelled glucose.)
3:11 Effect of the control 10-30kDa serum inhibitor fraction on radiolabelled glucose incorporation into triglycerides, fatty acids and glycerol in the presence of 0.25mM unlabelled glucose.

3:12 Effect of the control 10-30kDa serum inhibitor fraction on radiolabelled glucose incorporation into triglycerides, fatty acids and glycerol in the presence of 0.5mM unlabelled glucose.

3:13a Effect of pooled inhibitory serum fractions (0-1kDa to >50kDa) from NIDD patients on basal and insulin-stimulated rat adipocyte lipogenesis.

3:13b Effect of pooled non-inhibitory serum fractions (0-1kDa to >50kDa) from NIDD patients on basal and insulin-stimulated rat adipocyte lipogenesis.

3:14a Effect of pooled inhibitory serum fractions (0-1kDa to >50kDa) from IDD patients on basal and insulin-stimulated rat adipocyte lipogenesis.

3:14b Effect of pooled non-inhibitory serum fractions, (0-1kDa to >50kDa) from IDD patients on basal and insulin-stimulated rat adipocyte lipogenesis.

3:15 Rat adipocyte lipogenesis in the presence of serum fractions (inhibitory in the somatomedin bioassay) obtained from diabetic and control rats.
3:16 Effect of inhibitory fractions (0-1kDa) obtained from supernatants of ammonium sulphate precipitated rat sera (A: control; B: diabetic; C: insulin treated diabetic) on rat adipocyte lipogenesis.

4:1 Effect of DMSO on rat adipocyte lipogenesis.

4:2 Comparison of the effect of the phorbol esters, PDBu and PMA on rat adipocyte lipogenesis and the synergistic effect of the calcium ionophore A23187.

4:3 Effect of the calmodulin inhibitor, calmidazolium (CMZ), on basal and insulin-stimulated rat adipocyte lipogenesis.

4:4 Comparison of the effect of the protein kinase C inhibitors H7 & Polymixin B, the voltage dependent calcium channel blocker Verapamil, the calmodulin inhibitor, Calmidazolium (CMZ) & the calcium ionophore, A23187 on insulin- and IgG-stimulated rat adipocyte lipogenesis.

4:5 Effect of sodium fluoride (NaF) on basal and insulin-stimulated rat adipocyte lipogenesis.

4:6 Effect of inhibitory fractions (0-10kDa to >50kDa) obtained from supernatants of ammonium sulphate precipitated control sera on PMA-stimulated rat adipocyte lipogenesis.
4:7 Effect of unfractionated and fractionated supernatants obtained from ammonium sulphate precipitated control sera on cytochalasin B binding to erythrocyte membranes.

5:1 Effect of redissolved acid-ethanol precipitate from control sera on rat adipocyte lipogenesis.

5:2 Effect of increasing concentrations of exogenous unlabelled glucose on basal and insulin-stimulated rat adipocyte lipogenesis.

5:3 Effect of supernatants from acid-ethanol precipitated control sera in which glucose concentrations had been equalized on rat adipocyte lipogenesis.

5:4 Effect of gel filtrated, glucose free supernatants obtained from acid-ethanol precipitated control sera on rat adipocyte lipogenesis.

5:5 Effect of dialysed, glucose free supernatants of acid-ethanol precipitated control sera on rat adipocyte lipogenesis.

5:6 Effect of gel filtrated, dialysed and glucose free supernatants of acid-ethanol precipitated control sera on rat adipocyte lipogenesis.

5:7 Elution profile of supernatants of acid-ethanol precipitated control sera when applied to a C18 Sep-pak column. (By the method of Dean et al 1984).
5:8i Endogenous concentrations of glucose and insulin in acid-ethanol supernatant extracts prepared from control subjects and NIDDM & IDDM patients.

5:8ii Median (range) concentration of glucose and insulin in Preparations B.

5:9 Final median concentrations of glucose and insulin following the addition of supernatants of acid-ethanol precipitated sera to the rat adipocyte lipogenesis incubates.

5:10 Effect of supernatants of acid-ethanol precipitated sera before and after removal of glucose and insulin on insulin-stimulated rat adipocyte lipogenesis.

5:11a Insulin concentrations of pooled and fractionated supernatants of acid-ethanol precipitated control, NIDDM and IDDM sera.

5:11b Glucose concentrations of pooled and fractionated supernatants of acid-ethanol precipitated control, NIDDM and IDDM sera.

5:12 Effect of low molecular weight (0-1kDa & 1-5kDa) fractions obtained from supernatants of acid-ethanol precipitated control, NIDDM and IDDM sera on rat adipocyte lipogenesis.
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:2</td>
<td>Glycerol phosphate shuttle for the transport of reducing equivalents from cytosol into the mitochondrion.</td>
<td>37</td>
</tr>
<tr>
<td>1:3</td>
<td>The acetyl-CoA carboxylase reaction.</td>
<td>37</td>
</tr>
<tr>
<td>2:1</td>
<td>An example of an insulin radioimmunoassay standard curve.</td>
<td>82</td>
</tr>
<tr>
<td>2:2</td>
<td>An example of a protein standard line by the Folin Lowry method.</td>
<td>90</td>
</tr>
<tr>
<td>3:1</td>
<td>Effect of insulin on rat adipocyte lipogenesis.</td>
<td>95</td>
</tr>
<tr>
<td>3:2</td>
<td>Effect of unfractionated inhibitor (10mg.l⁻¹) obtained from supernatant of ammonium sulphate precipitated control serum on basal and insulin-stimulated glucose oxidation in rat adipocytes.</td>
<td>101</td>
</tr>
<tr>
<td>3:3</td>
<td>Effect of the 0-1kDa fraction obtained from supernatants of ammonium sulphate precipitated control sera on basal and insulin-stimulated rat adipocyte lipogenesis.</td>
<td>108</td>
</tr>
</tbody>
</table>
3:4 Effect of the 1-10kDa fraction obtained from supernatants of ammonium sulphate precipitated control sera on basal and insulin-stimulated rat adipocyte lipogenesis.

109

3:5 Effect of the 10-30kDa fraction obtained from supernatants of ammonium sulphate precipitated control sera on basal and insulin-stimulated rat adipocyte lipogenesis.

111

3:6 Effect of the 30-50kDa fraction obtained from supernatants of ammonium sulphate precipitated control sera on basal and insulin-stimulated rat adipocyte lipogenesis.

113

3:7 Effect of the 50-100kDa fraction obtained from supernatants of ammonium sulphate precipitated control sera on basal and insulin-stimulated rat adipocyte lipogenesis.

115

3:8 Effect of the >100kDa fraction obtained from supernatants of ammonium sulphate precipitated control sera on basal and insulin-stimulated rat adipocyte lipogenesis.

117

3:9 Effect of the 10-30kDa and 30-50kDa inhibitor fractions obtained from supernatants of ammonium sulphate precipitated sera on insulin stimulated rat adipocyte lipogenesis.

119

3:10 Effect of inhibitor fractions (10-30kDa and 30-50kDa) on the formation of triglycerides, fatty acids and glycerol in the absence of unlabelled glucose in rat adipocyte incubates.

123
3:11 The inhibitory effect of serum fractions 10-30kDa and 30-50kDa on 
\[^{35}S\]sulphate uptake by porcine cartilage.

3:12 Inhibition of serum-stimulated \[^{35}S\]sulphate uptake in porcine 
cartilage by 10-30kDa and 30-50kDa ammonium sulphate inhibitory serum 
fractions.

3:13 Effect of unfractionated inhibitor obtained from supernatants of 
ammonium sulphate precipitated control, NIDDM & IDDM sera on basal and 
insulin-stimulated rat adipocyte lipogenesis.

3:14 Effect of serum inhibitor preparations from control and obese 
(non-diabetic) subjects on basal and insulin-stimulated rat adipocyte 
lipogenesis.

4:1 Dose response histogram showing the effect of PMA on rat 
adipocyte lipogenesis.

4:2 Effect of the protein kinase C activator, PMA, on insulin-
stimulated rat adipocyte lipogenesis.

4:3 Effect of the protein kinase C inhibitor, H7, on PMA-stimulated rat 
adipocyte lipogenesis.

4:4 Effect of the protein kinase C inhibitor, polymyxin B (PB), on PMA-
stimulated rat adipocyte lipogenesis.
4:4 Effect of the protein kinase C inhibitor, polymyxin B (PB), on PMA-stimulated rat adipocyte lipogenesis. 154

4:5 Effect of the protein kinase C inhibitors, H7 and polymyxin B (PB), on insulin-stimulated rat adipocyte lipogenesis. 156

4:6 Effect of the calcium ionophore A23187, on PMA-stimulated rat adipocyte lipogenesis. 159

4:7 Effect of the protein kinase C inhibitor, H7, on PMA- and calcium ionophore A23187-stimulated rat adipocyte lipogenesis. 160

4:8 Effect of calcium ionophore A23187, on insulin-stimulated rat adipocyte lipogenesis. 162

4:9 Effect of the protein kinase C inhibitor H7, on insulin- and calcium ionophore A23187-stimulated rat adipocyte lipogenesis. 164

4:10 Effect of the calcium channel blocker, verapamil, on PMA-stimulated rat adipocyte lipogenesis. 166

4:11 Effect of the calcium channel blocker, verapamil, on insulin-stimulated rat adipocyte lipogenesis. 168

4:12 Effect of the calmodulin inhibitors W5 and W13 on insulin-stimulated rat adipocyte lipogenesis. 170
4:14 Effect of the calmodulin inhibitor, calmidazolium (CMZ), on PMA- and calcium ionophore A23187-stimulated rat adipocyte lipogenesis.

4:15 Effect of inhibitor fraction 10-30kDa obtained from supernatant of ammonium sulphate precipitated control serum on insulin- and calcium ionophore A23187-stimulated rat adipocyte lipogenesis.

4:16 Effect of inhibitor fraction 10-30kDa obtained from supernatants of ammonium sulphate precipitated control serum on PMA- and calcium ionophore A23187-stimulated rat adipocyte lipogenesis.

4:17 Effect of inhibitor fraction 30-50kDa obtained from supernatants of ammonium sulphate precipitated control serum on insulin- and calcium ionophore A23187-stimulated rat adipocyte lipogenesis.

4:18 Effect of inhibitor fraction (30-50kDa) obtained from supernatant of ammonium sulphate precipitated control serum on PMA- and calcium ionophore A23187-stimulated rat adipocyte lipogenesis.

5:1 Effect of dialysed, gel filtrated supernatants obtained from acid-ethanol precipitated serum on serum somatomedin stimulated $[^{35}S]$-sulphate uptake by porcine cartilage.

5:2 Effect of dialysed and non-dialysed supernatants obtained from acid-ethanol precipitated sera on basal $[^{35}S]$-sulphate uptake in porcine cartilage.
5:3 Correlation between the concentration of glucose in supernatants of acid-ethanol precipitated sera and the corresponding concentration in serum samples. 195

5:4 Effect of supernatants of acid-ethanol precipitated control, NIDDM & IDDM sera on basal rat adipocyte lipogenesis. 206

5:5 Effect of supernatants of acid-ethanol precipitated control, NIDDM & IDDM sera on insulin (10mU.l⁻¹)-stimulated rat adipocyte lipogenesis. 208

5:6 Insulin content of acid-ethanol supernatant extracts prepared from control subjects and diabetic (NIDD & IDD) patients before and after gel filtration on PD10 columns. 211

5:7 Effect of supernatants of acid-ethanol precipitated control, NIDDM and IDDM sera on basal [³⁵S]-sulphate uptake in porcine cartilage. 219

6:1 Hypothetical model of insulin receptor activation and second messenger system in rat adipocytes. 247
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>A23187</td>
<td>calcium ionophore</td>
</tr>
<tr>
<td>Ca</td>
<td>calcium</td>
</tr>
<tr>
<td>[Ca]i</td>
<td>intracellular calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CMZ</td>
<td>calmidazolium</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine-5’-triphosphate</td>
</tr>
<tr>
<td>H7</td>
<td>1-(5-isoquinolinylsulfonyl)-2-methyl piperizine</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>immunoglobulin A</td>
</tr>
<tr>
<td>IDD</td>
<td>insulin dependent diabetic</td>
</tr>
<tr>
<td>IDDM</td>
<td>insulin dependent diabetes mellitus</td>
</tr>
<tr>
<td>IGF I</td>
<td>insulin-like growth factor I</td>
</tr>
<tr>
<td>IGF II</td>
<td>insulin-like growth factor II</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo-dalton</td>
</tr>
<tr>
<td>KRB</td>
<td>Krebs Ringer bicarbonate buffer</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>LHRH</td>
<td>luteinizing hormone releasing hormone</td>
</tr>
<tr>
<td>MSA</td>
<td>multiplication stimulatory activity</td>
</tr>
<tr>
<td>NEFA</td>
<td>non-esterified fatty acid</td>
</tr>
</tbody>
</table>
NADPH  reduced nicotinamide adenine dinucleotide phosphate
NIDD  non-insulin dependent diabetic
NIDDM  non-insulin dependent diabetes mellitus
NSILA-a  non-suppressible insulin like activity - a
NSILA-p  non-suppressible insulin like activity - p
NSILA-s  non-suppressible insulin like activity - s
PMA  4β-phorbol 12β-myristate, 13α-acetate
PdBu  4β-phorbol 12, 13-dibutyrate
PKC  protein kinase C
PB  polymyxin B
RNA  ribonucleic acid
SE  standard error
W-7  N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide
W-13  N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide
CHAPTER 1

INTRODUCTION
HISTORICAL BACKGROUND AND RATIONALE

Insulin, a polypeptide hormone produced by the \( \beta \) cells of the islets of Langerhans, located in the pancreas, plays a crucial role in the regulation of intermediary metabolism of carbohydrates, fats and proteins. An absolute or relative lack of insulin leads to several metabolic abnormalities: 

a) an inability to transport and metabolize glucose and to suppress hepatic glucose production: this results in hyperglycemia,  
b) lipolysis of stored triglyceride from adipose tissue with the release of glycerol and fatty acids into the blood,  
c) increased hepatic synthesis of ketones from fatty acids which leads to ketosis and accumulation of \( H^+ \) ions,  
d) increased hepatic triglyceride synthesis which causes hypertriglyceridemia;  
e) an increase in gluconeogenesis which results in an enhanced conversion of aminoacids into glucose which is metabolised as an energy source; this leads to a loss of protein stores especially in the muscle and subsequently to weight loss. An absolute or relative lack of insulin results in changes described above and would cause diabetes.

Historically, the cardinal role of the pancreas in the pathogenesis of diabetes was first elucidated by von Mering & Minkowski (1890) showed that totally pancreatectomised dogs developed hyperglycaemia and glycosuria. Coma and death followed when the animals became ketotic. That insulin was the component of the pancreas which determined these devastating changes was first demonstrated in 1921 by Banting & Best, who extracted insulin from the pancreas and injected it into a pancreatectomized dog. This treatment induced a fall in blood glucose concentrations in the dog and relieved its diabetic symptoms. This
finding was quickly followed by clinical confirmation in man when a further purified extract of pancreas containing insulin was given to a young diabetic patient and then to a diabetic nurse with dramatically beneficial clinical results (Banting & Best, 1922).

INSULIN RESISTANCE

Following the institution of insulin therapy in diabetics it became apparent that in the diabetic population there were marked variations in response to insulin. Ketotic diabetics were sensitive to insulin whereas non-ketotic diabetics were relatively insensitive to the hormone. The evidence suggesting that resistance to the action of insulin also plays a causal role in the pathogenesis of diabetes in man has been derived from two different approaches. The first was a line of investigation pioneered by Himsworth, who in 1936, suggested that patients with diabetes could be differentiated into insulin sensitive and insulin insensitive types. This he determined by administering glucose orally followed by injection of insulin and subsequent measurement of blood glucose concentrations. He found that ketotic patients blood glucose levels declines sharply following insulin administration whereas in non-ketotic patients glucose remained elevated. In 1949, Himsworth published extensive data in support of the notion that insulin insensitivity, not deficiency, was present in many diabetics. Himsworth concluded that diabetics were divisible into two distinct groups according to their sensitivity to--insulin, and noted that intermediate degrees of sensitivity were rare. Intermediate degrees of insulin insensitivity were found to occur in the apparently healthy normal subjects, especially in the older age-groups.
It appeared from Himsworth's work that insensitivity to insulin must be of a certain magnitude for diabetes to develop. Since this initial concept this line of investigation has since been pursued by several other groups and considerable data indicating a diminished response to exogenous insulin of non-ketotic diabetics has been established (Bearn et al., 1951; Heller et al., 1958; Kalant et al., 1963; Zierler et al., 1963; Stocks & Martin, 1969; Alford et al., 1971; Ginsberg et al., 1974 & 1975.) In a key study Ginsberg et al. (1975) used a continuous intravenous infusion of glucose, insulin, adrenaline and propranolol to directly assess insulin resistance in maturity onset diabetes. During infusion endogenous insulin secretion was inhibited and steady state plasma glucose and insulin levels were achieved after 90 minutes. Plasma glucose concentration observed during the steady state period thus reflected sensitivity to exogenously infused insulin. Furthermore, it was demonstrated by Ginsburg et al. (1975) that not only non-ketotic diabetes was associated with insulin resistance but that diabetes due to chronic pancreatitis or other causes of insulinopenia was not associated with such resistance.

The second approach to the establishment of insulin resistance as a contributory factor in the pathogenesis of non-ketotic diabetes was based on the measurement of insulin and insulin like activity in plasma of diabetic patients. Bornstein & Trewhella (1950) measured insulin-like activity in undiluted plasma using a bioassay based on the uptake of glucose by rat diaphragm and the oxidation of labelled glucose to $[^{14}C]CO_2$ by rat adipose tissue. Using these bioassays they found that some patients with diabetes had normal levels of insulin-like activity. Bornstein & Lawrence (1951), Wallance-Owen et al. (1955) and Steinke et
al. (1961) also reported that not all patients with diabetes were insulin deficient. However, as experience with these biological methods increased and the limitation of these methods became more obvious, the situation became less clear. Thus there was no consensus regarding plasma levels of insulin-like activity in the various forms of human diabetes (Randle 1957). This uncertainty ended when Berson & Yalow (1961) used specific radioimmunoassay techniques to compare plasma immunoreactive insulin levels in normal subjects and non-ketotic diabetics. They found that insulin levels were significantly greater in patients with maturity onset diabetes, than those in normal subjects. They concluded that "the tissues of the subject with maturity onset diabetes do not respond to his insulin as well as the tissues of the non-diabetic subject respond to his insulin". Thus, patients with non-ketotic diabetes have been shown to be insensitive to the biological action of insulin; these patients have also been shown to have circulating levels of plasma immunoreactive insulin which are at least as high as those seen in normal subjects.

Reaven et al. (1976) demonstrated that the response of insulin to an oral glucose load is increased and that the glucose lowering effect of exogenous insulin is diminished in older patients with borderline glucose intolerance (the glucose tolerance test). Thus, insulin resistance may account for the abnormal glucose tolerance in these patients. In contrast, Reaven et al. (1976) considered relative insulin deficiency to be the primary lesion in non-ketotic patients with fasting hyperglycaemia; according to them insulin resistance could have developed in these patients as a secondary phenomenon. Thus, two
distinct pathological processes, insulin resistance and insulin deficiency may occur concurrently in non-ketotic diabetes mellitus.

Olefsky et al. (1985) observed that in subjects with impaired glucose tolerance and mild insulin resistance, the defect in insulin action is due to decreased numbers of cellular receptors for insulin; this leads to decreased insulin sensitivity. In patients with significant fasting hyperglycemia and severe insulin resistance, decreased insulin receptors and a post-receptor defect in insulin action co-exist, but the post-receptor defect is the predominant abnormality. They suggested that as insulin resistance increases, the post-receptor defect becomes more prominent. Impaired glucose uptake and subsequent increased hepatic glucose production in non insulin dependent diabetics are major contributing factors to fasting hyperglycaemia. The suggestion in some work that the reduction in β cell function is the primary lesion in non-insulin dependent diabetes mellitus (NIDDM) (Weir 1982), and that resistance to insulin stimulated glucose disposal evolves as a consequence of the insulin deficiency was rejected by Reaven et al. (1986). Rizza et al. (1985) also emphasised the complexity of the interactions between hyperinsulinaemia, insulin receptor binding and the resultant biological response to insulin. It had been proposed that hyperinsulinaemia may cause or exacerbate insulin resistance. By measuring glucose utilization, glucose production, overall glucose metabolism at submaximally and maximally effective plasma insulin concentrations and monocyte and adipocyte insulin binding studies, Rizza et al. (1985) found that hyperinsulinemia produced by infusion of insulin can cause insulin resistance in man and that this decrease in insulin action probably occurs at the post-receptor site. This implies
that hyperinsulinaemia, whether due to increased secretion or exogenous insulin administration (Type I diabetes), may also impair insulin action and may thus contribute to the pathogenesis of insulin resistance in a variety of disease states.

The precise mechanisms underlying the pathogenesis of insulin resistance are not well understood. However, several such mechanisms have been suggested: cellular defects including receptor and post-receptor abnormalities; circulating insulin inhibitory factors in insulin resistant diabetes and in diabetics with ketoacidosis; insulin antagonists associated with plasma albumin and those originating from the pituitary gland.

CELLULAR OR POST-RECEPTOR DEFECTS IN INSULIN RESISTANCE

Further confirmation of insulin resistance as a major factor in the pathogenesis of NIDDM has been obtained from studies on the evolution of diabetes among Pima Indians. This is an extensively studied model of insulin resistance as non-insulin dependent diabetes is extremely common in Pima Indians. Studies by Nagulesparan et al. (1980) on the evolution of diabetes among Pima Indians have shown marked insulin resistance in vivo. These studies show that hyperinsulinaemia is associated with impaired glucose tolerance in this community. The development of diabetes is associated with a fall in insulin concentrations (Nagulesparan et al. 1980). A progression of this insulin resistance results in diabetes. Extensive studies to demonstrate the existence of a cellular defect in terms of insulin action have been in vain. Howard et al. (1981) investigated fibroblasts from Pima Indians with NIDDM and without diabetes in terms of the following: hexose uptake, glycogen synthase activity and total cellular incorporation of glucose. Their
data did not demonstrate any difference in these indices when cells from non-diabetics and diabetics were compared. Kolterman et al. (1981) have also demonstrated a post-receptor defect in NIDDM patients in vivo. There is no convincing evidence for the presence of cellular defects of insulin action in studies conducted ex vivo in NIDDM patients. The absence of evidence for a definite intrinsic defect in insulin action at the cellular level in vitro (Howard et al., 1981; Berhanu et al., 1982), and the presence of a post receptor defect in insulin action in vivo raise the possibility of a role for a humoral inhibitor in the pathogenesis of diabetes.

Although the human fibroblast possesses a specific insulin receptor (Rechler et al., 1976; Mott et al., 1979) and several indices of carbohydrate metabolism respond to physiological levels of insulin (Fujimoto et al., 1974; Howard et al., 1979; Germinario et al., 1979; Hidaka et al., 1980; Podskalny & Kahn, 1980) it is yet to be determined if the mechanisms underlying the action of insulin in fibroblasts are similar to those in the major tissues involved in glucose homeostasis, i.e. the liver, the skeletal muscle and adipose tissue. If, indeed, the fibroblast is representative of other cells in terms of the mechanism of action of insulin, the results of this study indicate that the cells of patients with NIDDM have no intrinsic defect in terms of insulin sensitivity. It should be mentioned that the fibroblast has proved to be a useful model for eliciting insulin resistance in certain states like leprochaunism.

Apart from the insulin resistance due to receptor and post receptor abnormalities in NIDDM mentioned above, there are other causes of insulin resistance:
i) abnormalities of insulin molecule such that the intrinsic biological activity of the molecule is diminished while the molecule binds to the insulin receptor. This produces a state of insulin resistance. Hyperproinsulinaemia is one example of such a defect. In this condition the protease leading to the cleavage of proinsulin into insulin is missing and hyperinsulinaemia results (Tager et al., 1979).

ii) circulating autoantibodies to the insulin receptor which prevent the binding of insulin to its receptor. This results in marked insulin resistance and hyperinsulinaemia (Kahn & Rosenthal., 1979).

iii) hormonal antagonists such as cortisol, growth hormone, glucagon and catecholamines. An excess of these hormones is capable of producing states of insulin resistance, e.g. Cushings syndrome due to an excess of cortisol (Harrison & Flier., 1980).

**INSULIN ANTAGONISTS IN PLASMA AND SERUM**

The first indication that circulating factors play a role in the aetiology of diabetes was provided by Lowell (1944). He demonstrated that the convulsion inducing effect of insulin in mice was consistently reduced by a concurrent injection of serum from diabetic patients with insulin resistance. The interpretation of these results was difficult because of the variations in the response of the animals to insulin and the non-specific effects of the injection of serum.

Marsh & Haugaard (1952) used isolated rat hemidiaphragms to measure the synthesis of glycogen from glucose. This insulin bioassay system demonstrated that serum from normal and diabetic (NIDDM & IDDM) subjects contained significant insulin inhibitory activity. These authors defined insulin resistance as a dose requirement by the patient of greater than 300 to 600 units of insulin per day. This inhibition of insulin-
stimulated glycogen synthesis was dose dependent and was consistent
with a "non-competitive" effect on insulin action. The inhibitory
activity of sera from diabetic patients was also found to be greater in
diabetic patients than that in normal subjects. They concluded that
insulin resistance in patients with NIDDM was due to inhibitory factors
in the serum.

Some years later Vallance-Owen & Hurlock (1954) described methods
for insulin bioassay based on glucose uptake by the isolated rat
diaphragm. In this assay system plasma from normal subjects did not
inhibit insulin stimulated glucose uptake while plasma from untreated or
uncontrolled insulin-requiring diabetic patients inhibited insulin
stimulated glucose uptake. In 1956(a) Field & Stetten proposed that an
inhibitor was present in the sera of diabetics with ketoacidosis. Sera
from such patients inhibited insulin stimulated glycogen accumulation by
rat hemidiaphragms, a bioassay system similar to that used by Marsh &
Haugaard (1952). The inhibitory activity was stable, not attributable to
the concentration of proteins or to counter regulatory hormones like
ACTH and was present in the α-globulin fraction. Inhibitory activity did
not correlate with blood glucose but appeared to be an indicator of the
amount of insulin required to overcome "insulin resistance" during
ketoacidosis. It disappeared from the plasma after recovery of the
patient and it may have been acting at a "post-binding" site since the
binding of [131I]-insulin to tissues and the degradation of insulin were
both unaltered in the presence of this inhibitor (Field & Stetten
1956b). However, no further work has been done to fully characterise
this inhibitor.
Subsequent studies by Vallance-Owen & Lukens (1957) demonstrated that the presence of insulin antagonism in the plasma of pancreatectomised cats depended on the presence of both the pituitary gland and the adrenal cortex. The antagonistic activity was thus thought to relate, possibly indirectly, to the pituitary gland. In support of this concept Vallance-Owen et al. (1958b) detected no antagonism in serum albumin from hypophysectomised subjects.

Vallance-Owen et al. (1958a) suggested that the putative circulating inhibitor of insulin action resided in the albumin fraction of plasma. These conclusions were based on experiments with trichloracetic acid and ethanol precipitated plasma samples which were dialysed and freeze dried. These partly purified extracts from diabetic sera were found to inhibit glucose uptake in the rat hemi-diaphragm bioassay whereas preparations from control subjects were far less active. They subsequently named this antagonist synalbumin.

In order to clarify whether albumin itself or an associated substance was exerting the effects outlined above, Vallance-Owen et al. (1958b) eluted albumin prepared from plasma of insulin-requiring diabetics and normal subjects on a cellulose column and showed that it was not inhibitory in the rat hemi-diaphragm bioassay. These findings suggested that the anti-insulin activity of the albumin fraction from normal and diabetic subjects was not due to the albumin itself but to a substance associated with it. This antagonistic activity appeared to be present in large amounts in albumin from diabetic patients but not that obtained from normal subjects. These workers concluded that it was unlikely that the ability of albumin, from diabetic patients, to counteract the action of insulin on the rat diaphragm was due to a non-
specific action on the metabolism of the tissue because: (i) albumin at physiological concentrations was able to inhibit the glucose uptake of the diaphragm to levels comparable to those achieved by the tissue in the absence of added insulin, (ii) the addition of large concentrations of insulin partially counteracted the antagonism of the albumin.

Vallance-Owen & Lilley (1961) subsequently demonstrated that synalbumin antagonist activity was virtually absent in plasma samples obtained from hypophysectomised patients. They suggested that synalbumin antagonist production may be dependent upon the pituitary gland. In 1961 work by Vallance-Owen & Lilley demonstrated that this insulin antagonist was inactivated by heat and further observations suggested that the antagonist was of low molecular weight and was not likely to be a free lipid, a fatty acid or a steroid type compound. However, Vallance-Owens' work on the synalbumin antagonist was not confirmed by the studies of Lowy et al. (1961). While Lowy et al. found that synalbumin antagonist activity was demonstrable in the rat hemi-diaphragm preparation, it was not observed in the rat epidydimal fat pad bioassay of insulin. They also demonstrated that this insulin antagonistic activity was not dependent upon the pituitary gland. It is important to note that the method of preparation of albumin in Vallance-Owens work and that by Lowy et al. (1961) was the same (Debro et al., 1957).

The plasma antagonism changed during oral and intravenous glucose administration (Jervel & Vallance-Owen, 1967). These workers extracted albumin from plasma taken during oral glucose tolerance tests and examined the effect of this albumin on for insulin antagonism in the rat diaphragm in vitro. They observed a fall in insulin antagonism at 30 and/or 60 minutes. Intravenous glucose administration induced a fall in
antagonism after 5-15 minutes. The extracts had no effect on the glucose uptake of the rat diaphragm in the absence of insulin.

Although synalbumin antagonized the action of insulin on skeletal muscle, it did not inhibit the action of insulin on fat tissue (Alp & Recant, 1965; Lowy et al., 1961; Alp et al., 1966). The work of Alp & Recant (1965) and Alp et al. (1966) has not only shown unopposed insulin action in the presence of albumin but has also shown an insulin synergizing effect. Using the rat hemidiaphragm preparation (measuring glycogen synthesis from glucose) and an epidydimal fat preparation (glucose uptake and \( ^{14}C \) incorporation into lipid), Cameron et al. (1966) found that most TCA-ethanol extracts from the plasma of newly diagnosed diabetics demonstrated considerable insulin-like activity but showed no evidence of significant insulin antagonism. These findings were difficult to reconcile with those of Vallance-Owen and his colleagues (1958) whose extraction method had been followed by Cameron et al. (1966). Cameron et al. concluded that as synalbumin antagonism had not been demonstrated in whole plasma or serum, it arose during the extraction process. Keen (1963) suggested that the synalbumin antagonism was the result of an altered insulin molecule formed under conditions of the extraction process. If this was the case, a direct relationship between the amount of antagonist and the amount of insulin in the parent plasma might exist. This was indeed found to be so. Furthermore, the greatest amount of synalbumin antagonist was found in extracts of plasma of diabetics, pregnant women (Alp & Recant, 1965) in patients with myocardial infarction (Vallance-Owen & Ashton, 1963). An increased amount of circulating insulin has been described (Peters & Hales, 1965; Spellacy et al., 1965) in all these situations.
Following the work involving the synalbumin inhibitor there was a considerable time before Dean et al. (1984) re-examined the insulin inhibitory effects of plasma and plasma extracts from patients with NIDDM. The inhibitory effect of plasma and plasma extracts was assayed by assessing their effect on insulin stimulated lipogenesis by rat adipocytes. Dean et al. (1984) prepared their extracts by acid-ethanol extraction of heparinised plasma and by subsequently freeze-drying the supernatant. Plasma or extracts of plasma reconstituted in saline were incubated for 2 hours at 37°C with rat adipocytes in the presence of 3-[^3H]-glucose. The conversion of the radiolabelled glucose to the[^3H]-lipid was measured as the radioactivity extracted into a toluene-based scintillant. They observed that plasma and extracts from plasma of patients with NIDDM inhibited insulin action at a post receptor site. These authors ruled out inhibitory activity due to the presence of insulin antibodies, insulin receptor antibodies, other inhibitor of insulin binding or anti-insulin factors. They also demonstrated an absence of a correlation between the inhibitory activity and plasma glucose, insulin, C-peptide or HbA1c. Inhibitory activity was found to be heat stable, resistant to proteases and their data indicated an inhibitor of a molecular weight of less than 1000 daltons. More recently, Dean et al. (1987) have characterized this inhibitor further and have reported that it has a molecular weight of 300-400 daltons. Dean et al. (1984) also observed an inhibitor of insulin in plasma of patients with NIDDM, they also found a much less potent inhibitory activity in plasma of normal subjects, confirming the original observation of Marsh & Haugaard (1952).
Furthermore, in recent studies on the insulin-like stimulatory effect of human immunoglobulins on adipocyte lipogenesis, Khokher et al. (1981) demonstrated that human IgG and IgM stimulate adipocyte lipogenesis at sub-physiological concentrations whilst whole serum was either mildly stimulatory or neutral. They predicted the presence of potent inhibitor(s) of insulin action in human serum/plasma. The author of this thesis therefore investigated the presence and potency of this inhibitory activity in supernatants obtained from ammonium sulphate (2.5M) precipitation of globulins; such precipitation removes IgG and IgM totally. The author also investigated further the role of inhibitors described by Dean et al. (1984) and Khokher et al. (1981) in terms of their molecular size, their chemical characteristics, their inhibitory potency and their presence in patients with diabetes mellitus (type I and type II) and obesity.

AN INSULIN ANTAGONIST OF PITUITARY ORIGIN

Zimmet et al. (1971) reported the isolation of an "insulin antagonist" of pituitary origin in plasma of normal and diabetic subjects with both NIDDM & IDDM. This inhibitor was a polypeptide derived from and similar to growth hormone. It inhibited the enzymes glyceraldehyde-3-phosphate dehydrogenase and α-glycerophosphate dehydrogenase (Figure 1:2) and acetyl CoA-carboxylase (Figure 1:3). This antagonist was found to be virtually absent in subjects after hypophysectomy and in highest amount in diabetics on insulin therapy. For this work glyceraldehyde-P-dehydrogenase was prepared from rabbit muscle and the enzymatic activity assayed spectrophotometrically. Subsequent to these studies however, little work has been done on inhibitors of pituitary origin.
Glycerol phosphate shuttle for the transport of reducing equivalents from cytosol into the mitochondrion. It is likely that the FAD and cyt c are components of the inner mitochondrial membrane.

**Figure 1:3**

*Acetyl-CoA carboxylase reaction*

\[
\begin{align*}
\text{HCO}_3^- & \quad \text{biotin} & \quad \text{malonyl-CoA} \\
\text{ATP}^4^- & \quad \text{e-carboxybiotin} & \quad \text{CH}_3\text{CO.SCoA} \\
\text{ADP}^3^- + \text{P}_i^2^- + \text{H}^+ & \quad \text{Malonyl-CoA} & \quad \text{CH}_3\text{CO.SCoA}
\end{align*}
\]

Figures 1:2 & 1:3 are reproduced from Biochemistry for the Medical Sciences by E. A. Newsholme & A.R. Leech (copyright 1983) by permission of John Wiley & Sons Ltd.
More recently, interest has focussed on somatomedins. It is well established that insulin as well as growth hormone are involved in skeletal growth promoting effects. The growth promoting effects of the growth hormone appear to be mediated by a group of circulating peptides, the somatomedins, which are similar to insulin. Phillips & Scholz (1982) suggested that poor growth in uncontrolled experimental diabetes could be due at least in part to an increase in a circulating factor(s) which may inhibit the action of somatomedin(s). These workers studied the effect of diabetic rat serum and an inhibitory fraction prepared by gel filtration from diabetic rat serum, on the basal and insulin/somatomedin stimulated oxidation of glucose to carbon dioxide by rat epidydimal fat pads. Both diabetic rat serum and its inhibitory fraction inhibited the oxidation of glucose to carbon dioxide in a non-competitive fashion. Experiments with rat diaphragm also showed that diabetic sera and inhibitory fractions inhibited the incorporation of glucose into glycogen in muscle in basal and insulin stimulated states. Furthermore, rats given insulin with serum from diabetic rats exhibited decreased stimulation of glucose incorporation into muscle glycogen and into adipose tissue lipids when compared with animals given insulin alone. Thus, these workers suggested that the serum of diabetic rats contains an inhibitor of approximate molecular weight 30,000-50,000 daltons which can block anabolic processes in muscle (glycogen synthesis) and adipose tissue (lipid formation).

Phillips et al. (1983) have also studied somatomedin inhibitors in rats with streptozocin-induced diabetes but they have not published further work on the inhibitors of insulin action. These workers detected
no somatomedin inhibitors in sera of normal rats whilst in the sera of diabetic rats somatomedin inhibitors of molecular weight 8kDa - 27kDa were detected. This inhibitor appears to be similar to that reported by Salmon et al. (1983) of a molecular weight of approximately 30kDa in sera of starved rats. The authors concluded that these inhibitors of insulin/somatomedin may contribute to poor growth in diabetes by (a) inhibiting the anabolic effect of insulin and (b) inhibiting the growth promoting effect of somatomedins in cartilage.

Phillips et al. (1985) have recently extended their work to children with diabetes in whom, poor metabolic control is associated with diminished growth (Pond, 1970; Birkbeck, 1972; Williams & Savage, 1979). Such growth impairment occurs despite the presence of normal or elevated levels of growth hormone (Hansen & Johansen 1970). Circulating somatomedin activity as measured in bioassays is low while that measured by radioimmunoassays is normal. This suggests the presence of somatomedin inhibitors in plasma.

In the light of these findings work in this thesis also includes collaborative experimentation undertaken to determine whether inhibitors investigated in this work were inhibitory to the biological action of somatomedin.

MODE OF ACTION OF INSULIN

The first step in insulin action is the binding to specific receptors in the plasma membrane of the cell. This receptor is present on virtually all mammalian tissues, although the density varies from as few as 40 receptors on a circulating erythrocyte to more than 200,000
receptors on an adipocyte or hepatocyte. Although insulin is best known for its promotion of glucose metabolism and amino acid transport, insulin can also activate or inactivate cytoplasmic and membrane enzymes (Czech 1981), alter the rate of synthesis of protein and DNA and influence the processes of cell growth and differentiation. These multiple effects vary widely with respect to dose response and time course. Some effects, such as stimulation of glucose transport occur within seconds at very low insulin concentrations (10^-11M) whilst actions on DNA require much higher insulin concentrations (10^-7M). Several hypotheses exist on the mechanism of insulin action at the post receptor level:

a) There is evidence that an unidentified polypeptide of a low molecular weight is released from the cell membrane after binding of insulin to its receptor. The release mechanism can be blocked by trypsin inhibitors. It is possible that the released polypeptide functions as a second messenger (Lamer et al., 1979; Jarret & Seals, 1979; Seals & Czech, 1980).

b) The β subunit of the insulin receptor has been shown to be a tyrosine protein kinase (Shia & Pilch, 1983; Zick et al., 1983) which becomes phosphorylated upon binding of insulin to the receptor and is thereby activated. After internalisation of the insulin receptor, this kinase might bring about specific intracellular protein phosphorylation reactions that might account for some of the metabolic responses elicited by insulin.

c) A third mechanism of insulin action relates to the observation that insulin inhibits a calcium ATPase in cell membranes (Pershadsingh & McDonald, 1979; Schoenle & Froesch, 1981), possibly resulting in an
increase of cytosolic calcium which might function as a second messenger. Insulin stimulation of glucose transport through the cell membrane and the activation of calcium sensitive enzymes might ensue.

d) A fourth possible mechanism may be through insulin induced translocation of glucose carriers from the Golgi apparatus to the cell membrane 'recruitment hypothesis' which may mediate glucose transport into adipocytes (Suzuki & Kono, 1980; Cushman & Wardzala, 1980; Karnieli et al., 1981). The translocation might be accomplished by a calcium-mediated activation of the microtubular-microfilamentous system.

PROTEIN KINASE C AND CALCIUM AS MEDIATORS OF CELLULAR RESPONSE TO INSULIN

Much interest and investigation recently has been directed towards protein kinase C and calcium as mediators of cellular responses. To elucidate further the site and the mechanism of action of the serum inhibitor(s), the role of protein kinase C and calcium in mediating the action of insulin was examined in this thesis. Receptors for hormones, neurotransmitters and growth factors use a variety of mechanisms to transmit their message across the plasma membrane. Recent work has focused on the role of phosphoinositide turnover in the transduction of extracellular signals (Nishizuka, 1984a; Berridge & Irvine, 1984). This turnover of inositol phospholipids is associated with an increase in intracellular calcium ion which appears to mediate many of the subsequent physiological responses

PROTEIN KINASE C

When the enzyme protein kinase C was first discovered in 1977, as a proteolytically activated protein kinase in many tissues, it appeared to
have no obvious role in signal transduction (Inoue et al., 1977). Later, Takai et al. (1979a) showed it to be a Ca-activated, phospholipid-dependent enzyme, and it was firmly linked to signal transduction by the demonstration that diacylglycerol, one of the earliest products of signal-induced inositol phospholipid breakdown, greatly increased the affinity of protein kinase C for Ca thereby activating the enzyme. (Takai, 1979b; Kishimoto et al., 1980; Nishizuka, 1980; Nishizuka, 1983). Diacylglycerol is normally almost absent from membranes but is transiently produced from inositol phospholipids following receptor activation (Berridge, 1984). The active diacylglycerols appear to contain at least one unsaturated fatty acid irrespective of the chain length of the fatty acyl moiety. Triacylglycerol, monoglycerol and free fatty acids are thought to be totally inactive in the systems investigated.

The receptor for a peptide hormone or neurotransmitter serves firstly to recognize and bind its ligand with high affinity and specificity and secondly to initiate the intracellular response(s) to the ligand by generating a transmembrane signal. The biochemical effects of insulin may be rapid or slow: some may be observed at intervals of seconds (e.g. change in ion flux) while some are observed hours later (e.g. stimulation of protein, lipid, DNA and RNA synthesis) after insulin binds to its receptor (Kahn, 1985; Granner et al., 1986). The latter examples of effects probably mediate its growth promoting action.

Adipose tissue responds to insulin with a short term activation of phospholipase C which is responsible for the hydrolysis of phosphatidylinositol-4,5-bisphosphate (Koepfer-Hobelsberger & Wieland, 1984a). This observation accounts for the well known insulin-like
stimulatory effect of the enzyme when it is exogenously added to fat cells (Rosenthal, 1971; Honeyman et al., 1983). Furthermore there is stimulation by insulin of de novo synthesis of phosphotidylinositol and phosphotidylinositol 4,5-bisphosphate in isolated fat cells (Pennington & Martin, 1985). It has also been reported that inositol-trisphosphate acts as a physiological mediator of insulin action in the activation of the mitochondrial pyruvate dehydrogenase complex (Koepfer-Hobelsberger & Vieland, 1984b). Evidence that the insulin receptor-associated kinase acted as a phosphotidylinositol kinase was provided by Machicao & Vieland in (1984).

On the other hand, some studies showed that 4β-phorbol 12β-myristate,13α-acetate (PMA), a potent tumour promoting phorbol ester obtained from seeds of Croton tiglium L., Euphorbiaceae Const., substitutes for diacylglycerol to directly activate protein kinase C (Castagna et al., 1982). PMA also stimulated the phosphorylation of receptors for insulin and somatomedin C in IM-9 cells, a human B-lymphocyte line (Jacobs et al., 1983) and altered the phosphorylation of the insulin receptor in cultured hepatoma cells (Takayama 1984). Skogland et al (1985) demonstrated PMA and insulin induced translocation of protein kinase C activity from the soluble to the particulate fraction of isolated adipocytes. Furthermore, PMA was shown to increase basal lipogenesis while it antagonised submaximal stimulation by insulin of adipocyte lipogenesis. Van der Werve et al. (1985) suggested that protein kinase C might be implicated both in insulin action and modulation of the hormone bioactivity. Cherqui et al. (1986) presented the most comprehensive study to date to gain further insight into a possible role of protein kinase C in the action of insulin on glucose
and lipid metabolism in fat cells. Their experiments involved testing the insulin-like properties of PMA on (a) glucose transport, lipogenesis, and [3H]acetate incorporation into 3H-labelled lipids; (b) the effect of mellitin, a protein kinase C inhibitor on PMA and insulin induced stimulation of glucose metabolism and (c) the dependence of the above processes on calcium influx and energy expenditure. Their results showed that PMA had insulin-like effects on glucose metabolism in fat cells without any effect on lipid synthesis from acetate. They indicate that the biological effects of PMA and insulin may be mediated through a series of common steps implicating calcium- and energy-dependent processes. They concluded that the activation of protein kinase C was central to the issue of insulin stimulated lipogenesis in the adipocyte. The experiments of Cherqui et al. (1986) also implicated calcium as an intracellular messenger in the adipocyte. The intracellular calcium receptor protein is calmodulin, the most ubiquitous of the calcium binding protein as it is present in invertebrates, vertebrates and even in plants (Cheung, 1980; Manalan, 1984; Means, 1982; Rasmussen, 1984). Graves et al. (1986) observed that phosphorylation of calmodulin occurred in the presence of insulin and was both calcium and insulin concentration dependent. This supported the hypothesis that calcium and calmodulin participate in the molecular mechanisms coupling the binding of insulin to its receptor to changes in cellular metabolism which follow binding. The intracellular second messenger system involved in insulin action appears to involve protein kinase C and calcium but it is not fully established in terms of the sequence of action. We therefore conducted a series of experiments i) to build a framework of observations which allow us to hypothesize a sequence of events
following the binding of insulin to its receptor and ii) to use this framework to elucidate the site of action of the inhibitors investigated.

We have used several substances for this investigation, those that block voltage dependent calcium channels (verapamil), those that initiate calcium influx through the creation of artificial calcium channels, the ionophore A23187; inhibitors of the protein kinase C system such as polymyxin B and 1-(5-isoquinolinylsulfonyl)-2-methylpiperizine (H7); and stimulators of this system, the active phorbol esters, such as PMA; and inhibitors of the calmodulin pathway, trifluoperazine and calmidazolium. Sodium fluoride, a known G protein activator was also employed to investigate the possible roles for G proteins in rat adipocyte lipogenesis. It has been proposed that guanosine-5'-triphosphate (GTP)-activated proteins (G proteins) may play a role in linking receptors to Ca\textsuperscript{2+} mobilization (Goodhart et al., 1982; Gomperts, 1983.). Two recent studies have shown that: (i) G proteins activate phospholipase C (Guillon et al., 1983) and (ii) fluoride is a G protein activator (Bigay et al., 1981).

To investigate the role of protein kinase C in rat adipocyte lipogenesis the active phorbol esters 4\beta-phorbol 12,13-dibutyrate (PdBu) and 4\beta-phorbol 12\beta-myristate, 13\alpha-acetate (PMA) were used. PMA proved to be a more powerful and consistent stimulator than PdBu in this system. These esters activate protein kinase C (Nishizuka, 1984b), stimulate phophorylation of insulin and somatomedin C receptors (Jacobs et al., 1983), cause translocation of PKC activity from the soluble to the particulate fraction of rat adipocytes (Skoglund et al., 1985) and
have been implicated in rat adipocyte lipogenesis by van der Werve et al. (1985) and Cherqui et al. (1986).

Polymyxin B has been widely used in the recent past as an inhibitor of PKC and phorbol ester-induced C-kinase activity (Nel et al., 1985, Wakade et al., 1986). This compound, however, has several other actions. Isoquinolinesulfonamides, on the other hand, and specifically the derivative with the sulfonylpiperizine residue (H7) has a $K_i$ value of 6$\mu$M for H7 for protein kinase C (Hidaka et al., 1984). Kawamoto & Hidaka (1984) also demonstrated that while H7 inhibited PKC mediated phosphorylation in PMA stimulated platelets, this compound did not block the Ca-calmodulin-dependent phosphorylation in Ca ionophore A23187-stimulated cells. Thus, in intact cells, this inhibitor is selective for PKC. Nishikawa et al. (1986) suppressed the proliferation of HL-60 cells (human promyelocytic leukemia cell line) with H7 and also inhibited PMA induced cell differentiation of these cells. H7 has previously not been used to investigate the role of PKC in adipocyte lipogenesis.

CALCIUM

The calcium channel blocker verapamil is a phenylalkylamine, (5-[[N-3,4-dimethoxyphenethyl]methylamino]-2-[[3,4-dimethoxyphenyl]-2-isopropylvaleronitrile hydrochloride). Racemic verapamil has been used as a Ca$^{2+}$ channel blocker in several tissues (Miller & Freedman 1984) and specifically in isolated fat cells by Martin et al. (1975). It has also been used to investigate relationships between the exchange of calcium and phosphate to investigate modulation by calcium of the insulin action and of the insulin-like effect of (oxytocin) (Bonne et al., 1977) and to investigate stimulation of glucose transport by calcium (Taylor et al., 1979b).
The calcium ionophore A23187, has been used in many cell systems to permit calcium entry into cells or into membrane preparations. Pershadsingh et al. (1986) employed the ionophore in describing the presence of a Ca-activated K (Rb) channel in the adipocyte plasma membrane, Severson et al. (1976) used the ionophore to study its effect on pyruvate dehydrogenase activity and ATP content of fat cells. Bonne et al. (1977) and Taylor & Halperin (1979) also used A23187 in the investigation of oxytocin on isolated rat adipocytes and the investigation of the stimulation of glucose transport in rat adipocytes by calcium.

CALMODULIN

Shechter (1984) demonstrated that trifluoperazine, a calmodulin inhibitor, inhibited insulin induced stimulation of glucose metabolism in fat cells. Trifluoperazine however inhibits calmodulin at rather high concentrations (>1.0x10^-8M). These concentrations are several orders of magnitude greater than the drugs affinity for dopaminergic or serotinergic receptors (Landry et al., 1980; Van Belle, 1981). The V-compounds such as N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (V-7) and N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide (V-13) have also been used as potent calmodulin inhibitors (Hidaka et al., 1980), in human platelets (Nishikawa et al., 1980), tracheal smooth muscle contraction (Silver & Stull, 1983), and in fat cells (Shechter, 1984) but they are weak inhibitors of several other protein kinases (Tanaka et al., 1982; Schatzman et al., 1983).

R24571, a potent inhibitor of calmodulin, also known as calmidazolium, is a derivative of the antimycotic miconazole and has been shown to be a more specific tool for studying the involvement of
calmodulin in biological processes (Van Belle, 1981). Gietzen et al. (1981) used it to study calmodulin regulated functions of the red blood cell, Sullivan & Cooke (1985) blocked LH and LHRH-agonist-stimulated steroidogenesis in rat Leydig cells with calmidazolium. Carr et al. (1987) recently used calmidazolium to study its role on steroidogenesis by fetal zone cells of the human fetal adrenal gland. These three groups also used trifluoperazine and chlorpromazine another calmodulin inhibitor, in their experiments and concluded that the anti-calmodulin action of R24571 was specific. We therefore used calmidazolium as an inhibitor of calmodulin in basal and insulin stimulated adipocyte lipogenesis.

THE MONOSACCHARIDE TRANSPORT SYSTEM OF THE HUMAN ERYTHROCYTE

To assess the effect of the serum inhibitors on the glucose transporter system, their effect on the monosaccharide transport system of the human erythrocyte was studied in the present thesis. The monosaccharide transport system of the human erythrocyte has been characterized by means of its affinity for cytochalasin B (Zoccoli et al., 1978). Cytochalasin B is a potent, reversible inhibitor of monosaccharide transport (Bloch, 1973; Taverna & Langdon, 1973) and protects the transport system from irreversible inhibition by several protein-modifying reagents (Bloch, 1973). Erythrocyte membranes possess high affinity sites for cytochalasin B and monosaccharides; monosaccharides which are substrates for the transport system inhibit the binding of cytochalasin B to these sites (Taverna & Langdon, 1973; Lin & Spudich, 1974).
The protein involved in glucose transport in the erythrocyte is almost certainly exactly the same as that in the adipocyte. This protein is universal, (other than for the hepatocyte) the same transporter gene being expressed in other tissues. Erythrocytes are also technically the system of choice of inquiry in studies of membranes because of their ready availability in large quantities. They lack organelles and thus have only a single membrane, the plasma membrane. Nearly all the cytoplasmic contents of these cells can be released by osmotic hemolysis to give ghosts, which are nearly pure plasma membranes. In this study alkali stripped membranes [prepared by Dr. S.A. Baldwin, Dept of Biochemistry, Royal Free Hospital School of Medicine] were used. The alkali stripped membranes lose the spectrin and actin cytoskeleton, leaving behind only the cytochalasin B high affinity binding sites. In non-stripped membranes the actin also provides a high affinity binding site for cytochalasin B.
AIMS OF THE STUDY

1. To investigate presence and potency of the insulin inhibitor prepared by ammonium sulphate precipitation of serum from control, diabetic and obese subjects.

2. To study the effect of the above inhibitor on the metabolism of glucose (glucose oxidation, lipogenesis, lipogenic breakdown products) by isolated rat adipocytes.

3. To investigate the second messenger cascade in the isolated adipocyte and to study the effect of this inhibitor on this system.

4. To elucidate the effect of this inhibitor on the glucose transporter mechanism in erythrocyte membranes.

5. To investigate the methodology of preparation of the acid ethanol extracted serum insulin inhibitor.

6. To investigate presence and potency of the insulin inhibitor prepared by acid/ethanol extraction of serum obtained from the control and diabetic population, by the rat lipogenesis bioassay.
TECHNIQUES USED

Although the vital role of adipose tissue as a reservoir of fat which can be mobilized to provide energy has long been recognized, it was assumed until relatively recently that the tissue is, to a large extent, metabolically inert. By 1948 there was available a considerable body of data derived from physiological studies which indicated that adipose tissue is not an inactive storehouse. Wertheimer & Shapiro (1948) stressed this view despite absence of precise information concerning the enzymes and metabolic pathways of this tissue. Several investigators have compared specific aspects of the metabolism of rat white adipose tissue derived from different anatomical sites. In studies of fatty acid synthesis Shapiro & Wertheimer (1948) found equivalent activity in mesentery and in groin fat. Minced preparations of epididymal, perirenal and subcutaneous adipose tissue incorporated roughly equal amounts of glucose carbon into fatty acids (Hausberger & Milstein, 1955). Krahl (1951) and Ball & Cooper (1960) reported glucose uptake and responses to insulin to be quantitatively alike for adipose tissue from different regions. Since then, work done in the field pertains chiefly to the metabolism of the epididymal fat pad which is a particularly convenient source of adipose tissue for study in vitro. It exhibits high rates of glucose lipogenesis in the basal state (Jeanrenaud, 1961) and is sufficiently sensitive and reproducible in its response to insulin to serve as a bioassay for this hormone (Renold et al., 1960; Sheps et al., 1960).
Rodbell (1964) made an important methodological advance by treating adipose tissue with collagenase thus preparing a homogenous suspension of fat cells which maintained the intrinsic metabolic characteristics of this tissue. This was the tissue chosen for the basis of the experimental work in this thesis. This tissue responds to insulin in a dose dependant manner when looking at the metabolism of U-[\(^14\)C]-glucose and the incorporation of label into carbon dioxide and/or triglycerides. Moody et al. (1974) also found 3-[\(^3\)H]-glucose to be incorporated into lipids in a dose dependant manner, thus either label can be used as an index for insulin action although 3-[\(^3\)H]-glucose cannot be used for a glucose oxidation study as the 3-[\(^3\)H]-carbon is not incorporated into carbon dioxide. In our glucose oxidation work the universally labelled \([\(^14\)C]-glucose has been employed, the common premise being that it is the carbon labelled in the glucose-1 position and to a lesser extent the glucose-6 position that is incorporated into labelled carbon dioxide via the pentose cycle (Katz et al., 1966).

The presence and potency of the serum inhibitors of insulin was assessed by their effect on glucose metabolism in rat adipocytes, the binding of cytochalasin B to the glucose transporter in erythrocyte (pH12) membranes and on the \([\(^3\)S]-sulphate uptake by cultured porcine cartilage was also assessed. The latter was kindly carried out at the Institute of Child Health, London by A.M. Taylor. The incorporation of \([\(^3\)S]-sulphate into cartilage cells has been the basis of nearly all the assays for somatomedin since, as sulphation factor, it was discovered. Other indicators of metabolism are also used (the incorporation of \([\(^3\)H]-leucine and \([\(^3\)H]-thymidine into proteins and DNA respectively) but these usually supplement, rather than replace, the \([\(^3\)S]-sulphate
measurements. Many bioassays have been established for the estimation of somatomedin activity, but there are three major widely used bioassay systems: the rat cartilage bioassay (Daughaday et al., 1957), the chick pelvic rudiment bioassay (Hall, 1970) and the porcine costal cartilage bioassay (Van den Brande & Du Caju, 1974). All these methods are time-consuming and are not suitable for use in hospital routine laboratories or for occasional studies. Spencer & Taylor (1978) developed a simplified method for measuring somatomedin activity, based on the porcine costal cartilage bioassay. The method has not suffered any loss of precision or sensitivity (compared with the standard porcine cartilage bioassay) as a result of the modifications. A known standard, equivalent to 1U.ml⁻¹ somatomedin is incorporated in the assays. Inter-assay variation i.e. the value of an unknown sample assayed on a different day and with cartilage from a different animal is ≈ 20%. Intra-assay variation, i.e. that which is intrinsic to a single assay is ≈ 14%. The sensitivity of the assay is 3.125% (plasma diluted in medium). The average concentration of somatomedin in plasma is 200ng.ml⁻¹, thus the minimum sensitivity of the assay is 6.25ng.ml⁻¹.
CHAPTER TWO

MATERIALS AND METHODS
MATERIALS AND METHODS.

2:1 MATERIALS
a Chemicals
b Radiochemicals
c Proteins
d Gels
e Scintillants
f Dialysis tubing
g Kits

2:2 PREPARATIONS OF TEST SOLUTIONS

2:3 BUFFERS
a Krebs Ringer Buffer
b Phosphate Buffer
c Sodium Phosphate Buffer
d Coupling Buffer
e Tris Buffer

2:4 INSTRUMENTS

2:5 METHODS
a Selection of subjects and patients and details of serum samples collected for the preparation of inhibitory fractions by ammonium sulphate precipitation.
b Control and obese subjects: collection of blood samples for the preparation of inhibitory fraction by ammonium sulphate precipitation.

c Selection of subjects and serum samples collected for preparation of inhibitory fraction by acid-ethanol extraction.

d Preparation of inhibitory fractions by ammonium sulphate precipitation of serum.

e Ultrafiltration of inhibitor preparations to obtain fractions of varying molecular weight ranges.

f Trypsinization of inhibitor preparations

  i Tryptic proteolysis.

  ii Soya bean trypsin inhibitor inhibition of trypsin.

  iii Trypsinization by use of immobilized enzyme.

g Heat stability of inhibitory preparations.

h Effect of inhibitory preparations on cytochalasin B binding.

i Preparation of inhibitory fraction by acid-ethanol extraction of serum.

j Preparation of glucose free fractions.

k C18 Sep-pak chromatography.

l Preparation of insulin free fractions.

m Insulin radioimmunoassay.

n Preparation of adipocytes.

o Glucose oxidation by adipocytes.

p Insulin bioassay

q Analysis of lipogenesis breakdown products.
r $[^{35}\text{S}]$-sulphate uptake, a simplified bioassay for somatomedin.

s In vivo rat studies.

t Protein assay.

u Statistics.
2:1  Materials

a)  Chemicals  All analytical grade

**Sigma**

Ammonium sulphate

Sodium chloride

Potassium chloride

Calcium chloride

Sodium hydrogen carbonate

Sodium dihydrogen carbonate

Magnesium sulphate heptahydrate

Potassium dihydrogen phosphate

Sodium merthiolate

Ethylenediaminetetraacetic acid (Anhydrous disodium salt)

Glucose

Calmidazolium (R24571) 1-\(\text{bis}(4\text{-chlorophenyl})\text{-methyl}\)-3-\(\text{2-(2,4-dichlorophenyl)}\)-2-\(\text{2,4 dichlorophenylmethoxy1}\text{-ethyl}\)-1H-imidazolium chloride.

Calcium ionophore (freecid) (A23187)

(±) Verapamil hydrochloride

4ß-phorbol 12-myristate 13-acetate (phorbol monoacetate)

4ß-phorbol 12, 13,-dibutyrate

1-\(\text{isoquinolinylsulphony1})\)-2-methylpiperazine. (H7)

Polymixin B sulphate (8156 USP Units/mg)

N-\(\text{6- aminohexyl})\)- 1-naphthalene sulfonamide . (W5)

N-\(\text{4-aminobutyl})\)- 5-chloro-2-naphthalene-sulfonamide. ( W13)

Trypan blue
British Drug House (Enfield, U.K.)

Sulphuric acid 98.0%
Hydrochloric acid 35.4%
Heptane 99.5%
Propan-1-ol 99.5%
Toluene 99.5%
Triton X-100 (iso-Octylphenoxypolyethoxyethanol) 100%
Acetonitrile

Smith Kline & French

Trifluoperazine

James Burrough plc London, UK

Ethanol: Absolute Alcohol 100  99.86%v/v min

b) Radiochemicals Amersham International Radiochemical center (Amersham, UK)

D-[U-¹⁴C]-glucose
3-[³H]-glucose

New England Nuclear

[³H]- cytochalasin B
c) Proteins

**Armour Pharmaceuticals, Eastbourne, UK**

Bovine serum albumin fraction V.

**Novo Industries, Copenhagen, Denmark.**

Porcine insulin (monocomponent actrapid insulin)

**Sigma Chemical Company Ltd, Dorset, England.**

Immunoglobulin G, Human (Lyophilized; essentially salt-free)

trypsin,

Insoluble trypsin from Bovine Pancreas attached to cross-linked beaded agarose (100Units.ml⁻¹).

soya bean trypsin inhibitor.

Bovine serum albumin (highly purified A7030)

**Millipore Corporation**

Collagenase (clostridiopeptidase A; EC3.4.24.3)

from Clostridium histoyticyum, specific activity 130 units.mg⁻¹

d) **Gels Pharmacia, Uppsala, Sweden**

Sephadex G25 (PD10 columns)

CNBr activated sepharose
e) **Scintillants**

**National Diagnostics**

Exscint

**British Drug House**

PPO (4g.1⁻¹) in toluene/triton(2:1)

Cocktail T

f) **Dialysis tubing**

**Medicell International Ltd.**

Visking size 1-8/32".

g) **Kits**

**Novo Industri Copenhagen Denmark**

Novo diagnostic kit. - Product code 10:

Kit: 20μCi ['²⁵I]-insulin.

   Human insulin standard

   Anti porcine guinea-pig serum M8309.
Preparations of test solutions.

Appropriate dilutions of each of the test substances were prepared in Krebs Ringer Bicarbonate buffer solution on the day of assay.

Calmidazolium: A 10M stock solution in DMSO was prepared and stored at 4°C.

Calcium ionophore: A 10mM stock solution in DMSO was prepared and stored in 5μl aliquots at -20°C.

Phorbol monoacetate: A 10mg.ml⁻¹ (1.62×10⁻²M) stock solution in DMSO was prepared and stored in 10μl aliquots at -20°C.

Polymyxin B sulphate: This was prepared fresh in KRB before assay.

W5 & W13: 500μg.ml⁻¹ (W5: 1.46×10⁻³M, W13: 1.43×10⁻³M) stock solutions in ethanol were prepared and stored at 4°C.

H7: A 25mg.ml⁻¹ (8.58×10⁻²M) stock solution in DMSO was prepared and stored at 4°C.

(±)Verapamil hydrochloride: A 10mg.ml⁻¹ (2.03×10⁻²M) stock solution in 20% ethanol was prepared fresh each time before assay.
Buffers all made up in double distilled water.

a) Krebs Ringer Bicarbonate (KRB)

NaCl 7.000g
KCl 0.359g
KH₂PO₄ 0.164g
MgSO₄ 7H₂O 0.610g
KHCO₃ 2.176g
CaCl₂ 2H₂O 0.0946g
Glucose 0.050g
Bovine serum albumin 5.000g

Made up in 1 litre water, pH adjusted to 7.4 by aerating with 95% O₂, 5% CO₂, final pH adjustments made with 0.5M NaOH.

b) Phosphate Buffer

NaCl 8.500g
NaH₂PO₄ 1.185g
Na₂HPO₄ (anhydrous) 4.605g
Sodium merthiolate 0.240g
EDTA 3.720g
Bovine serum albumin (A7030) 1.000g

Made up in 1 litre water, pH adjusted to 7.4.

Used for diluting tracer and antibodies for the insulin radiocimmunoassay.
c) Na Phosphate Buffer
To 100ml Phosphate buffer (b) add:

\[
\begin{align*}
\text{NaCl} & \quad 0.6 \text{g} \\
\text{BSA(A7030)} & \quad 5.9 \text{g}
\end{align*}
\]

pH adjusted to 7.4.
Used to prepare standards and dilutions of specimens for the insulin radioimmunoassay.


d) Coupling Buffer

\[
\begin{align*}
\text{NaHCO}_3 & \quad 16.0 \text{g} \\
\text{NaCl} & \quad 8.0 \text{g}
\end{align*}
\]

Made up in 1 liter water, pH adjusted to pH 8.0.

e) Tris buffer 50mM pH 6.8
Dissolve 6.057g Tris (MW121.14) in approximately 850ml water and then adjust the pH to a value of 6.8 using 1M HCl. Make up volume to 1 litre with water.
2:4 Instruments.

a) Waterbath - Grant Instruments (Cambridge Ltd) Eng


c) Centrifuges - IEC CENTRA-7R- refrigerated centrifuge, Damon/IEC (UK) Ltd, Unit 7, Dunstable, Beds.

- MSE High Speed 18, Manor Royal, Crawley, Sussex, England.

d) Pipettes - Gilson Anachem, Luton, Beds.

e) Magnetic stirrer - MT3 Amicon Ltd, Upper Mill, Stonehouse, Glos, UK.

f) Stirred ultrafiltration cell Model No.8050 - Amicon Ltd, Upper Mill, Stonehouse, Glos, UK.


h) β scintillation counter Phillips PW4540 - Holland.

i) γ counter Nuclear enterprises 1600 - Sightill, Edinburgh, Scotland.

2:5 Methods.

a) Selection of subjects and patients and details of serum samples collected for the preparation of inhibitory fractions by ammonium sulphate precipitation.

Selection of patients is fraught with limitations because it is very difficult to standardise all factors such as age, weight, HbA1, blood glucose, drug therapy, presence and absence of complications and obesity in any diabetic population of NIDDs or IDDs. It would appear
that such studies would benefit from being conducted in newly diagnosed, untreated diabetics. These diabetics would therefore not have potential interference from drugs such as glibenclamide or injected hormones such as insulin. However, their metabolic profile will vary extensively; for example, in relation to blood glucose concentration, the extent of ketosis and the duration of diabetes preceding sampling. Therefore, the only ideal option remains that patients be standardised in terms of the variables listed in the above.

Blood was obtained in regular clinic sessions. Blood glucose in control subjects and diabetic (NIDD & IDD) patients can differ markedly and in the assay system utilised, which is affected by the concentration of glucose, this was controlled for by removing the glucose during the preparation of the inhibitor fractions. Insulin was similarly absent from these preparations. The absence of fasting blood collections for each investigation in this work may however remain a shortcoming of this part of the study. As for the treatment of patients with sulphonylureas and biguanides, it is worth mentioning that previous work from our laboratory has shown that both of these drugs, at therapeutic concentrations, do not have a significant effect on the adipocyte lipogenesis assay.

From this pilot experiment obesity was identified as a factor which affects the presence of insulin inhibitory activity in diabetic blood samples and therefore further investigations were pursued in these subjects.

12 control subjects were selected (5 females & 7 males). Their median age was 29 years (24-47) and median weight 70kg (55-85),
median glucose $5\text{mmol.l}^{-1}\ (4.5-6.5)$. None of these individuals was obese and none had glycosuria. Obesity was defined as a body weight 20% above Ideal Body Weight.

13 NIDDM patients were randomly selected. This group consisted of 6 females and 7 males with median age 60 years (43-80), median weight 72kg (40-96), median HbA1 12% (8.2-15) and median glucose at time of blood collection $10.1\ \text{mmol.l}^{-1}\ (7.6-17.8)$. Two of these individuals were controlled with diet alone while, eleven were on treatment with metformin and/or glibenclamide therapy. Five were obese and three had retinopathy.

14 IDDM patients were selected randomly. These 8 females and 6 males had a median age of 48 years (24-79), weight 68kg (52-99), HbA1 13% (8-15) and random blood sugar on presentation at the clinic of $12.9\text{mmol.l}^{-1}\ (4.7-20)$. 8 of these individuals were poorly controlled (HbA1 $>9.3\%$), 6 were well controlled (HbA1 $<7.5\%$), 5 were obese, all were on insulin therapy. Blood was collected in plain 10ml blood collection tubes and allowed to clot before centrifugation for 15 minutes at 1500g at 4°C. The serum was stored at $-70^\circ\text{C}$ until time of preparation of inhibitors.

b) Control and obese subjects: collection of blood samples for the preparation of inhibitory fraction by ammonium sulphate precipitation.

11 control subjects were selected for this study. 5 females and 6 males had a median age of 30 y (28-50) and weight 60kg (50-80). 8 obese subjects (5 females and 3 males) were randomly selected. Their median age was 30y (23-50) and weight 85kg (67-91). These individuals had urine free of glucose, and had no other illness. Blood was collected whilst
all subjects were fasting and subjected to ammonium sulphate precipitation as above. The preparations were stored at -70°C until assay.

c) **Selection of subjects and serum samples collected for preparation of inhibitory fraction by acid-ethanol extraction.**

Blood samples were collected in a random manner from control subjects and insulin dependent and non-insulin dependent diabetic patients attending clinics at the Royal Free Hospital. These subjects were not fasting.

9 control subjects, 6 male and 3 females whose median age was 30y (24-38) and weight was 65kg (60-76) were chosen. All had urine free of glucose and at time of blood collection their median glucose was 4.4 mmol.l⁻¹ (3-6.2).

10 non-insulin dependent diabetics (NIDD) (6 females, 4 males) were selected randomly. Their median age was 64y (50-78), body weight 81kg (50-90.2), HbA₁: 9.2% (6.7-12.0) and glucose on attendance at the clinic was 9.1 mmol.l⁻¹ (6.2-16.3). These individuals were on treatment with metformin and/or glibenclamide and had no retinopathy.

13 insulin dependent individuals were selected randomly (7 females, 6 males). The median age of this group was 61y (30-82), body weight 70kg (52.2-85), HbA₁: 8.8% (7.3-13.3) and glucose at attendance at the clinic was 8 mmol.l⁻¹ (2.2-16.4). These individuals were on treatment with Actrapid and Insultard, insulin injected twice daily. Two of these individuals have retinopathy.
Blood samples were collected in 10 ml plain glass tubes, allowed to clot and centrifuged for 15 mins at 4°C at 1500g. Serum was collected and stored at -70°C until required for extraction.

The population of diabetic patients studied in this group do not display a difference in any of the factors (e.g. blood glucose, body weight) listed above. The control group are not of a similar age to the patient groups however within the patient group studied there is no relationship between age and the presence of humoral insulin inhibitory activity. Previous studies involving this insulin inhibitory preparation did not control for the factors (glucose and insulin) which confound the lipogenesis assay (Dean et al 1984). This problem was addressed in this work.

d) Preparation of inhibitory fractions by ammonium sulphate precipitation of serum.

Neutral salts have pronounced effects on the solubility of globular proteins. This ability is a function of the ionic charge of the neutral salt. At sufficiently high ionic strength a protein may be almost completely precipitated from solution, an effect called SALTING OUT. The physicochemical basis of salting-out is complex, one factor is that the high concentration of salt may remove water of hydration from the protein molecules, thus reducing their solubility, but other factors are also involved. Salting out remains an important method for the preliminary purification of proteins and large peptides from crude extracts. Salts containing polyvalent anions such as sulphates and phosphate, e.g. ammonium and sodium sulphates and potassium phosphate have been used widely for the salting out of and purification of
proteins. Larger molecules are more readily precipitated, thus serum proteins are precipitated in the order, euglobulin, pseudoglobulin and albumin. Proteins precipitated by salting out retain their native conformation, and can be dissolved again, usually without denaturation. Ammonium sulphate is preferred for salting out proteins because it is soluble in water and very high ionic concentrations can be attained.

In this study supernatants were obtained from human serum by precipitation of serum proteins by the addition of 2.5M ammonium sulphate slowly with stirring. This mixture was left at 4°C mixing gently on a roller mixer for 2 hours. This was followed by centrifugation at 4°C at 3,500g for 30 minutes. The supernatant was decanted and desalted by extensive dialysis against doubly distilled water and saline. The increased volume of the inhibitor preparation was reduced to original volume by placing in dialysis tubing and subjecting the tubing to a stream of air in a cold room. The absence of a precipitate (barium sulphate) after the addition of barium chloride to an aliquot of the dialysed supernatant, was used to check for the complete elimination of ammonium sulphate.
e) **Ultrafiltration of inhibitory preparations to obtain fractions of varying molecular weight ranges.**

The inhibitor preparations produced from ammonium sulphate precipitation of sera were separated into size-graded macromolecular mixtures by fractionation through Amicon Diaflo ultrafiltration membranes using a stirred cell. The sera were sequentially ultrafiltered through membranes with the following nominal molecular weight "cut-offs": 1000; 10,000; 30,000; 50,000 daltons. This system is operated at 4°C under approximately 50 psi (3.7 atm) nitrogen. Ultrafiltration is the selective rejection of solutes by convective solvent flow through an anisotropic ("skinned") membrane. Solutes, colloids or particles of dimensions larger than the specified membrane "cut-off" are quantitatively retained in solution, while solutes smaller than the skin pores pass with solvent through the membrane substructure. For effective ultrafiltration, equipment must be designed to promote the highest transmembrane flow and selectivity. The major problem to be overcome is concentration polarization, the accumulation of retained macrosolute above the membrane. Its extent is determined by macrosolute concentration and diffusivity, temperature effects on solution viscosity, and system geometry. If left undisturbed, concentration polarization restricts solvent and solute transport through the membrane. It can alter membrane selectivity by forming a gel layer on the membrane. In other words a secondary membrane forms which increases rejection of normally permeating species. The magnetic stirring action used in this work controls concentration polarization of the retained macrosolutes under conditions of minimal potential shear denaturation.
The ultrafiltration membranes used in this work offer good filtration at modest pressure and also provide resistance to clogging because retained substances are rejected at the membrane surface. These membranes are made of inert polymers and do not denature biological materials.

Ultrafiltration of each group of pooled inhibitor preparations was carried out in an identical manner:-(i) fractions were filtered until 1% of the original volume remained in the ultrafiltration cell. (ii) Volume of the unfiltered solute was taken up to the original volume before filtration through the next filter.

f) Trypsinization of inhibitory preparations.

To determine whether the extracted species being studied is a protein or not, partial or selective hydrolysis of the polypeptide chain is accomplished by the use of proteases, enzymes that hydrolyse peptide bonds. The most specific protease is trypsin, a digestive enzyme secreted into the small intestine by the pancreas. Trypsin is readily obtained in pure crystalline form. It catalyses the hydrolysis of only those peptide bonds in a polypeptide chain whose carbonyl group is donated by either a lysine or an arginine residue, regardless of the length or amino acid sequence of the chain.
Trypsin cleaves peptide bonds where amino acid 1 = lysine or arginine.

(i) Trypsin at a 1% concentration is required for its optimum proteolytic activity to be manifested. The unfractionated inhibitor was incubated with 1% trypsin for one hour at 37°C before assay.

(ii) 1mg soyabean trypsin inhibitor inhibits 1.8g trypsin.
This concentration of trypsin inhibitor was assessed as a trypsin blocker. Thus after trypsinization of the unfractionated serum inhibitor, soyabean trypsin inhibitor was added to stop further tryptic activity.

(iii) Trypsinization was carried out by use of the immobilized enzyme. This is enzyme attached to cross-linked agarose bead obtained from Sigma No. T-1763. The gel suspension which was in 10mM acetic acid pH3.2 was washed repeatedly with water. Aliquots of this gel suspension containing approximately 17 units of enzyme were incubated with inhibitor and Krebs Ringer Bicarbonate buffer as a control, at 37°C for 1hr. The inhibitor was separated from the agarose by gentle centrifugation and filtration before assay.

[ 1 unit will hydrolyse 1.0µmol of Nα-bezoyl-l-arginine ethyl ester (BAEE) per minute at pH8.0 at 30°C.]
g) **Heat stability of inhibitory preparations.**

Inhibitor fractions were incubated at 60°C and 80°C in a water bath before assay.

h) **Effect of inhibitory preparation on cytochalasin B binding.**

The apparatus used for the cytochalasin B assay was similar to that described by Uhlenbeck (1972) and Zoccoli et al (1978). Each rack consisted of two pieces of perspex held firmly together by screws and wing nuts. Each half had six shallow circular chambers of 50μl capacity which were aligned with the other half when the apparatus was sealed. Between the two halves, a small piece of dialysis membrane separated the solutions in each chamber.

The procedure is as follows. Dialysis membrane was boiled in 20mM Na₂CO₃, 1mM Na₂ EDTA and then 3 times with water, and soaked in the same buffer as the sample to be assayed. The membrane, cut into small squares, was dried by pressing firmly between tissues, then placed in position and sealed by tightening the wing nuts firmly. Tritiated cytochalasin B (8x10⁻⁶ M in ethanol) was diluted to 8x10⁻⁶ M with the same buffer as the sample for assay (50mM Tris pH6.8). Using a glass 50μl syringe, 40μl was loaded into the chamber on one side of the membrane. Into the other side 40μl of the inhibitor and alkali stripped membrane mixture was loaded. The top of each chamber was sealed to prevent evaporation and the apparatus was shaken for 16-18h at room temperature on a rotary shaker. A volume of 25μl was then removed from each chamber and added to 4ml of Cocktail T (BDH Chemicals Ltd) for liquid scintillation counting. An automatic filler was used on the syringe to keep volumes consistent. The syringe was washed thoroughly.
with distilled water between additions. To determine the non-specific binding of cytochalasin B to the membranes, binding was performed in the presence of 400mM D-glucose in order to inhibit binding to the high affinity sites on the glucose transporters. D-glucose at this high concentration will competitively inhibit cytochalasin B binding. \( K_i \) for glucose inhibition of cytochalasin B binding is 20mM. Results are thus reported after correction for binding in the presence of 400mM D-glucose.

Calculation of bound to free ratios for cytochalasin B:

Let \( X \) be the radioactivity (cpm) measured by sampling that half of the dialysis chamber containing protein—this was contributed by both bound and free ligand (L).

\[ X = R + L \]

Where \( R \) = Glucose transporter, a high affinity site for cytochalasin B.

Let \( Y \) be the radioactivity (cpm) measured by sampling the other half of the chamber—this was contributed by free ligand alone and since equilibrium had been reached, it must also be identical to the radioactivity contributed by free ligand in the protein containing half of the chamber.

\[ Y = L \]

To convert cpm to concentrations, the total added cytochalasin B concentration must be taken into consideration:

\[ [R] + [L] = X / (X + Y) \quad \text{[cytochalasin B]} \]

and

\[ [L] = Y / (X + Y) \quad \text{[cytochalasin B]} \]
\[ \text{[RL]} = \frac{X - Y}{X + Y}. \text{[cytochalasin B]} \]

By definition, bound/free = \[ \text{[RL]} / [L] \]

\[ \text{bound/free} = \frac{X - Y}{Y} = \frac{X}{Y} - 1 \]

From this equation it can be seen that a measure of the concentration of cytochalasin B binding sites (i.e. bound/free or B/F) can be obtained by simply dividing the cpm on the side of the protein by the count on the side of the ligand and subtracting one.

The bound/free ratio is a good measure of concentration of binding sites only at low concentrations of cytochalasin B. Under these conditions of assay where binding is measured using a concentration of cytochalasin B \((4 \times 10^{-7} \text{ M})\) which is substantially less than the dissociation constant for cytochalasin B binding \((1.2 \times 10^{-7} \text{ M})\) it can be shown that the B/F ratio is approximately equal to the concentration of binding sites \((R)\) divided by the dissociation constant for binding (Zoccoli et al. 1978).

Binding of cytochalasin B to membranes is not very tight therefore washing the membranes to count the radiolabelled sites is not feasible as the cytochalasin B would wash off rapidly.

The ammonium sulphate inhibitor and its fractions were assayed in triplicate, controls for each fraction being the cytochalasin B binding measured in the absence of erythrocyte membranes.

1) Preparation of inhibitory fraction by acid-ethanol extraction of serum.

The addition of water miscible neutral organic solvents, particularly ethanol and acetone decreases the solubility of most
globular proteins in water to such an extent that they precipitate out of solution. Quantitative study of this effect shows that protein solubility at a fixed pH and ionic strength is a function of the dielectric constant of the medium. Since ethanol has a lower dielectric constant than water, its addition to an aqueous protein solution increases the attractive force between opposite charges, thus decreasing the degree of ionization of the R groups of the protein. As a result, the protein molecules tend to aggregate and precipitate. The variables which must be considered when using this method of precipitation are: a) Ionic strength of the medium from which precipitation occurs. At low ionic strength increasing amounts of organic solvents severely decrease protein solubility, and this may also affect protein stability. The use of serum in this work as the extraction medium provides a reasonably constant ionic strength to work from. b) The temperature at which precipitation occurs is an important factor. Solvents can denature proteins at higher temperatures and at low temperatures the yield of active proteins is greater. c) The manner of bringing the precipitation about mechanically. It is important to mix rapidly on addition of solvent to prevent localized concentrations of ethanol from reaching denaturation levels. Acid-ethanol extraction of sera was carried out by a modified method of Dean et al. (1984)

Serum and acid-ethanol (10ml distilled water, 200ml 1M HCl and 790ml ethanol) in a ratio 1:9 were mixed overnight at 4°C on a roller mixer followed by centrifugation at 3,500g for 30 minutes. The supernatant was decanted, adjusted to pH 7.4 with 1M NaOH and the resultant precipitate discarded following centrifugation. This supernatant was freeze-dried,
reconstituted to the original serum volume and tested for inhibitory activity.

j) **Preparation of glucose free fractions.**

Glucose was removed from the inhibitor fractions by passing the reconstituted acid-ethanol extracts through PD10 Pharmacia columns. (Exclusion limit of 5000 daltons). The process of gel filtration separates molecules according to size in a column packed with a porous gel medium. Molecules larger than the largest pores in the swollen gel beads, i.e. above the exclusion limit, cannot enter the gel and are eluted first. Smaller molecules which enter the gel are retarded. Molecules are eluted in order of decreasing molecular size.

The columns equilibrated with water were loaded with 2.5ml sample and the high molecular weight components eluted with 3.5ml water. The increased eluate volume was reduced to original volume by placing in dialysis tubing and subjecting the tubing to a stream of air in a cold room.

k) **C18 Sep-pak chromatography.**

Sep-pak C18 cartridges accomplish fractionation of the sample by Reverse Phase Liquid Chromatography. In this process, the various components of the sample are retained and separated from each other based on the strength of adsorption of each component to the active surface of the material in the cartilage. In general lipophilic components will be adsorbed 'strongly', hydrophilic (ionic) components will be adsorbed 'weakly' or not at all. Inorganic salts, amino acids, hydrophilic proteins and sugars are examples of compounds with weak or
no retention. Since most biological samples contain both hydrophilic and lipophilic components, elution of the desired component(s) is accomplished by selectively varying the composition of the eluting phase (mobile phase). Beginning with an aqueous mobile phase (water, buffers etc), increasing concentrations of miscible organic solvents permits fractionation and collection of sample components. Chromatography in this case was conducted by the method of Dean et al.

A primed Sep-pak column was washed with acidified NaCl (pH 3.0). The acid/ethanol extract was diluted 1:4 with acidified 0.9% NaCl (pH 3.0) and spiked with 10μCi (2μmol) [3H]-glucose per ml. This diluted preparation containing 2.1mmol.l⁻¹ glucose thus was spiked with 2μmol labelled glucose. The label elution will be representative of the sample elution profile. The column was then loaded with 2.5ml diluted acid/ethanol extract and washed with 5x1ml aliquots of acidified NaCl, following which it was eluted with 1ml aliquots of 25% 50% and 100% acetonitrile. 100μl of each fraction was added to 5ml Liquiscint (a scintillation liquid for aqueous radioactive samples) before counting on a β scintillation counter. A second sample was eluted in the same manner which had not been spiked with radioactive glucose. The eluates in this case were checked for glucose on a Clandon YSI 23AM glucose analyser.
1) **Preparation of insulin free fractions.**

Insulin was removed from the inhibitor fractions by affinity coupling to anti porcine insulin raised in guinea pigs (Miles Yeda Ltd) (Heding, 1975)

The insulin antibody was immobilized by covalent coupling with CNBr-activated Sepharose 4B (Pharmacia, Sweden). 21.4g CNBr-activated Sepharose 4B was allowed to swell overnight in 1mM HCl at 4°C. The anti insulin antibody which was supplied as porcine lyophilized serum was recostituted to 1ml and a 100\(\mu\)l aliquot (binding capacity 24,000\(\mu\)U insulin) was added to 25ml coupling buffer. This antibody mixture was added to the freshly prepared CNBr-activated Sepharose 4B (from which excess hydrochloric acid had been removed) and mixed overnight at 4°C on an end over end mixer. The remaining reactive groups were blocked by adding slowly 24mls redistilled ethanolamine (pH8) and incubating with end over end mixing for 2 hours at room temperature. The excess non-covalently bound ligand and excess blocking agent were then washed away overnight with distilled water, 0.5M sodium chloride in 60% ethanol and phosphate buffer. The gel was stored at 4°C until use.

Insulin binding capacity of the gel was assessed by incubating \([^{125}I]\)-insulin spiked range of cold insulin concentrations with aliquots of the gel. On washing several times the radiolabel in the gel and in the washes was counted. The optimum binding of radiolabel was considered to be an indication of the insulin binding capacity of that volume of gel. To remove insulin from the inhibitor fractions, aliquots were added to the gel, shaken, centrifuged at 4°C at 1000g for 5 minutes and the supernatant aspirated.
m) Insulin Radioimmunoassay. (A method using a 2' antibody)

1 Use Na Phosphate buffer for double dilutions of standards and dilutions of high value unknowns.
2 Incorporate 3 quality control sera at low (~7μU), medium (~20μU), and high (~60μU) insulin concentration. These are also serially diluted for the assay to assess dilution accuracy.
3 Non specific binding tubes are set up in triplicate. These contain 200μl buffer only. (i.e. no antibody)
4 Zero tubes in triplicate contain 100μl buffer + 100μl antibody.
5 Unknown samples are assayed in duplicate. 100μl of each is aliquoted into test tubes.
6 100μl 1' antibody is added to all tubes (except NSB), mixed and incubated overnight at 4°C.
7 Add 100μl tracer to all tubes, mix and incubate at 4°C for two hours.
8 Add 100μl 2' antibody (rabbit anti guinea-pig) to all tubes, mix and incubate at room temperature for 30 minutes.
9 Centrifuge at 4°C, at maximum r.p.m. for 30 minutes.
10 Leave tubes for 20 minutes at room temperature, allowing viscosity to decrease, thereby allowing efficient decanting.
11 Invert racks to void supernatant, leaving them inverted for 10 minutes.
12 Read activity in γ counter for 3 minutes each.

Results are interpreted in μU.ml⁻¹ but are reported in SI Units μU.l⁻¹.

A typical insulin standard line is shown in Fig 2:1.
Figure 2:1

A representative standard curve for the insulin R.I.A.
Sensitivity of this assay procedure as the range of the minimum detectable level varied between 0.12 - 1.61 mU.l⁻¹.

n) Preparation of adipocytes. (Rodbell 1964)

Epididymal fat pads were obtained from Sprague-Dawley rats (100-200g) and kept on an ad libitum diet (Grain Harvesters Brand 41B). Rats were killed by cervical dislocation. The fat pads were dissected out into a plastic vial containing 0.33 ng.ml⁻¹ collagenase in 3 ml KRB buffer pH 7.4. This was allowed to incubate for 1 hour at 37°C in a shaking water bath (Rodbell, 1964). The adipocyte suspensions were filtered through a nylon mesh with gentle agitation by means of a plastic Pasteur pipette. The cells were washed with 6x10 ml KRB buffer by flotation; the infranatant was removed and discarded after each wash. The adipocytes were resuspended in KRB buffer before aliquoting into the bioassay systems.

o) Glucose oxidation by adipocytes. (Khokher & Dandona 1985)

The conversion of D-[U⁻¹⁴C] glucose to ¹⁴C-carbon dioxide by rat adipocytes was used as the index of glucose oxidation. Plastic reaction tubes (Brunswick blood collection tubes) were set up to contain 100 µl insulin, 100 µl test substance, 700 µl adipocytes in KRB buffer and 100 µl radiolabelled glucose (0.4 µCi.ml⁻¹, final concentration 0.25 mmol.l⁻¹). Care was taken to deliver the radioactivity to the bottom of the tube without contaminating the rest of the tube.

A strip of filter paper (9x2 cm) was dipped in hyamine-10X-hydroxide, the excess blotted off and the paper rolled to fit the neck of the test
tube. The tubes were tightly capped and placed in a shaking water bath at 37°C for 90 minutes.

To stop the reaction, the cap of the incubation tube was pierced by a 21G needle attached to a 2ml syringe containing 1ml 6M sulphuric acid. The sulphuric acid was carefully added and the tubes incubated for a further 30 minutes in a shaking water bath at 37°C. The filter papers were then removed and placed in scintillation vials containing 15ml scintillation liquid (PPO 4g.l⁻¹ in toluene triton mixture 2:1). 200μl methanol was added to each vial to increase the solubility of hyamine-¹⁴CO₂ in the scintillation liquid.

p) **Insulin bioassay.**

Insulin was bioassayed by the adipocyte lipogenesis method as described by Moody et al. (1974) and adapted by Coulston & Dandona (1980) using D-[U-¹⁴C]-glucose to provide an index of incorporated glucose into lipid. For each test point five replicated samples were set up in every assay. The insulin standards were at final concentration of 2.5, 5, 10, 20μU.l⁻¹ from which a dose response curve was compiled. Lipogenesis under basal conditions was determined by incubating adipocytes without insulin and the non specific background (NSB) counts representing the radioactivity in the scintillant (organic phase) were obtained by incubating adipocytes as for basal conditions but lysed by the addition of 10ml scintillant.
Thus for a final volume of 1ml the experimental conditions were:

<table>
<thead>
<tr>
<th>TEST</th>
<th>INSULIN (µl)</th>
<th>LABEL (µl)</th>
<th>BUFFER (µl)</th>
<th>ADIPOCYTES (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(0.1 mU.ml⁻¹)</td>
<td>(0.2 µU.ml⁻¹)</td>
<td>KRB</td>
<td>(4x10 cells.ml⁻¹)</td>
</tr>
<tr>
<td>NSB</td>
<td>-</td>
<td>100</td>
<td>400</td>
<td>500</td>
</tr>
<tr>
<td>BASAL</td>
<td>-</td>
<td>100</td>
<td>400</td>
<td>500</td>
</tr>
<tr>
<td>INSULIN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 mU.ml⁻¹</td>
<td>25</td>
<td>100</td>
<td>375</td>
<td>500</td>
</tr>
<tr>
<td>5.0 mU.ml⁻¹</td>
<td>50</td>
<td>100</td>
<td>350</td>
<td>500</td>
</tr>
<tr>
<td>10.0 mU.ml⁻¹</td>
<td>100</td>
<td>100</td>
<td>300</td>
<td>500</td>
</tr>
<tr>
<td>20.0 mU.ml⁻¹</td>
<td>200</td>
<td>100</td>
<td>200</td>
<td>500</td>
</tr>
</tbody>
</table>

The reaction mixture was gassed with 95% O₂ : 5%CO₂ before a 2 hour incubation at 37°C in a shaking water bath. The reaction was stopped at the end of this period by the addition of 10ml scintillant which resulted in formation of two transparent phases. Radioactivity incorporated into lipids was extracted into the upper organic phase which was counted by a Phillips β scintillation spectrometer. The lower aqueous phase (1ml) is not counted and contains the major portion of quenching material.

The effect of test substances on the insulin dose response curve was assessed by addition of 100µl test substance to the reaction mixture and adjusting the buffer volume so that the final volume was one millilitre.

Results were expressed as percentage stimulation (or inhibition) over basal level of lipogenesis. To calculate this, the mean NSB c.p.m. value was subtracted from the mean c.p.m. value of all other tests.
Then \[ \text{test c.p.m.} - \text{basal c.p.m.} \times 100 = \text{percentage} \]
\[
\text{basal c.p.m.} \quad \text{stimulation/inhibition}
\]

To express results as percentage stimulation/inhibition over basal, basal lipogenesis was read as 100%.

q) **Analysis of lipogenesis breakdown products.**

Rat adipocyte lipogenesis was carried out as described and at the end of the 2 hour incubation period the 1ml incubation mixture was treated with 5ml extraction cocktail (784ml isopropanol, 196ml heptane, 20ml 1N sulphuric acid), 3ml heptane and 2ml water to extract long chain fatty acids as described by Dole & Meinertz (1960). The label incorporated into triglycerides was measured in 1ml of the heptane (upper) layer (Hansen et al., 1983). To determine \(^{14}\text{C}-\text{glucose}\) incorporation into fatty acids and glycerol, another 1ml aliquot of the heptane layer was subjected to hydrolysis with 0.5ml, 0.5M ethanolic KOH for 20 minutes at 60°C followed by addition of 1ml 1N sulphuric acid. Addition of 10ml toluene/PPO resulted in two layers, the upper organic layer was counted for fatty acid content and then the lower aqueous layer was counted with PPO-toluene/triton 2:1 for glycerol content.
r) \[^{38}S\] Sulfate uptake.— A simplified bioassay for somatomedin.

(Spencer & Taylor 1978)

These assays were carried out at: Dept. of Growth & Development, Institute of Child Health, London by Taylor.

The basis of this somatomedin assay is the incorporation of \[^{38}S\] Sulphate into cartilage. Somatomedin stimulates \[^{38}S\] sulphate uptake by cartilage. A simplified bioassay of somatomedin established by Spencer & Taylor uses porcine cartilage discs.

The effect of the serum inhibitor preparations on \[^{38}S\] sulphate uptake was tested in the presence and absence of serum somatomedin activity as used to test the effect of other agents on this system (Taylor et al 1983). All incubations of cartilage with inhibitor preparations were carried out for the entire incubation period of 24h at 37°C. After incubation the cartilage discs were washed in running water to remove all non-incorporated radioactive sulphate. The discs were transferred to counting vials containing 0.5ml 90% (w/v) formic acid and heated at 80°C for 30 minutes in a water bath. After cooling 10ml scintillation fluid was added before activity was measured in a liquid scintillation counter.

s) In vivo studies with streptozotocin-induced diabetic rats.

This was a collaborative study, the diabetic animals being set up by A.K. Sharma of the Department of Anatomy, Marischal College, University of Aberdeen, Aberdeen. Three groups of Sprague-Dawley rats, aged 11 weeks and weighing between 357 and 515g (mean: 456g) were rendered
diabetic by the intraperitoneal injection of streptozotocin (55mg.kg\(^{-1}\) body weight). Control, untreated diabetic and diabetic rats were treated with a daily subcutaneous injection of insulin (Novo Alle, Denmark). Blood glucose of all insulin-treated animals was estimated daily at midday. Insulin dosage was adjusted to maintain blood glucose level within the normal range (4-7m.mol.l\(^{-1}\)). Blood glucose concentrations were also estimated in the control and untreated rats at weekly intervals. Additionally, blood glucose and glycosylated haemoglobin (HbA1c) levels were measured for the three groups at 4-weekly intervals.

Skeletal growth was assessed in the control group at the beginning of the study and for all three groups in the final week. One hind limb was radiographed in a standard position with the animal under ether anaesthesia, and tibial length measured on X-ray plates using a magnifying eyepiece containing a graticule.

At the end of the study, animals were anaesthetized with ether and 7-10ml blood obtained by cardiac puncture, allowed to coagulate and centrifuged for 10 min at 1000rpm. The serum was removed and stored at -20°C.

To confirm the consistency of the data related to the appearance of an inhibitory activity in serum of diabetic rats and its disappearance following insulin therapy, the entire experiment was repeated three times. Detailed growth measurements, however, were carried out only once.

Frozen serum samples were sent to us to be processed. Pooled serum from diabetic treated, diabetic untreated and control rats was separated into size-graded macromolecular mixtures by fractionation through
Amicon's Diaflo ultrafiltration membranes as described in Materials & Methods.

These fractions and serum samples were then assayed for somatomedin activity, in a system using [³⁵S]-sulphate uptake as an index of stimulation (each fraction was assayed at serial concentrations of 50, 25, 12 and 6% in Hams F12 medium). This assay was performed in the Department of Growth & Development, Institute of Child Health, London. These fractions were also assayed for insulin-like activity in the rat adipocyte lipogenesis.

\( t \) Protein assay: The Folin Lowry method. (Lowry et al. 1951).

Protein reacts with the Folin-Ciocalteau reagent to give a coloured complex. The colour so formed is due to the reaction of the alkaline copper with the protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein.

Materials.

i Alkaline sodium carbonate solution (2% \( \text{Na}_2\text{CO}_3 \) in 0.1M \( \text{NaOH} \)).

ii Copper sulphate-sodium potassium tartarate solution (0.5% \( \text{CuSO}_4 \) in 1% \( \text{Na}^+, \text{K}^+ \) tartrate). Prepare fresh by mixing stock solutions.

iii "Alkaline solution". Prepare on day of use by mixing 50ml of (i) and 1ml of (ii).

iv Folin-Ciocalteau reagent. (Dilute the commercial reagent with an equal volume of water on the day of use. This is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acid.)

v Standard protein (albumin solution 0.2mg.ml⁻¹).
Method.
Add 5ml of the "alkaline solution" to 1ml of the test solution. Mix thoroughly and allow to stand at room temperature for 10min or longer. Add 0.5ml of diluted Folin-Ciocalteau reagent rapidly with immediate mixing. After 30min read the extinction against the appropriate blank at 750nm.
Estimate the protein concentration of an unknown solution after preparing a standard curve.
A typical protein standard line is shown in Fig 2.2.
u) **Statistics.**

Paired & Unpaired "Students" t test. (Swinscow 1978)

The "paired" version of this test can be used to assess the significance of differences between paired observations. For example the paired t test has been used to evaluate significance within one assay, and within one sample. The "unpaired" sample t tests were used to compare two means from different assays/samples.

Wilcoxon matched-pairs signed-ranks test. (Siegel 1956)

This test utilizes information about the direction and the relative magnitude of the differences considered within pairs. This test is preferred for the study of a small number of samples, the assumptions for this test are fewer than those associated with a parametric test (i.e. requirement for Gaussian distribution). This test was thus used to compare two sets of data within the same group.

Mann Whitney U test. (Siegel 1956)

This non-parametric test compares two independent samples. Like the non-parametric Wilcoxon test there is no requirement for normal distribution.

Chi Squared test. (Siegel 1956).

This test determines the significance of distribution/frequency of two populations. While there is no precise rule defining the circumstances in which to use Yates correction, a common practice is to incorporate it into \( \chi^2 \) calculations on tables with a total of under
100 or with any cell containing a value less than 10. It is probably wise to apply it for almost all Chi² tables.
CHAPTER THREE

RESULTS OF INSULIN INHIBITORY ACTIVITY FOUND IN SUPERNATANTS OBTAINED AFTER AMMONIUM SULPHATE PRECIPITATION OF SERUM
The results in this chapter, based on the lipogenesis and glucose oxidation bioassay, are reported as a representative example of a minimum of four experiments carried out for each table or figure shown. Each table and figure shows the results obtained from one experiment only, so that the values shown are directly comparable. Each assay point represents the mean of five observations. Dose-response curves for insulin standards were plotted for each experiment. Data from these curves are expressed with the results of each experiment presented in this work.

The glucose oxidation bioassay was employed as an additional index of glucose metabolism. This is a non-invasive procedure, the end point being the trapping of radiolabelled carbon dioxide onto a strip of filter paper. The incubation mixture remaining can thus be used to determine radiolabel incorporation into fatty acids and glycerol. The lipogenesis assay does not allow this as scintillant is added to the total incubation mixture to determine radiolabel incorporation into triglycerides.

Results are expressed in a manner such that 100% is basal lipogenesis, all values below 100% being an inhibition of the system.

Insulin (1.25-20mU.l\(^{-1}\)) was shown to stimulate lipogenesis in a dose dependant manner. During the process of lipogenesis \([U-^{14}C]\)-glucose or \(D-3-[^{3}H]\)-glucose are metabolized and label is incorporated into triglycerides. A typical dose response curve to standard doses of insulin is presented in Figure 3:1 demonstrating the levelling out of the lipogenic response at 20mU.l\(^{-1}\) insulin.
Figure 3:1

Effect of insulin on rat adipocyte lipogenesis

% stimulation ± S.E.

Insulin (mU l⁻¹)
The unfractionated inhibitor antagonized insulin (Table 3:1), IgG (Table 3:2), NSILA-s (Table 3:3) and NSILA-p (Table 3:4) stimulated lipogenesis. Each of these lipogenic substances stimulate lipogenesis in a dose dependent manner and these responses are comparable. The inhibition of these lipogenic responses is also dose dependent and statistically significant.

Insulin (5-20 mU.l⁻¹) stimulates oxidation of [U-¹⁴C]-glucose in rat adipocytes in a dose dependent manner. (Figure 3:2). This dose-response curve is shifted to the right by the 'crude' ammonium sulphate inhibitor preparation in a non-competitive fashion. (Figure 3:2).

Another inhibitor of glucose metabolism; ethanol (237mmol.l⁻¹) also caused inhibition of glucose oxidation and lipogenesis in rat adipocytes. (Table 3:5). A concentration of 1.5% ethanol in the incubate causes marked inhibition of basal and insulin stimulated glucose metabolism.

To assess the effect of heat treatment on this serum inhibitor, the inhibitor preparations were incubated for 20 minutes in waterbaths at 60°C and 80°C respectively. Incubation at 60°C resulted in 70% loss of insulin inhibitory activity as measured by the effect of the preparations on insulin (10mU.l⁻¹) stimulated lipogenesis. Incubation at 80°C resulted in total loss of activity. (Table 3:6).

Trypsin at varying concentrations has an inhibitory effect on lipogenesis, causing an inhibition of insulin stimulated lipogenesis at concentrations required for its proteolytic effect. Thus it is very difficult to use this protease (as well as any others) in this bioassay system. A trypsin dose response on lipogenesis demonstrated inhibitory
TABLE 3:1

Effect of unfractionated inhibitor obtained from supernatants of ammonium sulphate precipitated control sera on basal and insulin-stimulated rat adipocyte lipogenesis. (% stimulation ± SE; n=5)

[Basal counts: 454cpm (=100%)]

<table>
<thead>
<tr>
<th>Insulin (mU.l⁻¹)</th>
<th>Unfractionated inhibitor (mg.l⁻¹)</th>
<th>0</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>100</td>
<td>140±10</td>
<td>93±16</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>346±47</td>
<td>341±49</td>
<td>276±72</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>526±7</td>
<td>442±47</td>
<td>269±25</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>606±10</td>
<td>409±68</td>
<td>327±39b</td>
<td></td>
</tr>
</tbody>
</table>

Statistical comparison (Students t test)

a: p<0.025 when compared to corresponding insulin-stimulated lipogenesis.
b: p<0.001 when compared to corresponding insulin-stimulated lipogenesis.
TABLE 3:2

Effect of unfractionated inhibitor obtained from supernatants of ammonium sulphate precipitated control sera on IgG-stimulated rat adipocyte lipogenesis. (%stimulation ± SE; n=6)

[Basal counts: 257cpm (=100%)]

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Unfractionated inhibitor (mg.l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG(mg.l⁻¹)</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>152±8</td>
</tr>
<tr>
<td>100</td>
<td>182±10</td>
</tr>
<tr>
<td>200</td>
<td>817±13</td>
</tr>
</tbody>
</table>

Statistical comparisons  (Students t test)
a: p<0.001 when compared with corresponding IgG stimulated lipogenesis.
TABLE 3:3

Effect of unfractionated inhibitor obtained from supernatants of ammonium sulphate precipitated control sera on NSILA-s stimulated rat adipocyte lipogenesis. (% stimulation ± SE; n=5)

[Basal counts: 454cpm (=100%)]

<table>
<thead>
<tr>
<th>Insulin (mU.l⁻¹)</th>
<th>Unfractionated inhibitor (mg.l⁻¹)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>100</td>
<td>93±16</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>346±47</td>
<td>276±72</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>526±7</td>
<td>269±25*</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NSILA-s (g.l⁻¹)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>229±35</td>
<td>200±45</td>
</tr>
<tr>
<td>100</td>
<td>314±22</td>
<td>239±20p</td>
</tr>
<tr>
<td>200</td>
<td>418±54</td>
<td>256±44p</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)
a: p<0.01 when compared to corresponding insulin stimulated lipogenesis.
b: p<0.05 when compared to corresponding insulin stimulated lipogenesis.
TABLE 3:4

Effect of the unfractionated inhibitor obtained from supernatants of ammonium sulphate precipitated control sera on NSILA-p stimulated rat adipocyte lipogenesis. (% stimulation ± SE; n=4)

[Basal counts: 158cpm (=100%)]

<table>
<thead>
<tr>
<th>Insulin (mU.1⁻¹)</th>
<th>Unfractionated inhibitor (mg.1⁻¹)</th>
<th>0</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>100</td>
<td>105±3</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>357±28</td>
<td>234±20a</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>414±25</td>
<td>371±31</td>
</tr>
</tbody>
</table>

NSILA-p (g.1⁻¹)

| 0.5             | 290±9                           | 174±17b|
| 1.0             | 306±17                          | 188±30a|

Statistical comparisons  (Students t test)
a: p<0.02 when compared to corresponding NSILA-p stimulated lipogenesis.
b: p<0.001 when compared to corresponding NSILA-p stimulated lipogenesis.
Figure 3:2

Effect of unfractionated inhibitor (10mg.l⁻¹) obtained from supernatant of ammonium sulphate precipitated control serum on basal and insulin-stimulated glucose oxidation in rat adipocytes.

[Basal counts: 978cpm (=100%); n=4]

- ■ - Dose response to insulin
- □ - Dose response to insulin with serum insulin inhibitor (10mg.l⁻¹)

Statistical comparisons (Students t test)

* p<0.02 when compared to insulin-stimulated glucose oxidation.
** p<0.025 when compared to insulin-stimulated glucose oxidation.
* p<0.05 when compared to basal (0 insulin) glucose oxidation.
**TABLE 3.5**

a) Effect of ethanol on glucose oxidation in rat adipocytes.

(\% stimulation ± SE; n=5) [Basal counts: 800 cpm (=100%)]

<table>
<thead>
<tr>
<th>Insulin (mU.l⁻¹)</th>
<th>Ethanol (ml.l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>325±21</td>
</tr>
<tr>
<td>10</td>
<td>559±8</td>
</tr>
</tbody>
</table>

b) Effect of ethanol on lipogenesis in rat adipocytes.

(\% stimulation ± SE; n=5) [Basal counts: 350 cpm (=100%)]

<table>
<thead>
<tr>
<th>Insulin (mU.l⁻¹)</th>
<th>Ethanol (ml.l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>213±12</td>
</tr>
<tr>
<td>10</td>
<td>310±20</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)

p<0.001 for all inhibitions of insulin stimulated lipogenesis.
TABLE 3:6

Effect of heat treatment (37°, 60° & 80°C), on the action of inhibitors obtained from supernatants of ammonium sulphate precipitated control sera on basal and insulin-stimulated rat adipocyte lipogenesis.

(% stimulation ± SE; n=5) (Basal counts: 80cpm (=100%))

<table>
<thead>
<tr>
<th>Insulin alone</th>
<th>Insulin + Inhibitor (1mg.1⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37°C</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>188±33</td>
</tr>
<tr>
<td>10</td>
<td>222±20</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)

a: p<0.005 when compared to corresponding insulin stimulated lipogenesis.
activity at 0.01g.l⁻¹ trypsin (Table 3:7). For the proteolytic activity of trypsin to be apparent at least a 1% solution of trypsin must be used; since much smaller concentrations of trypsin exhibit insulin inhibitory effects. The use of a protease in this system is fraught with problems. These problems are enhanced by the fact that the reactions based on trypsinization have to be terminated by the addition of the soya bean inhibitor; this inhibitor resulted in the stimulation of adipocyte lipogenesis (Table 3:8). An attempt to use immobilized trypsin i.e. trypsin bound to an inert gel still resulted in tryptic activity appearing in the lipogenesis bioassay system (Table 3:9), probably because the enzyme was uncoupled from the gel during the trypsinization of the inhibitor. A control experiment where Krebs Ringer Bicarbonate buffer was incubated as above with immobilized trypsin also resulted in inhibitory tryptic activity appearing in the bioassay.

Fractionation of the ammonium sulphate inhibitor preparation into nominal molecular weight ranges: 0-1,000Da; 1,000-10,000Da; 10,000-30,000Da; 30,000-50,000Da; 50,000-100,000Da; and >100,000Da was carried out. The effect of these fractions on basal and insulin stimulated (10mU.l⁻¹) lipogenesis (Figures 3:3-3:8) led to the identification of two fractions with inhibitory activity. These were the 10,000-30,000Da (Figure 3:5) and 30,000-50,000 (Figure 3:6) moieties. These two inhibitor fractions caused the dose-response curve of insulin to shift to the right, indicative of inhibition in a non-competitive manner. (Figure 3:9) The fraction 10,000-30,000Da was inhibitory to basal and insulin stimulated lipogenesis at a protein concentration of 2mg.l⁻¹ and the 30,000-50,000Da fraction was similarly inhibitory at a protein
TABLE 3:7

Effect of trypsin on basal and insulin-stimulated rat adipocyte lipogenesis. (% stimulation ± SE; n=4)

[Basal counts: 226cpm (=100%)]

<table>
<thead>
<tr>
<th>Trypsin (g.l⁻¹)</th>
<th>0</th>
<th>0.0001</th>
<th>0.001</th>
<th>0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (mU.l⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>100</td>
<td>98±12</td>
<td>96±12</td>
<td>114±13</td>
</tr>
<tr>
<td>2.5</td>
<td>308±22</td>
<td>300±13</td>
<td>310±37</td>
<td>106±3*</td>
</tr>
<tr>
<td>5.0</td>
<td>478±24</td>
<td>414±19</td>
<td>445±18</td>
<td>116±19*</td>
</tr>
<tr>
<td>10.0</td>
<td>733±65</td>
<td>636±27</td>
<td>624±27</td>
<td>75±50*</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)

a: p<0.001 when compared to corresponding insulin stimulated value.
**TABLE 3:8**

Effect of trypsinisation and soya bean trypsin inhibitor on the action of inhibitors obtained from supernatants of ammonium sulphate precipitated control sera on rat adipocyte lipogenesis. (% stimulation ± SE; n=5)

[Basal counts: 155cpm (=100%)]

<table>
<thead>
<tr>
<th>Insulin (mU.1⁻¹)</th>
<th>Insulin alone</th>
<th>Inhibitor (15mg.1⁻¹)</th>
<th>Inhib / Tryp / SBTI*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>100</td>
<td>90±12</td>
<td>3519±157</td>
</tr>
<tr>
<td>2.5</td>
<td>620±27</td>
<td>562±18</td>
<td>3009±155</td>
</tr>
<tr>
<td>5.0</td>
<td>747±13</td>
<td>680±25*</td>
<td>3068±210</td>
</tr>
<tr>
<td>10.0</td>
<td>887±15</td>
<td>730±19*</td>
<td>2833±112</td>
</tr>
<tr>
<td>20.0</td>
<td>980±21</td>
<td>891±14*</td>
<td>3780±221</td>
</tr>
</tbody>
</table>

* Inhib / Tryp / SBTI = Trypsinised serum inhibitory preparation followed by treatment with soya bean trypsin inhibitor.

Statistical comparisons (Students t test)
a: p<0.05 when compared to corresponding insulin stimulated lipogenesis.
b: p<0.001 when compared to corresponding insulin stimulated lipogenesis.
c: p<0.01 when compared to corresponding insulin stimulated lipogenesis.
### TABLE 3:9

Effect of immobilized trypsin on the action of serum inhibitors (from control subjects) on basal and insulin stimulated rat adipocyte lipogenesis. (% stimulation ± SE; n=5)

[Basal counts: 200cpm (=100%)]

<table>
<thead>
<tr>
<th>Insulin response</th>
<th>Insulin + Inhibitor (10mg.l⁻¹)</th>
<th>Insulin + Trypsinized inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (mU.l⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>100</td>
<td>80±12</td>
</tr>
<tr>
<td>2.5</td>
<td>300±9</td>
<td>317±7</td>
</tr>
<tr>
<td>5.0</td>
<td>500±17</td>
<td>346±20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10.0</td>
<td>982±21</td>
<td>439±24&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)

a: p<0.001 when compared to basal lipogenesis.
b: p<0.001 when compared to corresponding insulin stimulated lipogenesis value.
c: p<0.01 when comparing responses of the inhibitor and the trypsinized inhibitor.
d: p<0.001 when comparing responses of the inhibitor and the trypsinized inhibitor.
Figure 3.3

Effect of the 0-1kDa fraction obtained from supernatants of ammonium sulphate precipitated control sera on basal and insulin-stimulated rat adipocyte lipogenesis. (mean ± SE; n=6)

1. Basal lipogenesis (cpm:368 =100%)
2. Insulin (5mU.l⁻¹)
3. Insulin (10mU.l⁻¹)
4. Insulin (20mU.l⁻¹)
5. 0-1kDa fraction (1mg.l⁻¹)
6. 0-1kDa fraction (2mg.l⁻¹)
7. 0-1kDa fraction (4mg.l⁻¹)
8. Insulin (10mU.l⁻¹) + 0-1kDa fraction (1mg.l⁻¹)
9. Insulin (10mU.l⁻¹) + 0-1kDa fraction (2mg.l⁻¹)
10. Insulin (10mU.l⁻¹) + 0-1kDa fraction (4mg.l⁻¹)

Statistical comparisons (Students t test)

5 vs 1  p<0.01
10 vs 3  p<0.01
Figure 3:4

Effect of the 1-10kDa fraction obtained from supernatants of ammonium sulphate precipitated control sera on basal and insulin-stimulated rat adipocyte lipogenesis. (mean ± SE; n=6)

1. Basal lipogenesis (cpm:368 =100%)
2. Insulin (5mU.l⁻¹)
3. Insulin (10mU.l⁻¹)
4. Insulin (20mU.l⁻¹)
5. 1-10kDa fraction (6.5mg.l⁻¹)
6. 1-10kDa fraction (13.0mg.l⁻¹)
7. 1-10kDa fraction (26.0mg.l⁻¹)
8. Insulin (10mU.l⁻¹) + 1-10kDa fraction (6.5mg.l⁻¹)
9. Insulin (10mU.l⁻¹) + 1-10kDa fraction (13.0mg.l⁻¹)
10. Insulin (10mU.l⁻¹) + 1-10kDa fraction (26.0mg.l⁻¹)

Statistical comparisons (Students t test)

5 vs 1 \( p < 0.02 \)
6 vs 1 \( p < 0.005 \)
Lipogenesis: % stimulation/inhibition
Figure 3:5

Effect of the 10-30kDa fraction obtained from supernatants of ammonium sulphate precipitated control sera on basal and insulin-stimulated rat adipocyte lipogenesis. (mean ± SE; n=6)

1. Basal lipogenesis (cpm:437 =100%)
2. Insulin (5mU.l⁻¹)
3. Insulin (10mU.l⁻¹)
4. Insulin (20mU.l⁻¹)
5. 10-30kDa fraction (2mg.l⁻¹)
6. 10-30kDa fraction (4mg.l⁻¹)
7. 10-30kDa fraction (8mg.l⁻¹)
8. Insulin (10mU.l⁻¹) + 10-30kDa fraction (2mg.l⁻¹)
9. Insulin (10mU.l⁻¹) + 10-30kDa fraction (4mg.l⁻¹)
10. Insulin (10mU.l⁻¹) + 10-30kDa fraction (8mg.l⁻¹)

Statistical comparisons (Students t test)

5 vs 1  p<0.001
6 vs 1  p<0.001
7 vs 1  p<0.001
8 vs 3  p<0.001
9 vs 3  p<0.001
10 vs 3 p<0.001
Lipogenesis: % stimulation/inhibition
Figure 3:6

Effect of the 30-50kDa fraction obtained from supernatants of ammonium sulphate precipitated control sera on basal and insulin-stimulated rat adipocyte lipogenesis. (mean ± SE; n=6)

1. Basal lipogenesis (cpm:437 =100%)
2. Insulin (5mU.l⁻¹)
3. Insulin (10mU.l⁻¹)
4. Insulin (20mU.l⁻¹)
5. 30-50kDa fraction (6.5mg.l⁻¹)
6. 30-50kDa fraction (13.0mg.l⁻¹)
7. 30-50kDa fraction (26.0mg.l⁻¹)
8. Insulin (10mU.l⁻¹) + 30-50kDa fraction (6.5mg.l⁻¹)
9. Insulin (10mU.l⁻¹) + 30-50kDa fraction (13.0mg.l⁻¹)
10. Insulin (10mU.l⁻¹) + 30-50kDa fraction (26.0mg.l⁻¹)

Statistical comparisons (Students t test)

5 vs 1  p<0.001
6 vs 1  p<0.001
7 vs 1  p<0.001
8 vs 3  p<0.001
9 vs 3  p<0.005
10 vs 3  p<0.001
Figure 3:7

Effect of the 50-100kDa fraction obtained from supernatants of ammonium sulphate precipitated control sera on basal and insulin-stimulated rat adipocyte lipogenesis. (mean ± SE; n=6)

1. Basal lipogenesis (cpm:648 =100%)
2. Insulin (5mU.l⁻¹)
3. Insulin (10mU.l⁻¹)
4. Insulin (20mU.l⁻¹)
5. 50-100kDa fraction (1mg.l⁻¹)
6. 50-100kDa fraction (2mg.l⁻¹)
7. 50-100kDa fraction (4mg.l⁻¹)
8. Insulin (10mU.l⁻¹) + 50-100kDa fraction (1mg.l⁻¹)
9. Insulin (10mU.l⁻¹) + 50-100kDa fraction (2mg.l⁻¹)
10. Insulin (10mU.l⁻¹) + 50-100kDa fraction (4mg.l⁻¹)

Statistical comparisons  (Students t test)

All comparisons to basal and insulin-stimulated lipogenesis were not significant.
Lipogenesis: % stimulation/inhibition

- 116 -
**Figure 3.8**

Effect of the >100kDa fraction obtained from supernatants of ammonium sulphate precipitated control sera on basal and insulin-stimulated rat adipocyte lipogenesis. (mean ± SE; n=6)

1. Basal lipogenesis (cpm: 648 = 100%)
2. Insulin (5mU.l⁻¹)
3. Insulin (10mU.l⁻¹)
4. Insulin (20mU.l⁻¹)
5. >100kDa fraction (4mg.l⁻¹)
6. >100kDa fraction (8mg.l⁻¹)
7. >100kDa fraction (16mg.l⁻¹)
8. Insulin (10mU.l⁻¹) + >100kDa fraction (4mg.l⁻¹)
9. Insulin (10mU.l⁻¹) + >100kDa fraction (8mg.l⁻¹)
10. Insulin (10mU.l⁻¹) + >100kDa fraction (16mg.l⁻¹)

**Statistical comparisons**  (Students t test)

5 vs 1  p<0.005
6 vs 1  p<0.001
7 vs 1  p<0.001
10 vs 3  p<0.001
Effect of the 10-30kDa and 30-50kDa inhibitor fractions obtained from supernatants of ammonium sulphate precipitated sera on insulin stimulated rat adipocyte lipogenesis. (mean ± SE; n=4)

[Basal counts: 560cpm (=100%)]

- o - Insulin dose response.
- ◆ - 30-50kDa (2mg.l⁻¹) inhibitory fraction + insulin
- □ - 10-30kDa (2mg.l⁻¹) inhibitory fraction + insulin.

Statistical comparisons (Students t test)

‡ p<0.001 when compared to basal lipogenesis.
* p<0.005 when compared to basal lipogenesis.
** p<0.01 when compared to insulin-stimulated lipogenesis.
† p<0.025 when compared to insulin-stimulated lipogenesis.
δ p<0.05 when compared to insulin-stimulated lipogenesis.
concentration of 6.5mg.l⁻¹. The other fractions assayed at varying protein concentrations did not exhibit inhibitory activity.

Investigation of the incorporation of glucose into triglycerides, fatty acids and glycerol was carried out with [U-¹⁴C]-glucose (Table 3:10b) since ethanolic hydrolysis of triglycerides to fatty acids and glycerol when using D-3-[³H]-glucose often led to a loss in radioactivity of approximately 35%. (Table 3:10a)

The unfractionated inhibitor antagonized both basal and insulin stimulated conversion of [U-¹⁴C]-glucose to triglycerides and fatty acids. In the presence of low concentrations of glucose (0.1mM.l⁻¹) in the buffer the incorporation of [U-¹⁴C]-glucose was largely in the glycerol moiety as observed by the counts per minute value (Table 3:10b), however inhibition by the serum inhibitory fraction of this moiety was not observed.

Figure 3:10 illustrates the effect of the 10-30kDa and 30-50kDa inhibitory fractions on triglyceride breakdown products in the absence of buffer glucose during adipocyte incubations. Inhibition of radiolabel incorporation into the fatty acid moiety but not the glycerol moiety is observed during basal lipogenesis. Under conditions of insulin stimulation although some inhibition of radiolabel incorporation into the glycerol moiety is observed, the inhibition of the fatty acid moiety is more marked.

On increasing the concentration of glucose in the incubation medium to 0.25mM, glucose uptake also increased and the fraction converted to glycerol (as detected by the counts per minute) decreased. (Table 3:11).
TABLE 3:10

a) Effect of insulin and unfractionated serum inhibitor on the incorporation of [3H]-glucose into triglycerides, fatty acids and glycerol in rat adipocytes. (Incubation media contained 0.1mM unlabelled glucose.) (% stimulation ± SE; n=5)

<table>
<thead>
<tr>
<th></th>
<th>TG</th>
<th>FA</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>100 (cpm:345)</td>
<td>100 (cpm:165)</td>
<td>100 (cpm:61)</td>
</tr>
<tr>
<td>Insulin (20mU.l⁻¹)</td>
<td>246±5</td>
<td>315±7</td>
<td>90±10</td>
</tr>
<tr>
<td>Inhibitor (1.2g.l⁻¹)</td>
<td>77±3*</td>
<td>50±5*</td>
<td>148±3</td>
</tr>
<tr>
<td>Insulin (20mU.l⁻¹) +Inhibitor (1.2g.l⁻¹)</td>
<td>207±4b</td>
<td>203±6b</td>
<td>189±6</td>
</tr>
</tbody>
</table>

b) Effect of insulin and unfractionated serum inhibitor on the incorporation of [¹⁴C]-glucose into triglycerides, fatty acids and glycerol in rat adipocytes. (Incubation media contained 0.1mM unlabelled glucose.) (% stimulation ± SE; n=5)

<table>
<thead>
<tr>
<th></th>
<th>TG</th>
<th>FA</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>100 (cpm:1195)</td>
<td>100 (cpm:242)</td>
<td>100 (cpm:919)</td>
</tr>
<tr>
<td>Insulin (20mU.l⁻¹)</td>
<td>215±9</td>
<td>330±10</td>
<td>187±6</td>
</tr>
<tr>
<td>Inhibitor (1.2g.l⁻¹)</td>
<td>94±2c</td>
<td>40±31*</td>
<td>99±3</td>
</tr>
<tr>
<td>Insulin (20mU.l⁻¹) +Inhibitor (1.2g.l⁻¹)</td>
<td>177±6b</td>
<td>120±2b</td>
<td>179±7</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)

a: p<0.001 when compared to basal lipogenesis.
b: p<0.001 when compared to insulin stimulated lipogenesis.
c: p<0.02 when compared to basal lipogenesis.
Effect of the inhibitor fractions (10-30kDa and 30-50kDa) on the formation of triglycerides, fatty acids and glycerol in the absence of unlabelled glucose in rat adipocyte incubates.

1: Basal
2: Insulin (5mU.l⁻¹)
3: Insulin (10mU.l⁻¹)
4: Insulin (20mU.l⁻¹)
5: Insulin (40mU.l⁻¹)
6: Inhibitory fraction 10-30kDa; (0.035g.l⁻¹)
7: Inhibitory fraction 10-30kDa; (0.070g.l⁻¹)
8: Inhibitory fraction 10-30kDa; (0.140g.l⁻¹)
9: Insulin (10mU.l⁻¹) + Inhibitory fraction 10-30kDa; (0.035g.l⁻¹)
10: Insulin (10mU.l⁻¹) + Inhibitory fraction 10-30kDa; (0.070g.l⁻¹)
11: Insulin (10mU.l⁻¹) + Inhibitory fraction 10-30kDa; (0.140g.l⁻¹)
12: Inhibitory fraction 30-50kDa; (0.035g.l⁻¹)
13: Inhibitory fraction 30-50kDa; (0.070g.l⁻¹)
14: Inhibitory fraction 30-50kDa; (0.140g.l⁻¹)
15: Insulin (10mU.l⁻¹) + Inhibitory fraction 30-50kDa; (0.035g.l⁻¹)
16: Insulin (10mU.l⁻¹) + Inhibitory fraction 30-50kDa; (0.070g.l⁻¹)
17: Insulin (10mU.l⁻¹) + Inhibitory fraction 30-50kDa; (0.140g.l⁻¹)

N.B. SEE PAGE 125 FOR STATISTICAL COMPARISONS (students t test)
Statistical comparisons (Students t test) on data shown in Figure 3:10

Bar chart 1. (Triglycerides)
10 vs 3  p<0.05
11 vs 3  p<0.05
12 vs 1  p<0.05
13 vs 1  p<0.02
14 vs 1  p<0.005

Bar chart 2. (Fatty acids)
6 vs 1   p<0.05
8 vs 1   p<0.05
11 vs 3  p<0.05
13 vs 1  p<0.02
14 vs 1  p<0.05

Bar chart 3. (Glycerol)
10 vs 3  p<0.001
11 vs 3  p<0.001
12 vs 1  p<0.005
13 vs 1  p<0.05
14 vs 1  p<0.01
15 vs 3  p<0.001
16 vs 3  p<0.005
17 vs 3  p<0.001
TABLE 3:11
Effect of the control 10-30kDa serum inhibitor fraction on radiolabelled glucose incorporation into triglycerides, fatty acids and glycerol in the presence of 0.25mM unlabelled glucose. (% stimulation ± SE; n=5)

<table>
<thead>
<tr>
<th></th>
<th>TG</th>
<th>FA</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Insulin (mU.l⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100 (cpm:240)</td>
<td>100 (cpm:95)</td>
<td>100 (cpm:112)</td>
</tr>
<tr>
<td>5</td>
<td>253±2</td>
<td>433±2</td>
<td>330±10</td>
</tr>
<tr>
<td>10</td>
<td>296±7</td>
<td>482±3</td>
<td>343±5</td>
</tr>
<tr>
<td>20</td>
<td>275±6</td>
<td>478±6</td>
<td>347±5</td>
</tr>
<tr>
<td><strong>Fraction 10-30KDa (g.l⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.035</td>
<td>95±9</td>
<td>93±10</td>
<td>159±4</td>
</tr>
<tr>
<td>0.070</td>
<td>88±8</td>
<td>89±19</td>
<td>146±7</td>
</tr>
<tr>
<td>0.140</td>
<td>85±7</td>
<td>74±11*d</td>
<td>163±6</td>
</tr>
<tr>
<td><strong>Insulin (10mU.l⁻¹) + Fraction 10-30KDa (g.l⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.035</td>
<td>272±9</td>
<td>421±6*a</td>
<td>326±2*c</td>
</tr>
<tr>
<td>0.070</td>
<td>260±5*b</td>
<td>436±4*a</td>
<td>336±4</td>
</tr>
<tr>
<td>0.140</td>
<td>237±6*a</td>
<td>340±4*a</td>
<td>329±4</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)
a: p<.001 when compared to insulin stimulated lipogenesis.
b: p<.005 when compared to insulin stimulated lipogenesis.
c: p<.02 when compared to insulin stimulated lipogenesis.
d: p<.05 when compared to basal lipogenesis.
This effect was apparent both for basal and insulin stimulated lipogenesis.

At 0.25mM buffer glucose concentration, antagonism of radiolabel incorporation into the fatty acid moiety by the two inhibitory fractions (10-30kDa & 30-50kDa) is observed.

Similarly, a further increase in buffer glucose to 0.5mM resulted in inhibition of the incorporation of radiolabel into the fatty acid moiety. No effect on radiolabel incorporation into the glycerol moiety was observed (Table 3:12).

The inhibitory effect of the 10-30kDa and 30-50kDa fractions on $^{35}$S-sulphate uptake by porcine cartilage also demonstrated significant inhibition, the 30-50kDa fraction being markedly more inhibitory than the 10-30kDa fraction. This inhibition was evident with (Figure 3:12) and without (Figure 3:11) the addition of the standard human serum which has a stimulatory effect on this preparation due to its intrinsic somatomedin content.

A study to investigate the presence and potency of these inhibitor(s) in sera of diabetics and normal subjects was carried out. The combined data of this study are represented in Figure 3:13. In this study the insulin concentrations of the inhibitor preparations was assessed by radioimmunoassay. For each group (control, NIDDs & IDDs) the median endogenous insulin was below the lowest limit of detection of the assay. These preparations were also glucose and immunoglobulin G free.

Within the control population of twelve subjects the serum inhibitor fraction was significantly inhibitory to both basal and insulin
TABLE 3:12

Effect of the control 10-30kDa serum inhibitor fraction on radiolabelled glucose incorporation into triglycerides, fatty acids and glycerol in the presence of 0.5mM unlabelled glucose. (% stimulation ± SE; n=5)

<table>
<thead>
<tr>
<th></th>
<th>TG</th>
<th>FA</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Insulin (mU.l⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100 (cpm:353)</td>
<td>100 (cpm:165)</td>
<td>100 (cpm:191)</td>
</tr>
<tr>
<td>5</td>
<td>314±9</td>
<td>399±7</td>
<td>191±7</td>
</tr>
<tr>
<td>10</td>
<td>408±4</td>
<td>467±5</td>
<td>232±4</td>
</tr>
<tr>
<td>20</td>
<td>427±8</td>
<td>524±6</td>
<td>260±3</td>
</tr>
<tr>
<td><strong>Fraction 10-30KDa (g.l⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.035</td>
<td>104±6</td>
<td>89±8</td>
<td>90±6</td>
</tr>
<tr>
<td>0.070</td>
<td>106±17</td>
<td>95±12</td>
<td>87±11</td>
</tr>
<tr>
<td>0.140</td>
<td>105±12</td>
<td>91±3</td>
<td>96±12</td>
</tr>
<tr>
<td><strong>Insulin (10mU.l⁻¹) + Fraction 10-30KDa (g.l⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.035</td>
<td>376±6</td>
<td>492±5</td>
<td>251±2</td>
</tr>
<tr>
<td>0.070</td>
<td>372±8</td>
<td>448±6</td>
<td>258±1</td>
</tr>
<tr>
<td>0.140</td>
<td>391±4</td>
<td>409±2</td>
<td>246±1</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)
a: p<0.001 when compared to insulin stimulated lipogenesis.
b: p<0.005 when compared to insulin stimulated lipogenesis.
c: p<0.02 when compared to insulin stimulated lipogenesis.
d: p<0.05 when compared to insulin stimulated lipogenesis.
Figure 3:11

The inhibitory effect of serum fractions 10-30kDa and 30-50kDa on $[^{35}S]$-sulphate uptake by porcine cartilage.

- - - 10-30kDa fraction
□ □ □ 30-50kDa fraction

% Inhibition
% Concentration.
Inhibition of Serum Stimulated $^{35}$S Sulphate Uptake in Porcine Cartilage by Serum Fractions

- O Normal Human Serum (NHS).
- • NHS + 30,000 Mw Serum Fraction (SF).
- □ NHS + 50,000 Mw SF.
Figure 3.13

Effect of unfracionated inhibitor obtained from supernatants of ammonium sulphate precipitated control, NIDDM & IDDM sera on basal and insulin-stimulated rat adipocyte lipogenesis. (Basal =100%)

1: 10mU.l⁻¹ insulin stimulation in assays of inhibitor fractions prepared from the control group.
2: 10mU.l⁻¹ insulin stimulation in assays of inhibitor fractions prepared from the NIDDM group.
3: 10mU.l⁻¹ insulin stimulation in assays of inhibitor fractions prepared from the IDDM group.

4: Inhibitory fractions (control group) on basal lipogenesis. (p<0.01)
5: Inhibitory fractions (NIDDM group) on basal lipogenesis.
6: Inhibitory fractions (IDDM group) on basal lipogenesis.

7: Inhibitory fractions (control group) on insulin (10mU.l⁻¹)-stimulated lipogenesis. (p<0.01)
8: Inhibitory fractions (NIDDM group) on insulin (10mU.l⁻¹)-stimulated lipogenesis.
9: Inhibitory fractions (IDDM group) on insulin (10mU.l⁻¹)-stimulated lipogenesis.
stimulated lipogenesis in rat adipocytes. (p<0.01; the Wilcoxon Rank test, a non-parametric test) was used in this statistical analysis. The inhibitor caused a 53% decrease over both basal and insulin stimulated lipogenesis respectively.

The inhibitor preparations obtained from sera of non-insulin dependent diabetic patients caused a 32% increase over basal lipogenesis and a 16% decrease of insulin stimulated lipogenesis. Both these effects were not significant.

The median insulin-stimulated (10mU.l⁻¹) response for all assays in this study were comparable and not statistically different. (Columns 1, 2, & 3 in Figure 3:13). The inhibitory activity from sera of NIDD patients was significantly less than that from sera of control subjects for both basal (p<0.002) and insulin stimulated lipogenesis (p<0.002). Statistical comparisons in this study were carried out by two-tailed Mann Whitney tests (non-parametric).

If it were required to definitively eliminate the possibility that variables such as age and sex were important factors in determining inhibitory status, a very large population would have to be investigated in order to keep all variables constant whilst varying only one at a time. Furthermore, the variables which identify diabetics would also have to be standardised. Although, as will be seen in the following paragraph, numbers in this work are small there is an indication that sex and age are equally distributed and do not determine presence of serum insulin inhibitory activity whereas body weight does. This work therefore resulted in general guidelines which will help the course of future studies.
To examine whether the activity of the inhibitory preparations from NIDDs was related to age, sex or obesity we divided the patients according to their age, sex and weight. Amongst the serum fractions which were inhibitory (n=7) 4 belonged to females and 3 to males whilst in the non-inhibitory group (n=6), 3 belonged to females and 3 to males. The median weight of the group with inhibitory preparations, [75kg (61-96)] was significantly different from the group whose preparations were not markedly inhibitory: [68kg (40-77.2)]. The median age of the group with inhibitory activity in their preparations [55 years (31-70)] was not significantly different from the median age of the non-inhibitory group [50 years (44-75)]. Thus, looking at the frequency of inhibitory sera in obese/non-obese patients by the $\chi^2$ test there is significant difference, $p<0.05$, suggesting that obesity status may play a role in determining inhibitory/non-inhibitory status in NIDD subjects.

Inhibitor preparations from insulin-dependent diabetic patients caused a 7% increase over basal lipogenesis and a 8% increase of insulin-stimulated lipogenesis. Both these effects were not statistically significant.

On comparing the inhibitor preparations from normal subjects and IDDs, the latter were significantly ($p<0.002$) less inhibitory than those from the former in terms of both basal and insulin-stimulated lipogenesis. There was no significant difference between the effects of NIDD and IDD preparations on basal or insulin stimulated lipogenesis.

To examine whether the activity of the inhibitory preparations from IDDs was related to age, sex or obesity we divided the patients according to their age, sex and weight. Amongst the serum fractions which were inhibitory (n=7), 3 belonged to females and 4 to males,
whilst in the non-inhibitory group (n=7) 5 belonged to females and 2 to males. These differences between the sexes was not significant. The median weight of the group with inhibitory preparations [70kg(52-89.7)] was not significantly different from the median weight of the group whose preparations were not inhibitory [65kg(52.3-99)]. The median age of the group with inhibitory activity in their preparations [58years(26-79)] was not significantly different from the median age of the non-inhibitory group [46years(24-68)]. In this population of IDDs there is therefore no significant difference between the frequency of obesity and the inhibitory/non-inhibitory status of sera.

The inhibitor preparations from the NIDD subjects which were found to have an antagonistic effect to basal- and insulin- stimulated lipogenesis were pooled and fractionated into various molecular weight fractions described above. Of these fractions the 10-30kDa inhibited insulin stimulated lipogenesis by 36% and the 30-50KDa inhibited insulin stimulated lipogenesis by 31%. (Table 3:13a). On pooling and fractionating the inhibitor preparations which were initially non-inhibitory, no inhibitory activity was detected in any fractions (Table 3:13b).

The IDD inhibitor preparations were similarly divided and assayed. The' inhibitory pool' resulted in the 30-50kDa fraction reducing insulin stimulated lipogenesis by 71% (Table 3:14a) whilst the non-inhibitory fractions did not cause statistically significant reduction of insulin-stimulated lipogenesis. (Table 3:14b).

It is important to state here that each molecular weight fraction in each group did not have exactly the same protein concentration even
**TABLE 3:13**

a) Effect of pooled inhibitory serum fractions (0-1kDa to >50kDa) from NIDD patients on basal and insulin-stimulated rat adipocyte lipogenesis. (% stimulation ± SE; n=6)

<table>
<thead>
<tr>
<th>Inhibitor fraction (KDa)</th>
<th>0</th>
<th>0-1</th>
<th>1-10</th>
<th>10-30</th>
<th>30-50</th>
<th>&gt;50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>100</td>
<td>101±8</td>
<td>155±34</td>
<td>107±11</td>
<td>117±36</td>
<td>440±1</td>
</tr>
<tr>
<td>Insulin (10mU.1⁻¹)</td>
<td>193±21*</td>
<td>196±28</td>
<td>239±21</td>
<td>124±14*</td>
<td>134±17</td>
<td>76±19</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)

a: p<0.02 when compared to basal lipogenesis.
b: p<0.05 when compared to insulin stimulated lipogenesis.

b) Effect of pooled non-inhibitory serum fractions (0-1kDa to >50kDa) from NIDD patients on basal and insulin-stimulated rat adipocyte lipogenesis. (% stimulation ± SE; n=6)

<table>
<thead>
<tr>
<th>Inhibitor fraction (KDa)</th>
<th>0</th>
<th>0-1</th>
<th>1-10</th>
<th>10-30</th>
<th>30-50</th>
<th>&gt;50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>100</td>
<td>197±24</td>
<td>218±20</td>
<td>110±48</td>
<td>182±21</td>
<td>197±17</td>
</tr>
<tr>
<td>Insulin (10mU.1⁻¹)</td>
<td>229±41*</td>
<td>314±20</td>
<td>339±19</td>
<td>345±7</td>
<td>235±13</td>
<td>314±28</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)
a: p<0.05 when compared to basal lipogenesis.
**TABLE 3:14**

a) Effect of pooled inhibitory serum fractions (0-1kDa to >50kDa) from IDD patients on basal and insulin-stimulated rat adipocyte lipogenesis. (% stimulation ± SE; n=6)

<table>
<thead>
<tr>
<th>Inhibitor fraction (KDa)</th>
<th>0</th>
<th>0-1</th>
<th>1-10</th>
<th>10-30</th>
<th>30-50</th>
<th>&gt;50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>100</td>
<td>326±29</td>
<td>293±42</td>
<td>346±27</td>
<td>100±19</td>
<td>593±13</td>
</tr>
<tr>
<td>Insulin (10mU.1⁻¹)</td>
<td>248±48*</td>
<td>313±27</td>
<td>286±27</td>
<td>293±21</td>
<td>71±20*</td>
<td>433±10</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)

a: p<0.05 when compared to corresponding basal value.
b: p<0.005 when compared to insulin stimulated lipogenesis.

b) Effect of pooled non-inhibitory serum fractions, (0-1kDa to >50kDa) from IDD patients on basal and insulin-stimulated rat adipocyte lipogenesis. (% stimulation ± SE; n=6)

<table>
<thead>
<tr>
<th>Inhibitor fraction (KDa)</th>
<th>0</th>
<th>0-1</th>
<th>1-10</th>
<th>10-30</th>
<th>30-50</th>
<th>&gt;50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>100</td>
<td>187±52</td>
<td>196±41</td>
<td>26±22</td>
<td>20±33</td>
<td>195±13</td>
</tr>
<tr>
<td>Insulin (10mU.1⁻¹)</td>
<td>284±43*</td>
<td>343±11</td>
<td>249±41</td>
<td>164±34</td>
<td>252±54</td>
<td>562±49</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)

a: p<0.02 when compared to basal lipogenesis.

- 137 -
though fractionation was carried out in a similar fashion each time. Although inhibitory activity of each inhibitory fraction (10-30kDa or 30-50kDa) has a concentration related effect, inhibitory activity of one particular fraction cannot at this stage of purification, be compared directly to the inhibitory activity of another fraction.

The presence and potency of the inhibitor prepared by ammonium sulphate precipitation of serum from non-diabetic obese subjects as compared to normal subjects was studied. The results of a group of 11 normal subjects and 8 obese subjects are shown in Figure 3:14. These inhibitor preparations were free of IgG and insulin.

Preparations from both normal (p<0.01) and obese (p<0.05) subjects were significantly inhibitory to basal lipogenesis. Insulin stimulated lipogenesis was inhibited significantly by the control inhibitor preparations from both controls (p<0.05) and the obese (p<0.01). The Mann Whitney test comparing inter group inhibitory effect on basal and insulin-stimulated lipogenesis demonstrated no significant differences between the inhibitory activities of preparations from normal subjects and obese patients. There is no difference in incidence of inhibitory activity when comparing the obese and non-obese groups by the $\chi^2$ test. This was true of both the basal and insulin stimulated lipogenesis. Furthermore, there
Figure 3:14

Effect of serum inhibitor preparations from control (●) and obese (non-diabetic) (○) subjects on basal and insulin-stimulated rat adipocyte lipogenesis. Basal = 100%

1: The effect of 10mU.l⁻¹ insulin on basal lipogenesis.
2: The effect of 10mU.l⁻¹ insulin on basal lipogenesis.

3: The effect of inhibitor preparations from control subjects on basal lipogenesis. (p<0.01)
4: The effect of inhibitor preparations from obese subjects on basal lipogenesis. (p<0.05)

5: The effect of inhibitor preparations from control subjects on insulin (10mU.l⁻¹)-stimulated lipogenesis. (p<0.05)
6: The effect of inhibitor preparations from obese subjects on insulin (10mU.l⁻¹)-stimulated lipogenesis. (p<0.01)
was no relationship with age or sex and in incidence of inhibitory activity in the obese and non obese groups.

Sera and various molecular weight fractions from rats with streptozotocin induced diabetes did not inhibit adipocyte lipogenesis. Fractions obtained from control, diabetic and insulin-treated diabetic rats, which had been found to be inhibitory in the $[^{35}\text{S}]$-sulphate uptake assay in porcine cartilage were assayed in the rat lipogenesis assay. The 30-50kDa and >300kDa fractions (from the untreated diabetic rats) and the >300kDa fraction from control rats were markedly inhibitory to $[^{35}\text{S}]$-sulphate uptake by cartilage but were each found to be stimulatory to rat adipocyte lipogenesis (Table 3:15). The >300kDa fractions contain immunoglobulins (e.g. IgM : ≈ 900kDa, IgA : ≈ 400kDa) and 30-50kDa fractions containing IgF bound to the carrier protein can be expected to have a stimulatory effect on rat adipocyte lipogenesis. Concomitant $[^{35}\text{S}]$-sulphate uptake experiments with these fractions however resulted in inhibitory activity being detected in the <1kDa, 1-10kDa, 30-50kDa and >300kDa fractions. Inhibitory activity in the >300kDa fraction was the greatest and was exhibited by the control, diabetic and insulin-treated diabetic animals (Taylor et al., 1987). The >300kDa fraction from diabetic rats was significantly more inhibitory than that seen in controls or insulin-treated animals. Insulin treatment reduced the inhibitory activity of this fraction but the inhibitory activity was still significantly lower than that in controls. The diabetic animals alone exhibited inhibitory activity in the <1kDa, 1-10kDa and 30-50kDa fractions. The 30-50kDa fraction in control and insulin-treated animals exhibited stimulatory activity consistent with the presence of somatomedin bound to its carrier protein.
Table 3:15

Rat adipocyte lipogenesis in the presence of serum fractions (inhibitory in the somatomedin bioassay) obtained from diabetic and control rats (% stimulation ± SE; n=5) [Basal counts: 420cpm (=100%)]

A: 30-50kDa fraction from untreated diabetic rats.
B: >300kDa fraction from untreated diabetic rats.
C: >300kDa fraction from control rats.

<table>
<thead>
<tr>
<th>Test Substances</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (mU.1⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>98 ± 14</td>
<td>3304 ± 22*</td>
</tr>
<tr>
<td>2.5</td>
<td>354 ± 19</td>
<td>975 ± 17*</td>
<td>4234 ± 25*</td>
</tr>
<tr>
<td>5.0</td>
<td>563 ± 15</td>
<td>815 ± 22*</td>
<td>4472 ± 20*</td>
</tr>
<tr>
<td>10.0</td>
<td>643 ± 28</td>
<td>1169 ± 23*</td>
<td>4482 ± 31*</td>
</tr>
<tr>
<td>20.0</td>
<td>996 ± 18</td>
<td>1040 ± 17</td>
<td>4890 ± 15*</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)

p<0.001 when comparing effect of test substances A, B & C to insulin-stimulated lipogenesis.
In the rat lipogenesis assay only the <1kDa fraction was found to be inhibitory and this most probably was the result of the endogenous glucose in the rat sera (Table 3:16). However the inhibition observed in the control (glucose 13.1mmol.l⁻¹) and insulin treated diabetic rat (glucose 6.4mmol.l⁻¹) fractions was more marked than that observed in the diabetic fractions (glucose 23.4mmol.l⁻¹). The remaining fractions did not exhibit insulin inhibitory activity probably because these sera were not subjected to ammonium sulphate precipitation due to the small volumes available, thus the presence of stimulatory substances such as insulin, immunoglobulins and somatomedins would mask the presence of any insulin inhibitory activity.
Table 3.16

Effect of inhibitory fractions (0-1kDa) obtained from supernatants of ammonium sulphate precipitated rat sera (A: controls, B: diabetic, C: insulin-treated diabetic) on rat adipocyte lipogenesis (% stimulation ± SE; n=5)

[Basal counts: 240cpm (=100%)]

<table>
<thead>
<tr>
<th>Test Substance</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (mU.l⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>327 ± 26*</td>
<td>10 ± 34*</td>
</tr>
<tr>
<td>5.0</td>
<td>507 ± 16</td>
<td>387 ± 20*</td>
<td>80 ± 20*</td>
</tr>
<tr>
<td>10.0</td>
<td>667 ± 25</td>
<td>387 ± 10*</td>
<td>190 ± 28*</td>
</tr>
</tbody>
</table>

Where A = control animals
B = diabetic animals
C = insulin-treated diabetic animals

Statistical comparisons (Students t test)
a: p<0.001 when compared to basal or insulin stimulated lipogenesis.
b: p<0.005 when compared to corresponding insulin stimulated lipogenesis.
CHAPTER 4

RESULTS OF INVESTIGATION INTO THE POST-RECEPTOR MECHANISMS MEDIATING INSULIN STIMULATED LIPOGENESIS IN RAT ADIPOCYTES AND THE EFFECT OF SERUM INSULIN INHIBITORY FRACTIONS PREPARED BY AMMONIUM SULPHATE PRECIPITATION ON THESE PATHWAYS.
There has been much interest lately in the investigation of the role of protein kinase C, calcium and calmodulin as intracellular messengers in a variety of cell types. To assess the possible site/mechanism of action of the insulin inhibitors prepared by ammonium sulphate precipitation of serum it was necessary to establish the role of the second messenger system in the adipocyte. Although some workers have investigated parts of the post receptor chain of reactions the whole framework is hitherto unestablished. This chapter of results includes investigations into the relationship between calcium and protein kinase C in mediating insulin-stimulated lipogenesis by isolated rat adipocytes. The abbreviations used in this section are:

PE : phorbol ester
PdBu : 4β-phorbol 12, 13, -dibutyrate
PKA : 4β-phorbol 12-myristate 13α-acetate
CaI : calcium ionophore (free acid) A23187
H7 : 1-(5-isoquinolinyl-sulfonyl)-2-methyl-piperazine
W5 : N-(6-aminobethyl)-1-naphthalenesulfonamide
W13 : N-(4-aminobutyl)-5-chloro-2-naphthalene-sulfonamide
CMZ : calmidazolium
PB : polymyxin B
V : verapamil
DMSO : dimethyl sulfoxide

To demonstrate clearly the stimulation and inhibition of the lipogenesis assay in the bar diagrams of this chapter, it must be noted that basal lipogenesis is taken as 100%. Synergism is considered to occur when the stimulation is greater than the sum of the two separate responses above the basal value.
The effect of one of the vehicles (DMSO) used in this work was initially studied, as it was important to establish that it did not interfere with basal/insulin stimulated lipogenesis. The data in Table 4:1 demonstrates that concentrations of DMSO used in our experiments did not alter adipocyte lipogenesis. Such concentrations could therefore be used in our experiments without the possibility of artefactual results.

A concentration dependent stimulation of lipogenesis occurs in response to PMA, the maximal response being obtained at $1.62 \times 10^{-7} \text{M}$ (Figure 4:1). The stimulatory effect of PMA was significantly greater than that of PDBu (Table 4:2). We therefore used PMA as a stimulator of protein kinase C in future work with adipocytes. Calcium ionophore ($1.9 \times 10^{-6} \text{M}$) potentiated both PDBu and PMA stimulated lipogenesis. This concentration of ionophore was not stimulatory to rat adipocyte lipogenesis when applied on its own. In this assay $10 \text{mU} \cdot \text{l}^{-1}$ insulin resulted in a stimulation of $306 \pm 25\%$ over basal lipogenesis.

PMA 25 ($4.06 \times 10^{-8} \text{M}$) and $50 \text{ng} \cdot \text{ml}^{-1}$ ($8.12 \times 10^{-8} \text{M}$) caused a parallel left shift of the dose response curve of insulin on lipogenesis. However PMA did not increase the responsiveness of the adipocyte to insulin since magnitude of the maximal response did not alter. (Figure 4:2).

PMA ($100 \text{ng} \cdot \text{ml}^{-1}$, $1.62 \times 10^{-7} \text{M}$)-stimulated lipogenesis was inhibited by both the protein kinase C inhibitors H7, (Figure 4:3) and polymyxin B (Figure 4:4) in a concentration dependent manner. In addition insulin stimulated lipogenesis was also significantly inhibited ($p<0.001$) by H7 (Figure 4:5) and polymyxin B (Figure 4:5). These inhibitors did not inhibit basal lipogenesis at concentrations which caused a significant inhibition of insulin/PMA stimulated lipogenesis.
TABLE 4:1

Effect of DMSO on rat adipocyte lipogenesis. (% stimulation ±SE; n=5)

[Basal counts: 320 cpm (=100%)]

<table>
<thead>
<tr>
<th>DMSO (%)</th>
<th>0.000</th>
<th>0.001</th>
<th>0.010</th>
<th>0.100</th>
<th>1.000</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>89±17</td>
<td>88±34</td>
<td>117±37</td>
<td>118±25</td>
<td></td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)

All comparisons were not significant when compared to basal lipogenesis (i.e. 0 DMSO)
Figure 4:1
Dose response histogram showing the effect of PMA on rat adipocyte lipogenesis. (mean ± SE; n=4)

[Basal counts: 161cpm]

1 Basal = 100%
2 PMA (1.62 × 10^{-10} M)
3 PMA (1.62 × 10^{-9} M)
4 PMA (1.62 × 10^{-8} M)
5 PMA (1.62 × 10^{-7} M)
6 PMA (1.62 × 10^{-6} M)
TABLE 4.2

Comparison of the effect of the phorbol esters, PdBu and PMA, on rat adipocyte lipogenesis and the synergistic effect of the calcium ionophore A23187. (% stimulation ± SE; n=4)

[Basal counts: 159cpm (=100%)]

Concentration of A23187 (CaI) used: 1.91 x 10^{-6}M

<table>
<thead>
<tr>
<th>[Phorbol ester]</th>
<th>(M)</th>
<th>10^{-10}</th>
<th>10^{-9}</th>
<th>10^{-8}</th>
<th>10^{-7}</th>
<th>10^{-6}</th>
</tr>
</thead>
<tbody>
<tr>
<td>PdBu</td>
<td></td>
<td>58±30</td>
<td>63±21</td>
<td>83±31</td>
<td>97±16</td>
<td>99±30</td>
</tr>
<tr>
<td>PdBu + Cal</td>
<td></td>
<td>55±17</td>
<td>69±18</td>
<td>135±33</td>
<td>170±20*</td>
<td>176±20</td>
</tr>
<tr>
<td>PMA</td>
<td></td>
<td>60±30</td>
<td>177±30</td>
<td>184±33</td>
<td>279±12</td>
<td>254±9</td>
</tr>
<tr>
<td>PMA + Cal</td>
<td></td>
<td>58±15</td>
<td>268±31</td>
<td>294±24*</td>
<td>360±7*</td>
<td>315±32</td>
</tr>
</tbody>
</table>

Statistical comparisons. (Students t test)

a: p<0.05 when compared to corresponding PE stimulated lipogenesis.

b: p<0.005 when compared to corresponding PE stimulated lipogenesis.
Figure 4:2

Effect of the protein kinase C activator, PMA, on insulin-stimulated rat adipocyte lipogenesis. (mean ± SE; n=5)

[Basal counts: 249cpm]

Insulin alone

Insulin + PMA (4.06x10^-5M)

Insulin + PMA (8.12x10^-5M)
Figure 4:3

Effect of the protein kinase C inhibitor, H7, on PMA-stimulated rat adipocyte lipogenesis. (mean ± SE; n=5)

[Basal counts: 262cpm]

1 Basal =100%
2 PMA (1.62x10^{-7}M)
3 H7 (4.29x10^{-5}M)
4 H7 (8.58x10^{-5}M)
5 H7 (1.72x10^{-4}M)
6 PMA (1.62x10^{-7}M) + H7 (4.29x10^{-5}M)
7 PMA (1.62x10^{-7}M) + H7 (8.58x10^{-5}M)
8 PMA (1.62x10^{-7}M) + H7 (1.72x10^{-4}M)

Statistical comparisons (Students t test)
2 vs 1 p<0.001
5 vs 1 p<0.005
6,7 & 8 vs 2 p<0.001
Figure 4:4
Effect of the protein kinase C inhibitor, polymyxin B (PB), on PMA-stimulated rat adipocyte lipogenesis. (mean ± SE; n=6)

[Basal counts: 258cpm]

1 Basal =100%
2 PMA (1.62×10⁻⁷M)
3 PB (7.5mg.l⁻¹)
4 PB (15mg.l⁻¹)
5 PB (30mg.l⁻¹)
6 PMA (1.62×10⁻⁷M) + PB (7.5mg.l⁻¹)
7 PMA (1.62×10⁻⁷M) + PB (15mg.l⁻¹)
8 PMA (1.62×10⁻⁷M) + PB (30mg.l⁻¹)

Statistical comparisons (Students t test)
2 & 5 vs 1 p<0.001
6,7 & 8 vs 2 p<0.001
Figure 4:5

Effect of the protein kinase C inhibitors, H7 and polymyxin B (PB), on insulin-stimulated rat adipocyte lipogenesis. (mean ± SE; n=5)

[Basal counts: 325 cpm]

1 Basal = 100%
2 Insulin (5 mU.l⁻¹)
3 Insulin (10 mU.l⁻¹)
4 H7 (4.29 x 10⁻⁶ M)
5 H7 (4.29 x 10⁻⁶ M) + insulin (5 mU.l⁻¹)
6 H7 (4.29 x 10⁻⁶ M) + insulin (10 mU.l⁻¹)
7 PB (15 mg.l⁻¹)
8 PB (15 mg.l⁻¹) + insulin (5 mU.l⁻¹)
9 PB (15 mg.l⁻¹) + insulin (10 mU.l⁻¹)

Statistical comparisons (Students t test)
2, 3 & 7 vs 1 p<0.001
5 & 8 vs 2 p<0.001
6 & 9 vs 3 p<0.001
Lipogenesis: % stimulation / inhibition
As can be seen from Table 4:1 A23187 potentiates PMA and PdBu stimulated lipogenesis. Diagramatically, Figure 4:6 shows A23187 1.34×10⁻⁶M synerizes the stimulatory effect of PMA on lipogenesis. H7 inhibited the stimulation caused by A23187 alone and A23187+PMA (Figure 4:7) suggesting the absolute requirement for protein kinase C in PMA stimulated rat adipocyte lipogenesis. A23187 did not significantly stimulate basal lipogenesis consistently (Figure 4:8) but significantly synergized insulin stimulated lipogenesis in a fashion similar to that observed with PMA. The protein kinase C inhibitor, H7, inhibited A23187+insulin stimulated lipogenesis, again demonstrating the direct and absolute requirement for protein kinase C (Figure 4:9) in the stimulation of lipogenesis by insulin and PMA.

The calcium channel blocker verapamil (8.13×10⁻⁶M) inhibited basal (p<0.02) and PMA stimulated lipogenesis significantly (Figure 4:10). Insulin stimulated lipogenesis was similarly significantly inhibited (Figure 4:11) by verapamil. These data, in association with the synergistic effect of A23187 indicate that both PMA and insulin stimulated lipogenesis involve the influx of extracellular calcium.

The calmodulin inhibitors V5 (2.92×10⁻⁶M) and V13 (2.86×10⁻⁶M) both inhibited insulin stimulated lipogenesis, V13 being the slightly more potent inhibitor. (Figure 4:12).

CMZ, a more specific calmodulin inhibitor, also antagonized basal and insulin stimulated lipogenesis (Table 4:3) in a dose dependent manner. The 50% inhibitory concentration lies between 10⁻⁶M and 10⁻⁷M CMZ. At 10⁻⁶M CMZ complete antagonism of lipogenesis occurs. This calmodulin inhibitor also antagonized calcium ionophore + insulin stimulated lipogenesis (Figure 4:13) in a dose dependent manner.
Figure 4.6

Effect of the calcium ionophore A23187 on PMA-stimulated rat adipocyte lipogenesis. (mean ± SE; n=4)

[Basal counts: 393 cpm]
Figure 4:7

Effect of the protein kinase C inhibitor H7 on PMA- and calcium ionophore A23187-stimulated rat adipocyte lipogenesis. (mean ± SE; n=4)

[Basal counts: 384cpm (=100%)]

1 Basal
2 PMA (1.62x10^{-7} M)
3 Calcium ionophore A23187 (10^{-6} M)
4 Calcium ionophore A23187 (10^{-7} M)
5 H7 (8.58x10^{-6} M)
6 PMA (1.62x10^{-7} M) + A23187 (10^{-6} M)
7 PMA (1.62x10^{-7} M) + A23187 (10^{-7} M)
8 PMA (1.62x10^{-7} M) + H7 (8.58x10^{-6} M)
9 A23187 (10^{-6} M) + H7 (8.58x10^{-6} M)
10 A23187 (10^{-7} M) + H7 (8.58x10^{-6} M)
11 PMA (1.62x10^{-7} M) + A23187 (10^{-6} M) + H7 (8.58x10^{-6} M)
12 PMA (1.62x10^{-7} M) + A23187 (10^{-7} M) + H7 (8.58x10^{-6} M)

Statistical comparisons (Students t test)

2 vs 1 p<0.05
3 vs 1 p<0.01
4 vs 1 p<0.001
6 & 7 vs 2 p<0.001
8 vs 2 p<0.05
9 vs 3 p<0.005
10 vs 4 p<0.005
11 vs 6 p<0.001
12 vs 7 p<0.001
Lipogenesis: % stimulation / inhibition

Bar chart showing the percentage of lipogenesis stimulation or inhibition across different conditions. The y-axis represents the percentage (%) ranging from 0 to 300, and the x-axis represents different conditions labeled from 1 to 12.
Figure 4.8
Effect of calcium ionophore A23187, on insulin-stimulated rat adipocyte lipogenesis. (mean ± SE; n=5)
[Basal counts: 358cpm (=100%)]

1 Basal
2 Insulin (5mU.l⁻¹)
3 A23187 (1.91x10⁻⁸M)
4 A23187 (1.91x10⁻⁴M)
5 Insulin (5mU.l⁻¹) + A23187 (1.91x10⁻⁸M)
6 Insulin (5mU.l⁻¹) + A23187 (1.91x10⁻⁴M)

Statistical comparisons (Students t test)
2 vs 1 p<0.02
5 vs 2 p<0.05
6 vs 2 p<0.01
Figure 4:9

Effect of the protein kinase C inhibitor, H7, on insulin- and calcium ionophore A23187-stimulated rat adipocyte lipogenesis. (mean ± SE; n=4)

(Basal counts: 199cpm (=100%))

1 Basal
2 Insulin (10mU.l⁻¹)
3 Calcium ionophore A23187 (10⁻⁶M)
4 Calcium ionophore A23187 (10⁻⁷M)
5 H7 (8.58x10⁻⁶M)
6 Insulin (10mU.l⁻¹) + A23187 (10⁻⁶M)
7 Insulin (10mU.l⁻¹) + A23187 (10⁻⁷M)
8 Insulin (10mU.l⁻¹) + H7 (8.58x10⁻⁶M)
9 A23187 (10⁻⁶M) + H7 (8.58x10⁻⁶M)
10 A23187 (10⁻⁷M) + H7 (8.58x10⁻⁶M)
11 Insulin (10mU.l⁻¹) + A23187 (10⁻⁶M) + H7 (8.58x10⁻⁶M)
12 Insulin (10mU.l⁻¹) + A23187 (10⁻⁷M) + H7 (8.58x10⁻⁶M)

Statistical comparisons (Students t test)

2 & 4 vs 1 p<0.001
6, 7 & 8 vs 2 p<0.001
9 vs 3 p<0.001
10 vs 4 p<0.001
11 vs 6 p<0.001
12 vs 7 p<0.001
Lipogenesis: % stimulation / inhibition
Figure 4:10

Effect of the calcium channel blocker, verapamil, on PMA-stimulated rat adipocyte lipogenesis. (mean ± SE; n=4)

[Basal counts: 249cpm (=100%)]

1 Basal
2 PMA (1.62x10^-3M)
3 PMA (1.62x10^-5M)
4 PMA (1.62x10^-7M)
5 Verapamil (8.13x10^-6M)
6 PMA (1.62x10^-3M) + Verapamil (8.13x10^-6M)
7 PMA (1.62x10^-5M) + Verapamil (8.13x10^-6M)
8 PMA (1.62x10^-7M) + Verapamil (8.13x10^-6M)

Statistical comparisons (Students t test)
2 vs 1 p<0.05
3 vs 1 p<0.02
4 vs 1 p<0.001
5 vs 1 p<0.005
6 vs 2 p<0.001
7 vs 3 p<0.005
8 vs 4 p<0.001
Figure 4:11

Effect of the calcium channel blocker, verapamil, on insulin-stimulated rat adipocyte lipogenesis. (mean ± SE; n=4)

[Basal counts: 389cpm (=100%)]

1 Basal
2 Insulin (10mU.l⁻¹)
3 Insulin (20mU.l⁻¹)
4 Verapamil (8.13×10⁻⁶M)
5 Verapamil (8.13×10⁻⁶M) + insulin (10mU.l⁻¹)
6 Verapamil (8.13×10⁻⁶M) + insulin (20mU.l⁻¹)

Statistical comparisons (Students t test)

2 vs 1  p<0.05
3 vs 1  p<0.001
4 vs 1  p<0.001
5 vs 2  p<0.005
6 vs 3  p<0.001
Figure 4.12
Effect of the calmodulin inhibitors V5 and V13 on insulin-stimulated rat adipocyte lipogenesis. (mean ± SE; n=5)

(Basal counts: 214cpm)

- Insulin alone
- Insulin + V5 (2.92x10^{-8}M)
- Insulin + V13 (2.86x10^{-8}M)
**TABLE 4:3**

Effect of the calmodulin inhibitor, calmidazolium (CMZ), on basal and insulin-stimulated rat adipocyte lipogenesis. (% stimulation ± SE; n=5)

[Basal counts: 256cpm (=100%)]

<table>
<thead>
<tr>
<th>Insulin (mU.l⁻¹)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMZ (M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>290±12</td>
<td>346±12</td>
<td>439±25</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>98±15</td>
<td>256±50</td>
<td>344±39</td>
<td>311±12b</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>84±17</td>
<td>186±54</td>
<td>267±17c</td>
<td>228±10a</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>4±3a</td>
<td>7±23a</td>
<td>50±12a</td>
<td>0±2a</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>26±14a</td>
<td>0±11a</td>
<td>40±19a</td>
<td>8±6a</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)

a: *p*<0.001 when compared to corresponding basal insulin stimulated lipogenesis.

b: *p*<0.005 when compared to corresponding basal insulin stimulated lipogenesis.

c: *p*<0.01 when compared to corresponding basal insulin stimulated lipogenesis.
Effect of the calmodulin inhibitor, calmidazolium (CMZ), on insulin- and A23187-stimulated rat adipocyte lipogenesis. (mean ± SE; n=5)

[Basal counts: 450cpm (=100%)]

1 Basal
2 Insulin (5mU.1⁻¹)
3 Calcium ionophore A23187 (9.55×10⁻⁶M)
4 Insulin (5mU.1⁻¹) + A23187 (9.55×10⁻⁶M)
5 CMZ (10⁻⁶M)
6 CMZ (10⁻⁸M)
7 Insulin (5mU.1⁻¹) + A23187 (9.55×10⁻⁶M) + CMZ (10⁻⁶M)
8 Insulin (5mU.1⁻¹) + A23187 (9.55×10⁻⁶M) + CMZ (10⁻⁸M)

Statistical comparisons (Students t test)
2 vs 1  p<0.001
3 vs 1  p<0.02
4 vs 2  p<0.025
6 vs 1  p<0.01
7 vs 4  p<0.005
8 vs 4  p<0.001
Similarly PMA and PMA+A23187 stimulated lipogenesis was abolished by CMZ (Figure 4:14).

Although the calcium ionophore A23187 potentiated the effect of PMA and insulin, it did not have this effect on another known stimulator of lipogenesis: IgG. (Table 4:4). The effects of the protein kinase C inhibitors H7 and polymyxin B, the calcium channel blocker, verapamil and the calmodulin inhibitor, calmidazolium on IgG stimulated lipogenesis were on the other hand similar to their effects on insulin-stimulated lipogenesis. These antagonists were more potent in their effect on IgG stimulated lipogenesis than on insulin-stimulated lipogenesis. A comparison of the effects of these probes on insulin and IgG stimulated lipogenesis is shown in Table 4:4.

Another compound often used in the study of second messenger systems is sodium fluoride. Sodium fluoride activates G proteins forming a complex with aluminium which mimics the phosphate group of GTP (an endogenous G protein intracellular activator). Increasing concentrations of NaF had an inhibitory effect on basal and insulin stimulated lipogenesis (Table 4:5).

This outline of events in the adipocyte was then utilized to assess the possible site/mechanism of action of the inhibitory fractions of insulin prepared by ammonium sulphate precipitation of serum. The inhibitory activity of the two fractions (10-30kDa and 30-50kDa) on PMA stimulated lipogenesis was assayed. (Table 4:6). The 0-10kDa fraction had no effect on PMA stimulated lipogenesis whilst the >50kDa fraction was mildly but not significantly synergistic to PMA stimulated lipogenesis. (Table 4:6)

The 10-30kDa fraction inhibited both A23187+insulin stimulated lipogenesis (Figure 4:15) and PMA+A23187 stimulated lipogenesis (Figure 4:16). Where a
Figure 4:14

Effect of the calmodulin inhibitor, calmidazolium (CMZ), on PMA- and calcium ionophore A23187-stimulated rat adipocyte lipogenesis. (mean ± SE; n=5)

[Basal counts: 384cpm (=100%)]

1 Basal
2 Insulin (10mU.1⁻¹)
3 Calcium ionophore A23187 (10⁻⁷M)
4 PMA (1.62x10⁻⁸M)
5 PMA (1.62x10⁻⁷M)
6 PMA (1.62x10⁻⁸M) + A23187 (10⁻⁷M)
7 PMA (1.62x10⁻⁷M) + A23187 (10⁻⁷M)
8 CMZ (10⁻⁶M)
9 PMA (1.62x10⁻⁸M) + CMZ (10⁻⁶M)
10 PMA (1.62x10⁻⁷M) + CMZ (10⁻⁶M)
11 PMA (1.62x10⁻⁸M) + A23187 (10⁻⁷M) + CMZ (10⁻⁶M)
12 PMA (1.62x10⁻⁷M) + A23187 (10⁻⁷M) + CMZ (10⁻⁶M)

Statistical comparisons (Students t test)

4 vs 1 p<0.005 
2 & 5 vs 1 p<0.001 
6 vs 4 p<0.02 
7 vs 5 p<0.05 
9 vs 4 p<0.001 
10 vs 5 p<0.001 
11 vs 6 p<0.005 
12 vs 7 p<0.005
Lipogenesis: % stimulation / inhibition

Lipogenesis.

- 176 -
TABLE 4:4

Comparison of the effect of the protein kinase C inhibitors H7, Polymixin B, the voltage dependent calcium channel blocker, Verapamil, the calmodulin inhibitor, Calmidozolium (CMZ) & the calcium ionophore, A23187 on insulin- and IgG-stimulated rat adipocyte lipogenesis. (% stimulation ± SE; n=5)

[Basal counts: 145cpm (=100%)]

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>H7</th>
<th>Polymixin B</th>
<th>Verapamil</th>
<th>CMZ</th>
<th>A23187</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>100</td>
<td>62±12</td>
<td>94±26</td>
<td>30±9</td>
<td>20±18</td>
<td>76±24</td>
</tr>
<tr>
<td>Insulin</td>
<td>284±10</td>
<td>154±15*</td>
<td>118±31*</td>
<td>15±32*</td>
<td>0±4*</td>
<td>340±16b</td>
</tr>
<tr>
<td>(10mU.l⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>180±15</td>
<td>56±20*</td>
<td>44±14*</td>
<td>0±16*</td>
<td>0±3*</td>
<td>93±14c</td>
</tr>
<tr>
<td>(300mg.l⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)

a: p<0.001 when compared to corresponding insulin/IgG stimulated lipogenesis.
b: p<0.02 when compared to corresponding insulin stimulated lipogenesis.
c: p<0.005 when compared to corresponding IgG stimulated lipogenesis.
### TABLE 4:5

Effect of sodium fluoride (NaF) on basal and insulin-stimulated rat adipocyte lipogenesis. (% stimulation ± SE; n=5)

[Basal counts: 200cpm (=100%)]

<table>
<thead>
<tr>
<th>NaF (mM)</th>
<th>0.000</th>
<th>0.625</th>
<th>1.250</th>
<th>2.500</th>
<th>5.000</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basal</strong></td>
<td>100</td>
<td>99±22</td>
<td>112±12</td>
<td>63±24*</td>
<td>35±14*</td>
</tr>
<tr>
<td>**Insulin (10mU.1⁻¹)</td>
<td>192±13</td>
<td>7±3b</td>
<td>2±11b</td>
<td>0±12b</td>
<td>0±28b</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)

a: p<0.001 when compared to basal lipogenesis.

b: p<0.001 when compared to insulin stimulated lipogenesis.
TABLE 4:6

Effect of inhibitory fractions (0-1kDa to >50kDa) obtained from supernatants of ammonium sulphate precipitated control sera on PMA-stimulated rat adipocyte lipogenesis. (% stimulation ± SE; n=5)

[Basal counts: 170cpm (=100%)]

<table>
<thead>
<tr>
<th>Inhibitor fractions</th>
<th>Control</th>
<th>0-10KDa</th>
<th>10-30KDa</th>
<th>30-50KDa</th>
<th>&gt;50KDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA (M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100±22</td>
<td>119±25</td>
<td>109±23</td>
<td>98±15</td>
<td>110±10</td>
</tr>
<tr>
<td>1.62x10^{-5}</td>
<td>202±24*</td>
<td>203±27</td>
<td>140±12c</td>
<td>150±13*</td>
<td>193±17</td>
</tr>
<tr>
<td>1.62x10^{-7}</td>
<td>263±12c</td>
<td>253±18</td>
<td>143±29c</td>
<td>113±9*</td>
<td>281±13</td>
</tr>
<tr>
<td>1.62x10^{-8}</td>
<td>350±27c</td>
<td>357±27</td>
<td>172±11c</td>
<td>215±6c</td>
<td>373±37</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)

a: p<0.001 when compared to corresponding PMA-stimulated lipogenesis.
b: p<0.005 when compared to corresponding PMA-stimulated lipogenesis.
c: p<0.01 when compared to corresponding PMA-stimulated lipogenesis.
d: p<0.05 when compared to corresponding PMA-stimulated lipogenesis.
e: p<0.1 when compared to corresponding PMA-stimulated lipogenesis.
f: p<0.02 when compared to basal lipogenesis.
g: p<0.001 when compared to basal lipogenesis.
**Figure 4:15**

Effect of inhibitor fraction (10-30kDa) obtained from supernatant of ammonium sulphate precipitated control serum on insulin- and calcium ionophore A23187-stimulated rat adipocyte lipogenesis. (mean ± SE; n=5)

[Basal counts: 273cpm (=100%)]

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Basal</td>
</tr>
<tr>
<td>2</td>
<td>Insulin (5mU.l⁻¹)</td>
</tr>
<tr>
<td>3</td>
<td>Insulin (10mU.l⁻¹)</td>
</tr>
<tr>
<td>4</td>
<td>Calcium ionophore A23187 (10⁻⁷M)</td>
</tr>
<tr>
<td>5</td>
<td>Inhibitory fraction (10-30kDa)</td>
</tr>
<tr>
<td>6</td>
<td>Insulin (5mU.l⁻¹) + A23187 (10⁻⁷M)</td>
</tr>
<tr>
<td>7</td>
<td>Insulin (10mU.l⁻¹) + A23187 (10⁻⁷M)</td>
</tr>
<tr>
<td>8</td>
<td>Insulin (5mU.l⁻¹) + 10-30kDa (0.13g.l⁻¹)</td>
</tr>
<tr>
<td>9</td>
<td>Insulin (10mU.l⁻¹) + 10-30kDa (0.13g.l⁻¹)</td>
</tr>
<tr>
<td>10</td>
<td>A23187 (10⁻⁷M) + 10-30kDa (0.13g.l)</td>
</tr>
<tr>
<td>11</td>
<td>Insulin (5mU.l⁻¹) + A23187 (10⁻⁷M) + 10-30kDa (0.13g.l⁻¹)</td>
</tr>
<tr>
<td>12</td>
<td>Insulin (10mU.l⁻¹) + A23187 (10⁻⁷M) + 10-30kDa (0.13g.l⁻¹)</td>
</tr>
</tbody>
</table>

**Statistical comparisons**  
(Student t test)

<table>
<thead>
<tr>
<th>Comparison</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 &amp; 3 vs 1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>6 vs 2</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>9 vs 3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>11 vs 6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>12 vs 7</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Figure 4:16

Effect of inhibitor fraction (10-30kDa) obtained from supernatant of ammonium sulphate precipitated control serum on PMA- and calcium ionophore A23187-stimulated rat adipocyte lipogenesis. (mean ± SE; n=5)

[Basal counts: 396cpm (=100%)]

1 Basal
2 PMA (1.62×10⁻⁶M)
3 PMA (1.62×10⁻⁷M)
4 Calcium ionophore A23187 (10⁻⁷M)
5 Inhibitory fraction (10-30kDa) (0.13g.l⁻¹)
6 PMA (1.62×10⁻⁶M) + A23187 (10⁻⁷M)
7 PMA (1.62×10⁻⁷M) + A23187 (10⁻⁷M)
8 PMA (1.62×10⁻⁶M) + 10-30kDa (0.13g.l⁻¹)
9 PMA (1.62×10⁻⁷M) + 10-30kDa (0.13g.l⁻¹)
10 A23187 (10⁻⁷M) + 10-30kDa (0.13g.l⁻¹)
11 PMA (1.62×10⁻⁶M) + A23187 (10⁻⁷M) + 10-30kDa (0.13g.l⁻¹)
12 PMA (1.62×10⁻⁷M) + A23187 (10⁻⁷M) + 10-30kDa (0.13g.l⁻¹)

Statistical comparisons (Students t test)

2 & 3 vs 1 p<0.05
4 & 5 vs 1 p<0.005
6 vs 2 p<0.005
7 vs 3 p<0.01
8 vs 2 p<0.001
9 vs 3 p<0.001
10 vs 4 p<0.01
11 vs 6 p<0.001
12 vs 7 p<0.05
Lipogenesis: % stimulation / inhibition

- 183 -
stimulation over basal lipogenesis was obtained with A23187 alone (Figure 4:16), the 10-30kda fraction again significantly \((p<0.01)\) inhibited this action.

The 30-50kDa fraction similarly inhibited A23187+insulin (Figure 4:17) and A23187+PMA (Figure 4:18) stimulated lipogenesis.

To assess further the site of action of the serum inhibitory fractions, their effect on the monosaccharide transport system of the human erythrocyte was studied. The 0-1kDa, 10-30kDa and 30-50kDa fractions did not affect cytochalasin B binding to erythrocyte membranes (Table 4:7). The B/F ratios for cytochalasin B in the presence of the inhibitory fractions were similar to those achieved after incubation with buffers. The unfractionated 'crude' ammonium sulphate inhibitor however showed high cytochalasin B binding activity, as did the >50kDa fraction. This possibly represents binding to human albumin. Albumin has hydrophobic sites for the binding of fatty acids, and cytochalasin B is a small hydrophobic molecule.
Figure 4:17

Effect of inhibitor fraction (30-50kDa) obtained from supernatant of ammonium sulphate precipitated control serum on insulin-and calcium ionophore A23187-stimulated rat adipocyte lipogenesis. (mean ± SE; n=5)

[Basal counts: 265cpm (=100%)]

1 Basal
2 Insulin (5mU.l⁻¹)
3 Insulin (10mU.l⁻¹)
4 Calcium ionophore A23187 (10⁻⁷Μ)
5 Inhibitory fraction 30-50kDa (0.13g.l⁻¹)
6 Insulin (5mU.l⁻¹) + A23187 (10⁻⁷Μ)
7 Insulin (10mU.l⁻¹) + A23187 (10⁻⁷Μ)
8 Insulin (5mU.l⁻¹) + 30-50kDa (0.13g.l⁻¹)
9 Insulin (10mU.l⁻¹) + 30-50kDa (0.13g.l⁻¹)
10 A23187 (10⁻⁷) + 30-50kDa (0.13g.l⁻¹)
11 Insulin (5mU.l⁻¹) + A23187 (10⁻⁷Μ) + 30-50kDa (0.13g.l⁻¹)
12 Insulin (10mU.l⁻¹) + A23187 (10⁻⁷Μ) + 30-50kDa (0.13g.l⁻¹)

Statistical comparisons (Students t test)

2 & 3 vs 1 p<0.005
6 vs 2 p<0.05
7 vs 3 p<0.05
9 vs 3 p<0.01
11 vs 6 p<0.01
12 vs 7 p<0.01
Lipogenesis: % stimulation / inhibition

![Bar chart showing lipogenesis results](chart.png)
Figure 4:18

Effect of inhibitor fraction (30-50kDa) obtained from supernatant of ammonium sulphate precipitated control serum on PMA- and calcium ionophore A23187-stimulated rat adipocyte lipogenesis. (mean ± SE; n=5)

[Basal counts: 249cpm (=100%)]

1 Basal
2 PMA (1.62×10^{-6}M)
3 PMA (1.62×10^{-7}M)
4 Calcium ionophore A23187 (10^{-7}M)
5 Inhibitory fraction 30-50kDa (0.13g.l^{-1})
6 PMA (1.62×10^{-6}M) + A23187 (10^{-7}M)
7 PMA (1.62×10^{-7}M) + A23187 (10^{-7}M)
8 PMA (1.62×10^{-6}M) + 30-50kDa (0.13g.l^{-1})
9 PMA (1.62×10^{-7}M) + 30-50kDa (0.13g.l^{-1})
10 A23187 (10^{-7}M) + 30-50kDa (0.13g.l^{-1})
11 PMA (1.62×10^{-6}M) + A23187 (10^{-7}M) + 30-50kDa (0.13g.l^{-1})
12 PMA (1.62×10^{-7}M) + A23187 (10^{-7}M) + 30-50kDa (0.13g.l^{-1})

Statistical comparisons (Students t test)

<table>
<thead>
<tr>
<th></th>
<th>Diff</th>
<th>Test 1</th>
<th>Test 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3</td>
<td>p&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>p&lt;0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>p&lt;0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>p&lt;0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>p&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>p&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>p&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>
Lipogenesis: % stimulation / inhibition

1 2 3 4 5 6 7 8 9 10 11 12
Table 4:7

Effect of unfractionated and fractionated supernatants obtained from ammonium sulphate precipitated control sera on cytochalasin B binding to erythrocyte membranes. (n=4)

<table>
<thead>
<tr>
<th>Test fraction</th>
<th>In the presence of erythrocyte membranes</th>
<th>In the absence of erythrocyte membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated inhibitory preparation</td>
<td>9.14</td>
<td>5.16</td>
</tr>
<tr>
<td>0-1kDa</td>
<td>4.07</td>
<td>0.02</td>
</tr>
<tr>
<td>10-30kDa</td>
<td>4.29</td>
<td>0.03</td>
</tr>
<tr>
<td>30-50kDa</td>
<td>4.56</td>
<td>0.00</td>
</tr>
<tr>
<td>&gt;50kDa</td>
<td>16.80</td>
<td>12.07</td>
</tr>
<tr>
<td>Buffer control</td>
<td>4.17</td>
<td>-</td>
</tr>
</tbody>
</table>

Results are corrected for binding in the presence of 4mM D-glucose.
CHAPTER 5

RESULTS OF INSULIN INHIBITORY ACTIVITY FOUND IN SUPERNATANTS

OF ACID-ETHANOL PRECIPITATED SERUM
The preparation procedure of the serum insulin inhibitory activity in acid-ethanol extracts requires the neutralization of the acid with 2M sodium hydroxide. This resultant precipitate if separated from the supernatant and redissolved in double distilled water results in an inactive salt in the lipogenesis bioassay for insulin (Table 5:1). It is also inactive in the bioassay for somatomedin which is based on [³⁵S]-sulphate uptake by porcine cartilage for both the basal (Figure 5:1) and serum somatomedin (Figure 5:2) stimulated assays. We therefore discarded this precipitate for future preparations of insulin inhibitory activity.

The acid-ethanol extract containing the serum insulin inhibitory activity also contains both glucose and insulin. There was a highly significant correlation (p<0.002) between the glucose concentrations of the acid-ethanol extracts and the initial glucose concentrations in serum from which these extracts were prepared. Thus high serum glucose results in high concentrations of glucose in the acid-ethanol preparation. (Figure 5:3)

The effect of varying glucose concentrations in the lipogenesis incubation mixtures was assessed. Significant inhibition of lipogenesis (p<.005) was noted with increasing glucose concentrations (Table 5:2).

Acid-ethanol preparations prepared from six subjects were assayed for their glucose and insulin content. The highest glucose content in one of these preparations was 4.2mmol.l⁻¹. The remaining five preparations were spiked with varying negligible volumes of 10g.l⁻¹.
### TABLE 5:1

Effect of redissolved acid-ethanol precipitate from control sera on rat adipocyte lipogenesis. (% stimulation ± SE; n=5)

[Basal counts: 275 cpm (=100%)]

<table>
<thead>
<tr>
<th>Insulin (mU.l⁻¹)</th>
<th>Test 1</th>
<th>Test 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>100</td>
<td>89±5</td>
</tr>
<tr>
<td>2.5</td>
<td>198±13</td>
<td>205±11</td>
</tr>
<tr>
<td>5.0</td>
<td>400±11</td>
<td>384±12</td>
</tr>
<tr>
<td>10.0</td>
<td>570±13</td>
<td>511±19</td>
</tr>
<tr>
<td>20.0</td>
<td>616±17</td>
<td>569±10</td>
</tr>
</tbody>
</table>

Test 1: dose response to insulin.

Test 2: redissolved precipitate obtained on neutralization of acid-ethanol extract.

**Statistical comparisons** (Students t test)

All comparisons were non-significant.
Figure 5:1

Effect of dialysed, gel filtrated supernatants obtained from acid-ethanol precipitated serum on serum somatomedin stimulated $[^{35}S]$-sulphate uptake by porcine cartilage.

--- Basal

--- Acid-ethanol extract after removal of precipitate formed on neutralisation to pH 7.4 with 2M NaOH. (Non-dialysed).

--- Dialysed acid-ethanol extract.

--- Gel filtrated acid-ethanol extract.

--- Redissolved precipitate formed on neutralisation of acid-ethanol extract with 2M NaOH.
Figure 5:2

Effect of dialysed and non-dialysed supernatants obtained from acid-ethanol precipitated sera on basal $[^{35}S]_s$-sulphate uptake in porcine cartilage.

- Basal
- Acid-ethanol extract containing precipitate formed on neutralisation to pH 7.4 with 2M NaOH.
- Acid-ethanol extract after removal of precipitate formed on neutralisation to pH 7.4 with 2M NaOH. (Non-dialysed).
- Dialysed acid-ethanol extract.
Correlation between the concentration of glucose in supernatants of acid-ethanol precipitated sera and the corresponding concentration in serum samples.

\[ y = 0.0933 + 0.7328x \]

\[ R = 0.99 \]

\[ p < 0.001 \]
### TABLE 5:2

Effect of increasing concentrations of exogenous unlabelled glucose on basal and insulin-stimulated rat adipocyte lipogenesis. (% stimulation ± SE; n= 4)

[Basal counts: 267cpm (=100%)]

<table>
<thead>
<tr>
<th>Final concentrations of glucose in incubations (mmol.l⁻¹)</th>
<th>0.45</th>
<th>0.55</th>
<th>1.45</th>
<th>2.45</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (mU.l⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>135±5</td>
<td>95±5*</td>
<td>45±1*</td>
<td>43±3*</td>
</tr>
<tr>
<td>2.5</td>
<td>252±19</td>
<td>112±1*</td>
<td>54±9*</td>
<td>48±13*</td>
</tr>
<tr>
<td>5.0</td>
<td>342±15</td>
<td>197±29*</td>
<td>97±14*</td>
<td>44±23*</td>
</tr>
<tr>
<td>10.0</td>
<td>237±9</td>
<td>187±9*</td>
<td>56±18*</td>
<td>34±9*</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)

a: p<0.005 when compared with corresponding insulin-stimulated lipogenesis in the presence of glucose concentrations of 0.45 or 0.55 mmol.l⁻¹.
glucose so that they all contained 4.2mmol.l⁻¹ glucose. The effect of these preparations on basal and insulin stimulated lipogenesis is shown in Table 5:3. Some preparations containing high insulin concentrations still expressed significant insulin inhibitory activity, whilst others with lower insulin levels did not.

Acid-ethanol extracts prepared from normal subjects inhibited both basal and insulin stimulated lipogenesis in rat adipocytes (Tables 5:4, 5:5 & 5:6). They also inhibited basal (Figure 5:1) and serum somatomedin stimulated uptake of [³⁵S]-sulphate uptake (Figure 5:2) by porcine cartilage.

Glucose was removed from the acid-ethanol extract by gel filtration on a PD10 column (Table 5:4 & 5:6) and by dialysis against double distilled water (Table 5:5 & 5:6). In both cases insulin inhibitory activity was significantly reduced. A comparison of the effect on inhibitor activity of the different methods of glucose removal did not reveal significant differences; a slight but not significantly greater loss in inhibitory activity was noticed when glucose was removed by dialysis. (Table 5:5 ). A similar effect is shown by the somatomedin assay (Figures 5:1 & 5:2). The acid-ethanol extract is seen to be inhibitory to normal human serum stimulated [³⁵S]-sulphate uptake in porcine cartilage. This inhibitory effect is lost after the process of dialysis or gel filtration on a PD10 column.

Enzymatic removal of glucose by the use of glucose oxidase attached to polyacrylamide was not possssible because when this procedure was carried out on control samples (Krebs Ringer Bicarbonate buffer),
TABLE 5:3

Effect of supernatants from acid-ethanol precipitated control sera in which glucose concentrations had been equalized, on rat adipocyte lipogenesis. (% stimulation ± SE; n=4)

[Basal counts: 157cpm (=100%)]

<table>
<thead>
<tr>
<th>Insulin (mU.l⁻¹)</th>
<th>0</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>100</td>
<td>198±16</td>
</tr>
<tr>
<td>1 (7.4)</td>
<td>183±17a</td>
<td>138±25</td>
</tr>
<tr>
<td>2 (7.8)</td>
<td>125±24</td>
<td>195±29</td>
</tr>
<tr>
<td>3 (15.9)</td>
<td>176±36</td>
<td>216±26</td>
</tr>
<tr>
<td>4 (19.0)</td>
<td>145±21</td>
<td>126±13c</td>
</tr>
<tr>
<td>5 (21.5)</td>
<td>165±30</td>
<td>122±25d</td>
</tr>
<tr>
<td>6 (25.0)</td>
<td>174±11b</td>
<td>432±33e</td>
</tr>
</tbody>
</table>

Bracketed numbers: insulin content (mU.l⁻¹) of acid-ethanol preparation.

Statistical comparisons (Students t test)
a: p<0.02 when compared to basal lipogenesis.
b: p<0.01 when compared to basal lipogenesis.
c: p<0.02 when compared to insulin stimulated (10mU.l⁻¹) lipogenesis.
d: p<0.05 when compared to insulin stimulated (10mU.l⁻¹) lipogenesis.
e: p<0.001 when compared to insulin stimulated (10mU.l⁻¹) lipogenesis.
**TABLE 5:4**

Effect of gel filtrated, glucose free, supernatants obtained from acid-ethanol precipitated control sera on rat adipocyte lipogenesis. (% stimulation ± SE; n=4)

[Basal counts: 279cpm (=100%)]

<table>
<thead>
<tr>
<th>Insulin (mU. l⁻¹)</th>
<th>0.0</th>
<th>2.5</th>
<th>5.0</th>
<th>10.0</th>
<th>20.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>100</td>
<td>198±12</td>
<td>400±13</td>
<td>570±8</td>
<td>616±17</td>
</tr>
<tr>
<td>Test substance 1</td>
<td>80±14*</td>
<td>70±8*</td>
<td>80±10*</td>
<td>89±17*</td>
<td>95±23*</td>
</tr>
<tr>
<td>Test substance 2</td>
<td>477±7*</td>
<td>555±9*</td>
<td>610±12*</td>
<td>654±16b</td>
<td>769±11*</td>
</tr>
</tbody>
</table>

Test substance 1: acid-ethanol extract containing 23mU. l⁻¹ insulin and 22mmol.l⁻¹ glucose.

Test substance 2: acid-ethanol extract after elution on PD10 containing 16.2mU. l⁻¹ insulin and non-detectable glucose.

Statistical comparisons  (Students t test)
a: p<0.001 when compared to corresponding insulin stimulated lipogenesis.
b: p<0.01 when compared to corresponding insulin stimulated lipogenesis.
TABLE 5.5

Effect of dialysed, glucose free, supernatants of acid-ethanol precipitated control sera on rat adipocyte lipogenesis. (% stimulation ± SE; n=4)

[Basal counts: 146cpm (=100%)]

Test substance 1: acid-ethanol extract (15mmol. l⁻¹ glucose).
Test substance 2: dialysed acid-ethanol (glucose: <lowest limit of detection).

<table>
<thead>
<tr>
<th>Insulin (mU.l⁻¹)</th>
<th>0.0</th>
<th>2.5</th>
<th>5.0</th>
<th>10.0</th>
<th>20.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>100</td>
<td>304±8</td>
<td>453±13</td>
<td>557±7</td>
<td>656±18</td>
</tr>
<tr>
<td>Test substance 1</td>
<td>122±32</td>
<td>134±3*</td>
<td>113±27*</td>
<td>123±23*</td>
<td>110±30*</td>
</tr>
<tr>
<td>Test substance 2</td>
<td>817±7*</td>
<td>865±9*</td>
<td>871±32*</td>
<td>937±12*</td>
<td>952±13*</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)
a: p<0.001 when compared to corresponding insulin-stimulated lipogenesis.
TABLE 5:6

Effect of gel filtrated, dialysed and glucose free supernatants of acid-ethanol precipitated control sera on rat adipocyte lipogenesis. (% stimulation ± SE; n=4)

[Basal counts: 246cpm (=100%)]

Test substance 1: acid-ethanol extract (17.8 mmol.l⁻¹ glucose).
Test substance 2: acid-ethanol extract after gel filtration on a PD10 column (glucose: < lowest limit of detection).
Test substance 3: dialysed acid-ethanol extract (glucose: < lowest limit of detection).

<table>
<thead>
<tr>
<th>Insulin (mU.l⁻¹)</th>
<th>0.0</th>
<th>2.5</th>
<th>5.0</th>
<th>10.0</th>
<th>20.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>100</td>
<td>193±23</td>
<td>280±26</td>
<td>360±20</td>
<td>375±20</td>
</tr>
<tr>
<td>Test substance 1</td>
<td>30±11*</td>
<td>47±8*</td>
<td>89±13*</td>
<td>169±12*</td>
<td>202±18*</td>
</tr>
<tr>
<td>Test substance 2</td>
<td>280±28*</td>
<td>308±26²</td>
<td>300±9</td>
<td>310±9</td>
<td>344±21</td>
</tr>
<tr>
<td>Test substance 3</td>
<td>263±10*</td>
<td>316±21⁵</td>
<td>326±17</td>
<td>367±25</td>
<td>385±19</td>
</tr>
</tbody>
</table>

Statistical comparisons  (Students t test)

a: p<0.001 when compared to corresponding insulin-stimulated lipogenesis.
b: p<0.02 when compared to corresponding insulin-stimulated lipogenesis.
c: p<0.01 when compared to corresponding insulin-stimulated lipogenesis.
inhibitory activity resulted in the lipogenesis bioassay. Thus it is possible that the products of the oxidation process, hydrogen peroxide and gluconic acid interfere with the bioassay system used.

Another method of purification of the inhibitory preparation used by Dean et al (1984) was by application of the acid-ethanol extract (diluted 1:4 in double distilled water) to C_{18} Sep-pak (Waters) columns. We repeated this procedure in two ways: i) having spiked a sample with a negligible amount of tritiated glucose, and ii) a "cold" sample of the same acid-ethanol extract. (Table 5:7). In the first case 19% of the radioactivity was lost in the flow through fraction whilst 54% of the radioactivity was eluted in the first millilitre of eluate. A similar pattern was apparent when unlabelled glucose concentration was measured: 66% of the glucose was eluted with the first millilitre of the eluate after the flow through fraction. A protein assay of these eluates does show half the acid-ethanol proteins being eluted in the first millilitre of acidified sodium chloride whilst the rest is eluted in the next fraction. Thus half of the proteins contained in the acid-ethanol fraction were eluted with glucose after chromatography on Sep-pak columns. Further elution with increasing concentrations of acetonitrile resulted in background detection of radiolabelled glucose and none detected by the glucose analyser.

A comparison of basal and insulin serum inhibitory activity in acid-ethanol extracts from the control and diabetic populations was conducted. Insulin was removed from acid ethanol preparations by affinity binding to an insulin antibody linked gel. The insulin content
TABLE 5:7

Elution profile of supernatants of acid ethanol precipitated control sera when applied to a C₁₈ Sep-pak column. (By the method of Dean et al 1984).

<table>
<thead>
<tr>
<th>[³H]-glucose (cpm)</th>
<th>Protein (mg.l⁻¹)</th>
<th>Glucose (mmol.l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid ethanol inhibitor preparation</td>
<td>690968</td>
<td>68.00</td>
</tr>
<tr>
<td>Flow through fraction</td>
<td>132738</td>
<td>0.00</td>
</tr>
<tr>
<td>1ml acidified NaCl</td>
<td>370031</td>
<td>28.00</td>
</tr>
<tr>
<td>1ml acidified NaCl</td>
<td>17221</td>
<td>30.25</td>
</tr>
<tr>
<td>1ml acidified NaCl</td>
<td>1459</td>
<td>4.00</td>
</tr>
<tr>
<td>1ml acidified NaCl</td>
<td>228</td>
<td>0.00</td>
</tr>
<tr>
<td>1ml acidified NaCl</td>
<td>89</td>
<td>0.00</td>
</tr>
<tr>
<td>25% acetonitrile</td>
<td>120</td>
<td>0.00</td>
</tr>
<tr>
<td>50% acetonitrile</td>
<td>128</td>
<td>0.00</td>
</tr>
<tr>
<td>100% acetonitrile</td>
<td>64</td>
<td>0.00</td>
</tr>
</tbody>
</table>
of acid-ethanol extracts was measured by radioimmunoassay. The lowest limit of detection of these radioimmunoassays ranged from 0.12 - 1.61\mu l^{-1} of insulin. Thus the expression 'none detected' indicates insulin concentrations below the minimum detectable level of that assay. As cartilage is not sensitive to insulin these preparations with insulin removed were not assayed in the cartilage assay.

The data on insulin concentration of acid-ethanol extracts are shown in Table 5:8. It indicates that insulin concentrations in control and NIDD acid-ethanol extracts are similar whilst the IDD extracts have very much higher levels. Another serum constituent which would effect the lipogenesis bioassay is immunoglobulin G (IgG). It was important to establish if (i) the acid-ethanol extraction procedure of serum removed IgG and (ii) if the following cleaning procedures i.e. removal of glucose and insulin affected these IgG concentrations. IgG levels were measured by nephrometry in a Beckman Immunochemistry Analyser. The lowest limit of detection for this system is 0.1gl^{-1} IgG. In a series of randomly collected acid-ethanol preparations there was no detectable IgG. There was therefore no lipogenesis stimulatory activity due to IgG in these acid-ethanol extracts.

A study of the presence and lipogenesis inhibitory activity of acid-ethanol extracts in the diabetic and control populations was carried out. The combined results of this study are shown diagramatically; the different columns are directly comparable (Figures 5:4 & 5:5). Abbreviations used in this study represent (i). the acid-ethanol extract (Preparation A), (ii). the glucose free acid-ethanol extract Preparation
### TABLE 5.8

Endogenous concentrations of glucose and insulin in acid-ethanol supernatant extracts prepared from control subjects and NIDDM & IDDM patients.

1) Median (range) concentration of glucose and insulin in Preparations A.

<table>
<thead>
<tr>
<th></th>
<th>Glucose (mmol. l&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Insulin (mU. l&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.4 (1.0-4.3)</td>
<td>9.8 (6.8-23.2)</td>
</tr>
<tr>
<td>NIDDM</td>
<td>4.3 (1.9-6.4)</td>
<td>8.9 (6.9-32.0)</td>
</tr>
<tr>
<td>IDDM</td>
<td>5.5 (2.5-16.2)</td>
<td>&gt;160 (18.6-&gt;160)</td>
</tr>
</tbody>
</table>

ii) Median (range) concentration of glucose and insulin in Preparations B.

<table>
<thead>
<tr>
<th></th>
<th>Glucose (mmol. l&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Insulin (mU. l&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt;LLD</td>
<td>3.0 (1.0-18.0)</td>
</tr>
<tr>
<td>NIDDM</td>
<td>&lt;LLD</td>
<td>2.7 (1.0-18.2)</td>
</tr>
<tr>
<td>IDDM</td>
<td>&lt;LLD</td>
<td>71.4 (4.2-&gt;160)</td>
</tr>
</tbody>
</table>

iii) Preparation C was free of detectable glucose and insulin.

Where
<LLD: Less than the lowest level of detection.
Figure 5:4

Effect of supernatants of acid-ethanol precipitated control, NIDDM & IDDM sera on basal rat adipocyte lipogenesis.

A: Effect of acid-ethanol extracts on basal lipogenesis; where basal response = 100%.

B: Effect of acid-ethanol extracts after removal of glucose (small molecular weight substances), on basal lipogenesis.

C: Effect of acid-ethanol extracts after removal of glucose and insulin on basal lipogenesis.
Figure 5:5

Effect of supernatants of acid-ethanol precipitated control, NIDDM & IDDM sera on insulin (10mU.l⁻¹)-stimulated rat adipocyte lipogenesis.

A: Effect of acid-ethanol extracts on insulin-stimulated lipogenesis; where insulin (10mU.l⁻¹) stimulated response = 350%.

B: Effect of acid-ethanol extracts after removal of glucose (small molecular weight substances) on insulin stimulated-lipogenesis.

C: Effect of acid-ethanol extracts after removal of glucose and insulin on insulin stimulated-lipogenesis.
B), (iii). the glucose and insulin free acid-ethanol extract (Preparation C). There is considerable scatter in the results because there is considerable intra-assay variation in bioassay systems. Statistical analysis is however valid since all tests are two tailed and paired.

The concentrations of insulin present in acid-ethanol extracts prepared from normal subjects and diabetic patients, before and after gel filtration on the PD10 columns are shown in Figure 5:6. Thus some insulin (MW:6000) in the acid-ethanol extracts is retarded by the gel filtration process and the concentration of insulin is diminished following PD10 chromatography.

Acid-ethanol extracts (Preparation A) from control subjects had no significant effect on basal lipogenesis. The acid-ethanol extracts did not affect basal lipogenesis even after the removal of glucose (Preparation B) and on removal of insulin (Preparation C). These three preparations (A, B and C) did however inhibit insulin stimulated (10mUl⁻¹) lipogenesis significantly (p<0.01 in each case).

The removal of glucose from the acid-ethanol preparations did not result in a significant difference between these Preparations B and the initial Preparation A. Similarly the next step i.e. removal of insulin, (Preparation C), did not cause a significant change in the effect of Preparation A on basal or insulin stimulated lipogenesis.

Acid-ethanol extracts from normal subjects and NIDD patients were found to contain similar concentrations of glucose and insulin (Table 5:8); thus the final concentrations of these in the incubation mixture
Figure 5:6

Insulin content of acid-ethanol supernatant extracts prepared from control subjects and diabetic (NIDD & IDD) patients before and after gel filtration on PD10 columns.

A: Acid-ethanol extracts prepared from control subjects.

B: Acid-ethanol extracts prepared from control subjects, after filtration on PD 10 columns.

C: Acid-ethanol extracts prepared from NIDDM subjects.

D: Acid-ethanol extracts prepared from NIDDM subjects, after filtration on PD 10 columns.

E: Acid-ethanol extracts prepared from IDDM subjects.

F: Acid-ethanol extracts prepared from IDDM subjects, after filtration on PD 10 columns.
of the lipogenesis assay were similar (Table 5:9) and as in the control data, no significant effect of Preparations A, B and C on basal lipogenesis was detected. As in the control group insulin-stimulated lipogenesis was inhibited (p<0.01) by all three preparations.

The comparison of Preparations A and B in the NIDD group shows no significant differences in their effect on basal- or insulin-stimulated lipogenesis. Preparations A and C had similar effect on basal lipogenesis whilst Preparation C has significantly (p<0.05) less inhibitory activity on insulin-stimulated lipogenesis than Preparation A.

Acid-ethanol extracts prepared from IDD patients contained higher glucose concentrations and extremely high insulin concentrations (>160mU.l⁻¹). Preparation A significantly stimulated (p<0.05) basal lipogenesis; the removal of glucose (Preparation B) increased this stimulation further (p<0.01). The removal of insulin resulted in preparations which had no significant effect on basal adipocyte lipogenesis. Preparation A was not significantly different when compared to Preparation B but on removal of insulin (Preparation C) was significantly more inhibitory than Preparation A (p<0.01). Preparation A was significantly inhibitory (p<0.01) to insulin stimulated lipogenesis. On removal of glucose however, Preparations B had no significant effect on insulin stimulated lipogenesis but removal of insulin (Preparation C) resulted in a preparation which was significantly inhibitory to insulin stimulated lipogenesis. (p<0.01).
TABLE 5:9

Final median concentrations of glucose and insulin following the addition of supernatants of acid-ethanol preipitated sera to the rat adipocyte lipogenesis incubates.

Results are expressed as final concentrations in incubates.

<table>
<thead>
<tr>
<th></th>
<th>Glucose (mmol·L⁻¹)</th>
<th>Basal insulin (mU·L⁻¹)</th>
<th>Insulin stimulated (mU·L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.8</td>
<td>1.0</td>
<td>11.0</td>
</tr>
<tr>
<td>NIDDM</td>
<td>0.9</td>
<td>0.9</td>
<td>10.9</td>
</tr>
<tr>
<td>IDDM</td>
<td>1.1</td>
<td>&gt;16.0</td>
<td>&gt;26.0</td>
</tr>
</tbody>
</table>
There was a significant difference between Preparations A and B (p<0.01) and Preparations A and C (p<0.05) as far as their action on insulin stimulated lipogenesis is concerned. On removal of low molecular weight substances (<5kDa) and insulin from these acid-ethanol extracts the residual insulin-inhibitory activity was not a non-specific effect as it has a dose-dependent effect on insulin stimulated lipogenesis (Table 5:10).

In the preparation of glucose-free acid-ethanol extracts, the low molecular weight substances which were initially retarded on the gel were also eluted and assayed. Pooled acid-ethanol extracts (Preparations A) from controls (glucose:2.7mmol.l⁻¹), NIDDM (glucose:4mmol.l⁻¹) and IDDM (5mmol.l⁻¹) were gel filtrated, the low molecular weight eluate collected and separated into nominal 0-1kDa and 1-5kDa fractions by ultrafiltration. Glucose was present in the 0-1kDa fractions whilst insulin was present in the 1-5kDa fractions (Table 5:11). Therefore, on assaying these fractions, the 0-1kDa fractions were inhibitory to insulin stimulated lipogenesis whilst the 1-5kDa fractions increased the insulin stimulated response (Table 5:12).

Acid-ethanol extracts from the control, NIDDM and IDDM populations were also inhibitory to basal (Figure 5:7) uptake of [³⁵S]-sulphate by porcine cartilage. There were no significant differences between the inhibitory activities of these acid-ethanol extracts detectable by the [³⁵S]-sulphate bioassay. The cartilage assay however is not sensitive to glucose, thus the inhibitory response by the cartilage must be due to an inhibitory factor.
Table 5:10

Effect of supernatants of acid-ethanol precipitated sera before and after removal of glucose and insulin on insulin-stimulated rat adipocyte lipogenesis. (% stimulation ± SE; n=5)

[Basal counts: 330cpm (=100%)]

A: Insulin alone
B: Acid-ethanol extract.
C: Acid-ethanol extract after removal of glucose and insulin.

<table>
<thead>
<tr>
<th>Insulin (mU.l⁻¹)</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>5±7*</td>
<td>87±21</td>
</tr>
<tr>
<td>2.5</td>
<td>299±15</td>
<td>37±27*</td>
<td>237±22*</td>
</tr>
<tr>
<td>5.0</td>
<td>441±17</td>
<td>146±19*</td>
<td>255±19*</td>
</tr>
<tr>
<td>10.0</td>
<td>625±25</td>
<td>181±18*</td>
<td>304±16*</td>
</tr>
<tr>
<td>20.0</td>
<td>635±20</td>
<td>221±23*</td>
<td>298±27*</td>
</tr>
</tbody>
</table>

Statistical comparisons (Students t test)
a: p<0.001 when compared to corresponding insulin-stimulated lipogenesis.
b: p<0.05 when compared to corresponding insulin-stimulated lipogenesis.
Table 5:11

a) Insulin concentrations of pooled and fractionated supernatants of acid-ethanol precipitated control, NIDDM & IDDM sera.

Results are expressed as insulin concentration (mU.l⁻¹)

<table>
<thead>
<tr>
<th>Acid-ethanol fractions</th>
<th>0-5kDa</th>
<th>0-1kDa</th>
<th>1-5kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.9</td>
<td>-</td>
<td>12.5</td>
</tr>
<tr>
<td>NIDDM</td>
<td>11.8</td>
<td>-</td>
<td>10.1</td>
</tr>
<tr>
<td>IDDM</td>
<td>&gt;240</td>
<td>-</td>
<td>177</td>
</tr>
</tbody>
</table>

b) Glucose concentrations of pooled and fractionated supernatants of acid-ethanol precipitated control, NIDDM & IDDM sera

Results are expressed as glucose concentration (mmol.l⁻¹)

<table>
<thead>
<tr>
<th>Acid-ethanol fractions</th>
<th>0-5kDa</th>
<th>0-1kDa</th>
<th>1-5kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.7</td>
<td>2.6</td>
<td>0</td>
</tr>
<tr>
<td>NIDDM</td>
<td>4.0</td>
<td>3.7</td>
<td>0</td>
</tr>
<tr>
<td>IDDM</td>
<td>5.0</td>
<td>5.0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 5:12

Effect of low molecular weight (0–1kDa & 1–5kDa) fractions obtained from supernatants of acid-ethanol precipitated control, NIDDM and IDDM sera on rat adipocyte lipogenesis. (% stimulation ± SE; n=5)

[Basal counts: 264cpm (=100%)]

<table>
<thead>
<tr>
<th></th>
<th>0-5kDa</th>
<th>0-5kDa + Insulin</th>
<th>0-1kDa</th>
<th>0-1kDa + Insulin</th>
<th>1-5kDa</th>
<th>1-5kDa + Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30±17*</td>
<td>24±12*</td>
<td>96±23</td>
<td>171±19*</td>
<td>203±23*</td>
<td>344±27*</td>
</tr>
<tr>
<td>NIDDM</td>
<td>148±20b</td>
<td>112±15*</td>
<td>136±23</td>
<td>118±13*</td>
<td>207±11c</td>
<td>366±17c</td>
</tr>
<tr>
<td>IDDM</td>
<td>192±21*</td>
<td>138±14*</td>
<td>108±21</td>
<td>84±17*</td>
<td>351±19c</td>
<td>374±18c</td>
</tr>
</tbody>
</table>

Where:
Insulin = 10mU.1^-1, which gave a stimulation of 234 ± 17%

Statistical comparisons (Students t test)

a: p<0.005 when compared to basal lipogenesis.
b: p<0.05 when compared to basal lipogenesis.
c: p<0.001 when compared to basal lipogenesis.
d: p<0.001 when compared to insulin-stimulated lipogenesis.
e: p<0.005 when compared to insulin-stimulated lipogenesis.
f: p<0.05 when compared to insulin-stimulated lipogenesis.
g: p<0.01 when compared to insulin-stimulated lipogenesis.
Effect of supernatants of acid-ethanol precipitated control NIDDM & IDDM sera on basal (\(35\)S)-sulphate uptake in porcine cartilage.
CHAPTER SIX

DISCUSSION
Since Rodbell (1964) introduced a method for the isolation of fat-cells from rat epidydimal adipose tissue, studies investigating glucose metabolism and fatty acid synthesis have been facilitated. It has become possible to detect the biological responses to physiological (2.5mU/l) concentrations of insulin using adipocyte lipogenesis as the index of stimulation (Moody et al., 1974). The biological activity of other insulin like substances and that of inhibitors of this system can also be investigated.

The precipitation of globulins from normal sera resulted in supernatants which had an inhibitory activity not only against insulin but also NSILA-s, NSILA-p and immunoglobulins, all of which stimulate adipocyte lipogenesis. Insulin-like activity (ILA) of plasma is only partially suppressed by anti-insulin serum, whereas the biological activity of crystalline insulin is completely neutralized (Ramseier et al., 1962; Leonards & Landau, 1962). The fraction of ILA which is not inhibited by insulin antibodies and is not measurable by immunoassay procedures has been termed "nonsuppressible ILA" (NSILA) by Froesch et al. (1963) and "atypical insulin" by Samaan et al. (1963).

A protein with nonsuppressible ILA and a molecular weight of 6 - 10 kDa was extracted and partially purified by Jakob et al. (1968). This protein accounted for 5-10% of total NSILA and it was designated NSILA-s because of its solubility in acid-ethanol. Most of the NSILA present was insoluble in acid-ethanol (NSILA-p) and had a molecular weight of approximately 150kDa. NSILA-s has been shown to consist of at least two polypeptides, factors I and II (IGF I & IGF II). Both these polypeptides exert insulin-like effects on fat cells in the presence of an excess of insulin antibodies; both are sulfation factors and both are mitogens.
(Zapf et al. 1978). These workers showed that these proteins were 54-68
times less potent than insulin when comparing biological activity in the
fat pad assay. Isolated rat adipocytes possess IGF II receptors but do
not have IGF I receptors (Zapf et al., 1981; Massague & Czech, 1982).
This fact probably explains the difference in potencies between factors I
(IGF I) and II (IGF II) in stimulating glucose oxidation, lipogenesis and
3-O-methylglucose transport in fat cells, as observed by Zapf et al.
(1978).

Schoenle et al. (1976,1977) and later Zapf et al. (1978) confirmed
that fat cells contain at least two separate binding sites for insulin
and for insulin-like growth factors I and II. Whereas unlabelled factors
I and II compete for binding of labelled insulin to receptors, unlabelled
insulin does not compete for binding of either factor. On the contrary,
Schoenle et al (1976,1977) found that insulin induced an increase of the
binding of factor II to receptors of approximately 200% and that of factor
I by 120%. This effect of insulin may (a) protect insulin-like growth
factor(s) from degradation or (b) make more NSILA binding sites available
by changing some characteristics of the plasma membranes. Zapf et al.
(1978) also concluded that the effects of factor I and II on glucose
metabolism in fat cells are likely to be mediated via the insulin
receptor but at high concentrations (10^{-7}M).

Another growth factor, the multiplication stimulatory activity (MSA)
is a homologue of human IGFI. Isolated from rat liver cells, it has been
shown to mimic the metabolic effects of insulin (King et al. 1982) in
rat adipocytes. This action is mediated by the binding of MSA to the
insulin receptor whereas the growth promoting activity of MSA and insulin
is mediated via another receptor. These workers also noted that
unlabelled insulin does not displace labelled MSA from adipocytes, but that it enhances binding of labelled MSA to these cells (King et al. 1980). King et al. (1982) characterized this effect of insulin on the MSA receptor and found that it was mediated via the insulin receptor, suggesting an interaction between the receptors of insulin and MSA at the cellular level. King et al. (1980) showed that the weak affinity of MSA for the insulin receptor is important in the production of its insulin-like metabolic effect and that blockade of the insulin receptor with a specific anti-receptor antibody inhibits the stimulatory effect of both insulin and MSA on glucose oxidation to a similar extent.

Khokher et al. (1985) demonstrated that IgG, Fc fragments and IgM produced dose dependent stimulation of oxidation of glucose by adipocytes. However these proteins did not alter specific binding of insulin to adipocytes. This suggests that the stimulatory effect of immunoglobulins G and M is exerted independently of the insulin receptor and is probably mediated through the interaction of Fc fraction of immunoglobulins with putative Fc receptors on the adipocyte membrane. Fab fragments did not have lipogenic activity, whilst previous workers (King et al. 1982) found that treatment of cells with Fab fragments of insulin receptor antibody shifted the dose-response to the right i.e. Fab fragments acted as competitive inhibitors at the insulin receptor level. However bivalent antireceptor IgG to the insulin receptor was able to mimic insulin in enhancing MSA binding, which supported the notion that the insulin receptor is involved i.e. enhancement of MSA binding by insulin is mediated by the insulin receptor.

Incorporation of [¹⁴C] from [¹⁴C]-glucose into metabolites represents total metabolism of the tissue only if endogenous substrates are not
metabolized. Two sources of endogenous carbon are glycogen and lipid. Whereas muscle contains significant reserves of glycogen, adipose tissue from animals fed a routine diet does not (Tuerkischer & Wertheimer, 1946). Shafrir & Kerpel (1964) determined glycogen content at the beginning and at the end of epididymal fat pad incubations and showed no pronounced changes during the incubation. Thus, the contribution from this source to over-all metabolism under our conditions is probably not significant. However, with tissues of rats fed ad libitum, dilution of the acetyl pool from endogenous sources is probably extensive (Katz et al., 1966).

A significant fraction of the insulin-induced conversion of glucose to CO₂ in rat adipose tissue reflects enhanced function of the pentose cycle (Jeanrenaud, 1961). When glucose is metabolized by way of the pentose cycle, the first carbon of glucose is oxidized and removed by decarboxylation with the resulting formation of carbon dioxide and a pentose phosphate. This cycle facilitates fatty acid synthesis from glucose (Ko & Royer, 1967) and it is noteworthy that this cycle is absent/inactive in human fat cells. In rat adipose tissue when glucose (in the presence of insulin) is the substrate for FA synthesis, the pentose phosphate pathway appears to supply about 60% of the reducing power (Katz et al., 1966; Kather et al., 1972). However, this pathway is not maximally stimulated under these conditions (i.e. in the presence of glucose and insulin) since it can be increased by the addition of an artificial electron acceptor e.g. phenazine methosulphate (McLean, 1960). The remainder of the reducing power is thought to be provided by NADP-malate dehydrogenase; (Smith & Prior, 1981).
Vaughan (1961) described the suppression of FFA accumulation resulting from the availability of glucose for metabolism because of an increase in the rate of esterification of FFA. Adipose tissue triglycerides continuously undergo lipolysis to free fatty acids, and the fatty acids are in turn re-esterified, particularly if glucose is present in the medium. In this regard it is important to remember that the products of lipolysis, FFA and glycerol are not the immediate substrates for the esterification reactions. Firstly the FFA must be activated to form fatty acyl coenzyme A derivatives and these in turn react with α-glycerophosphate, not glycerol in the first steps of glyceride synthesis. White adipose tissue has very low glycerol kinase activity, thus α-glycerophosphate cannot be formed by the phosphorylation of glycerol and therefore must be formed from dihydroxyacetone phosphate derived from glycolysis. Thus glycerol produced by lipolysis accumulates providing an index of triglyceride breakdown and re-esterification of the fatty acids constantly produced by lipolysis depends on a continuing supply of glucose (or other equivalent substrate) to provide α-glycerophosphate. It is not possible to differentiate between the glyceride glycerol (resulting from ethanolic hydrolysis of triglycerides) and glycerol produced by lipolysis during the incubation period. However since lipolysis is inhibited by very low concentrations of insulin, labelled glycerol in our insulin-stimulated system may be considered as having been derived mainly from ethanolic hydrolysis of triglycerides.

In the presence of higher glucose concentrations there is sufficient formation of the three precursors necessary for triglyceride formation, long chain acyl CoA, NADPH and glycerol phosphate. Thus the rate of esterification of fatty acids is increased. During insulin stimulated
lipogenesis glyceride-glycerol synthesis from glucose increases two-fold (Cahill, Leboeuf & Renold, 1959), and even in the absence of insulin, three times as much carbon is recovered in glyceride-glycerol than in fatty acids; there is thus an excess of glyceride-glycerol synthesis.

The serum inhibitor(s), prepared by the precipitation of globulins by ammonium sulphate had an inhibitory effect on insulin, NSA, NSILA-p, and IgG stimulated adipocyte metabolism. Since each of these factors act through separate receptor mechanisms our data suggest that the inhibitory effect of these inhibitors is exerted at a post receptor site(s). An index of inhibitory activity to glucose metabolism was the shift of the glucose oxidation curve to the right by the 'crude' ammonium sulphate inhibitor. This non-competitive effect indicates that increasing agonist (insulin) in the concentration range used, did not restore lipogenic activity in the presence of fixed concentration of inhibitor. The serum inhibitor was found to be extremely heat labile and it was also unstable at 4°C probably due to the presence of a proteolytic enzyme in the preparation. The use of trypsin (a protease) to determine the protein character of the inhibitor preparation was not possible since trypsin itself caused an inhibition of lipogenesis. However when this inhibitor has been further purified it should be possible to detect structural changes after proteolysis by several other methods (e.g. high pressure liquid chromatography) which do not rely on a bioassay procedure to detect inhibitory activity.

On fractionation inhibitory activity resulted in two nominal molecular weight species, the larger one of 30 - 50kDa and a smaller one of 10 - 30kDa. This molecular weight range again suggests a protein character for the inhibitor.
To study the effect of these two molecular weight species on the incorporation of the label into glycerol and fatty acids, D-[U²⁴C]-glucose incorporation into ¹⁴C-lipids was used. In concomitant experiments with D-3-[³H]-glucose substantial losses were detected in radioactivity during ethanolic hydrolysis of triglycerides. However the incorporation of [³²C]-glucose into triglycerides, fatty acids and glucose was comparable to that of [¹⁴C]-glucose in the lipogenesis assay procedure. The method of extraction used is sensitive, reproducible and rapid. Absolute specificity in terms of fatty acid formation is not required and provided interfering materials are constant and relatively low in concentration, the interpretations depend only on changes in concentration of fatty acid.

The present evaluation of the effect of varying concentrations of glucose in the incubation buffer on the incorporation of radiolabelled glucose into fatty acids and glyceride-glycerol is in essential agreement with the observations of earlier workers. In the absence of or in the presence of low glucose concentrations we have shown that a large fraction of the total uptake is recovered in the glyceride-glycerol. As the concentration of glucose is increased glucose uptake rises, providing a supply of energy for the esterification process and the proportion of glucose converted to glyceride-glycerol decreases. When α-glycerophosphate production becomes sufficiently limited, esterification no longer keeps pace with lipolysis and net accumulation of FFA results. Conversely, when glucose utilization reaches a certain level the rate of esterification exceeds that of lipolysis and there is a net decrease of FFA in the system. The inhibition of basal lipogenesis by the two inhibitor species (10-30kDa & 30-50kDa) as detected by radiolabelled
glucose incorporation into the fatty acid and glycerol moieties is most apparent in the absence of or in the presence of extremely low concentrations of glucose in the incubation mixture. In the presence of 0.5mM or more glucose in the incubation mixture these inhibitors do not demonstrate significant antagonistic activity over basal lipogenesis, inhibition of incorporation of radiolabelled glucose in fatty acids alone being observed. This is probably what occurs in vivo, since such concentrations of glucose are similar to the physiological situation i.e. incorporation of glucose into the glycerol moiety of triglycerides is not markedly affected. At these glucose concentrations there is an increase in fatty acid turnover, and therefore the inhibition of this process appears to be more marked. Kather et al. (1972) have shown that a high rate of fatty acid synthesis is coupled with a high pentose phosphate cycle activity on the one hand and a low rate of glycerol formation from triglyceride on the other.

The inhibition of insulin-stimulated lipogenesis (radiolabel incorporation into fatty acid and glycerol moieties) is also detected markedly in the absence of or in the presence of low concentrations of glucose. In the insulin-stimulated state and in the presence of 0.5mM glucose in the incubation mixture, inhibition of radiolabelled glucose incorporation into the fatty acid moiety is observed, whilst inhibition of incorporation into the glycerol moiety is not.

The inhibition of [35S]-sulphate uptake into cartilage by the fractions found inhibitory to lipogenesis highlighted the fact that the inhibitors have an effect on metabolic processes other than those affected by insulin and at sites other than the adipocyte. The inhibitors antagonized both basal and serum stimulated uptake of [35S]-sulphate.
Human serum stimulates $[^{35}S]$-sulphate uptake because of its intrinsic somatomedin content. Cartilage is not sensitive to insulin at physiological concentrations but like the adipose tissue is responsive to NSILA-s (somatomedin C or insulin-like growth factor 1) which stimulates $[^{35}S]$-sulphate uptake by the cartilage.

The detection of insulin inhibitory activity in sera of normal subjects led us to investigate the possibility that these inhibitors may be increased in diabetic patients. Our investigation demonstrates that some diabetics exhibited insulin inhibitory activity in their sera whilst others did not. In our studies, of the twelve normal sera, eleven were inhibitory to basal lipogenesis whilst nine were inhibitory to insulin-stimulated lipogenesis. Of the thirteen sera from NIDDM patients investigated, four were inhibitory to basal lipogenesis whilst seven were inhibitory to insulin stimulated lipogenesis and of the fourteen IDDM sera investigated four were inhibitory to basal lipogenesis whilst five were inhibitory to insulin stimulated lipogenesis. Pooling of crude inhibitor preparations and fractionation of the preparations confirmed the presence of inhibitory activity in the 10 - 30kDa and 30 - 50kDa ranges. There was no significant difference in the inhibitor activity from control, NIDDM and IDDM sera in our work. The only group of patients which had increased inhibitory activity in their sera was that of obese NIDDMs, whilst that in the non-diabetic obese was similar to that in normal subjects.

A large number of obese individuals have metabolic derangements. Hyperinsulinism is now generally accepted as an effect rather than the cause of obesity whilst mild carbohydrate intolerance is associated with a relative impairment of insulin secretion. The pathogenesis of these
changes is probably based on the occurrence of insulin resistance in these patients which is attributable to a diminution in the number of insulin receptors and certain post-receptor defect(s) (Olefsky et al., 1985). Our data show no difference in the inhibitory preparations from the normal subjects and non-diabetic obese patients. However, the inhibitory preparations from obese patients with non-insulin dependent diabetes mellitus were significantly more inhibitory than either those from non-obese NIDDMs or from non-diabetic obese patients. The significance of and the mechanism underlying this observation is not clear. It is nevertheless possible that the increased occurrence of this inhibitory activity may play a role in the pathogenesis of diabetes in obese patients.

Other workers have detected differences in insulin inhibitory activity from control and diabetic populations. Our inhibitor does not seem to be the same as that detected by Marsh & Haugeard (1952). They described an insulin inhibitory activity in sera of insulin-resistant diabetics, presumably patients with NIDDM. Sera from normal subjects and 'non-resistant' diabetic patients (IDDM) demonstrated significant inhibitory activity whilst sera from insulin resistant diabetics (NIDDM) contained significantly greater amounts of this inhibitory activity. The inhibitor described by Field & Stetten (1956a & b) was also different from the one we studied in that theirs was detected only transiently during diabetic acidosis and was stable after heating for 15 minutes at 60°C. In their experiments sera of normal subjects had little if any similar inhibitory activity. Serum inhibitors described in this work lost 70% of their activity at 60°C. Our inhibitor, (molecular weight 10 - 50kDa) is also unlikely to be the MSILA-carrier protein (MW:200kDa) described by
Kaufmann et al. (1977) and investigated further by Meuli et al. (1978) since carrier proteins do not inhibit basal lipogenesis or basal $^{35}$S uptake. Similarly the insulin antagonist of pituitary origin described by Zimmet et al. (1971) which has a molecular weight <8kDa was found mainly in the NIDDM and IDDM populations and not in normal subjects.

Some comments regarding the selection of patients for this study and their relationships to our results are necessary. The patients with NIDDM were either being treated by sulphonylureas or metformin or a combination of both. Some patients were on dietary restriction alone. The quality of control of diabetes as reflected in their serum glucose concentrations was also variable. These variations in the diabetic control and drug treatment may affect the inhibitory activity in sera of these patients. There are several possible mechanism(s) underlying these effects. Firstly sulphonylureas and metformin may affect adipocyte lipogenesis. However, previous unpublished studies in our laboratory have shown that sulphonylureas do not affect either basal or insulin stimulated lipogenesis. Metformin, on the other hand, inhibits both basal and insulin stimulated lipogenesis but at extremely high concentrations; these concentrations are much greater than therapeutic concentrations of this drug. Taylor et al. (1982) also demonstrated that metformin inhibits $[^{35}S]$-sulphate uptake in porcine cartilage but once again at very high concentrations. Thus, even if sulphonylureas and metformin are found in supernatants prepared by ammonium sulphate precipitation they are unlikely to directly affect adipocyte lipogenesis. Whether these drugs affect the synthesis or release of these inhibitors into plasma is not clear. Secondly variable blood glucose concentrations may also affect adipocyte lipogenesis. Glucose may compete with radioactive labelled
glucose for entry into adipocytes and thus produce an 'inhibitory' effect. Such inhibition was unlikely in ammonium sulphate precipitated preparations since the supernatants were repeatedly dialysed and were therefore free of glucose. However, glucose was present in the <1kDa acid-ethanol extract fraction. This would particularly affect the observation made on this fraction. However, we have conducted experiments after the removal of glucose from these extracts and have described and discussed in detail the methodology involved in achieving this. We cannot, however, rule out the possibility that the quality of control of diabetes may have a 'biological' effect on the synthesis and release of this inhibitor into serum. The other reservation regarding the interpretation of our data is the fact that blood samples were not obtained in the fasting state. In most patients blood samples were obtained after breakfast during the hours of the diabetic clinic. Intake of breakfast may affect serum insulin inhibitory activity but since a majority of diabetic patients and control subjects had their blood samples obtained in this state, there is consistency in the manner in which these samples were obtained. However, subtle differences between various groups may have been shown up if the samples had been obtained in the fasting state. These 'reservations' to the interpretation of our data do not affect the fact that inhibitory activity in sera of our patients and control subjects was consistently observed in certain molecular fractions. However, they may cause variability in the magnitude of inhibition by these fractions in sera of diabetics on different drugs and with variable diabetic control.

Other inhibitors of somatomedin from rats with streptozotocin-induced diabetes of molecular weight ~21-24kDa, ~250kDa, ~940kDa were described by
Phillips et al. (1983). Salmon et al. (1983) described a somatomedin inhibitor of molecular weight 27-40kDa from starved rats. The molecular weights and other characteristics of the inhibitors described by us and by Phillips et al. suggest that they are probably proteins.

Taylor et al. (1987) in the study of inhibition of somatomedin-like activity in diabetic rats found four inhibitory molecular fractions, <1kDa, 1-10kDa, 30-50kDa and >300kDa. The inhibitory activity of the >300kDa fraction was the greatest.

The somatomedin inhibitory activity in sera of rats with streptozotocin-induced diabetes corresponded in part with the inhibitory activity detected in inhibitory preparations from human sera i.e. the 30 - 50kDa fraction. Sera from control or diabetic rats did not inhibit adipocyte lipogenesis. The somatomedin inhibitor from streptozotocin-induced diabetic rats described by Phillips et al. (1979) was non-competitive in its behaviour, in that it inhibited both the basal and somatomedin stimulated [35S]-sulphate uptake of cartilage. This non-competitive behaviour is similar to that exhibited by our human serum insulin/somatomedin inhibitors. Phillips & Scholz (1982) described an inhibitory activity in sera of rats with streptozotocin induced diabetes to glucose oxidation in rat adipocytes and to [35S]-sulphate uptake by cartilage. Although our cartilage data ([35S]-sulphate uptake) is comparable to that of Phillips & Scholz, that on the effect on rat adipocyte metabolism is not. There are, however, some differences between the two studies. Phillips & Scholz used 1-[14C]-glucose as a substrate for fat segments and their inhibitory fraction was obtained from rats weighing 120-170g which had been rendered diabetic with a dose of 241mg.kg⁻¹ streptozotocin. In comparison we used D-U[14C]-glucose as a
substrate for isolated fat cells to detect inhibitory activity of fractions obtained from rats weighing 357-515g which had been rendered diabetic with a dose of 55mg.kg⁻¹ streptozotocin. Furthermore different end points for detection of glucose metabolism inhibition were employed. Phillips & Scholz showed inhibition of glucose metabolism via the pentose cycle i.e. they detected an inhibition of glucose oxidation. The inhibitor in the work of Phillips & Scholz (1982) may be an antagonist of the pentose cycle. It is possible that in our system the pentose pathway has been antagonized but sufficient metabolism via the Embden Meyerhof pathway occurs so that inhibition of lipogenesis is not detected. However the production of the pentose-phosphate produces reduced NADP which is essential for lipid synthesis. If the cells had sufficient NADPH initially, lipogenesis may not be affected by the antagonism of the pentose cycle. If one assumes that glucose metabolism by the non-triose pathways (leading to formation of compounds such as glycogen and uronic acids) are of little quantitative importance in adipose tissue, one can tentatively estimate that 75% of glucose metabolism proceeds by the EMP, 20% by the PC and 5% by the non-triose pathways. (Katz & Wood, 1961). This ratio is not modified by the presence of insulin (Jeanrenaud & Renold 1959; Winegrad & Renold 1958; Cahill; Leboeuf & Flinn, 1960). It may be concluded, tentatively, that insulin stimulates both the EMP and PC without changing to any major extent the relative activity of the two metabolic pathways. If this is true, it is probable that the mechanism underlying this stimulation is the facilitation of the conversion of extra cellular glucose to intracellular glucose-6-phosphate which is a metabolic precursor of both pathways.
The inhibitory effect of the insulin inhibitor(s) described on stimulators of adipocyte lipogenesis other than insulin and the non-competitive nature of this inhibition indicated that the inhibitors affect lipogenesis and 35S-sulphate uptake by cartilage at a post receptor site. We therefore investigated the effect of these inhibitors on some post-receptor pathways. The role of calcium and protein kinase C in mediating the effect of insulin in adipocytes hitherto not fully elucidated was therefore investigated using (1) the PKC activator 4β-phorbol 12β-myristate, 13α-acetate and various inhibitors of PKC and (ii) calcium ionophore and various calcium blockers.

The initial task was to determine whether dimethylsulfoxide (DMSO), used as a carrier for PMA, affected adipocyte lipogenesis. DMSO, was found to have no effect on adipocytes in the concentrations used in the final incubation mixtures. DMSO is a dipolar aprotic solvent. It is able to act as a strong hydrogen bond acceptor but possesses no hydrogens suitable to act as hydrogen bond donors. Thus, DMSO is able to compete with water molecules where water is acting as a hydrogen bond acceptor. These factors have been used to explain the ability of DMSO to act as a solvent for many nonpolar molecules and its ability to rapidly penetrate biological membranes at high concentrations. Wieser (1983) studied the effect of DMSO on glucose metabolism in isolated fat cells and concluded that 0.8% (0.12M) DMSO had no significant effect on basal or insulin stimulated glucose oxidation. Our results with the highest dose (1%) of DMSO also demonstrate that this solvent is acceptable as a carrier in lipogenesis assays.

The phorbol esters have been found to be PKC stimulators in the order of their tumour promoting potency (Castagna, 1982; Yamanishi, 1983).
These tumour promoters directly activate the enzyme in vivo and in vitro. Our data show that 4β- phorbol 12β-myristate, 13α-acetate is a more potent stimulator of protein kinase C and adipocyte lipogenesis than 4β phorbol 12,13, dibutyrate.

The calcium ionophore, A23187 potentiated PdBu, PMA, and insulin stimulated lipogenesis while it had little effect on basal lipogenesis. It is increasingly apparent that calcium functions as an intracellular messenger in many cells (Rasmussen, 1981; Campbell, 1983; Berridge, 1984). Calcium was originally proposed as the mediator of insulin action by Clausen et al. (1974) and Kissebah et al. (1975a). The evidence was largely indirect. Since then many observations have provided evidence for the modification of transplasma membrane calcium concentrations through the inhibition of the high affinity Ca extrusion pump enzyme, Ca-Mg-ATPase (Pershadsingh & McDonald, 1981; Schoenle & Froesch, 1981). Evidence which suggests the essential importance of Ca in the mechanism of action of insulin on fat cells includes:

i) a requirement for extracellular Ca for some of the effects of insulin on cellular metabolism (Bonne et al., 1977; Desai & Hollenberg, 1975).

ii) several insulin-sensitive enzymes are Ca dependent (Hope-Gill et al., 1976, Kissebah et al., 1974).

iii) some Ca-altering compounds (e.g. local anaesthetics and heavy metals) mimic the effects of insulin (Siddle & Hales, 1974; Saggerson et al., 1976; Coulston & Dandona, 1980).

iv) insulin alters Ca fluxes in intact cells (Clausen & Martin, 1977).

v) insulin is known to increase plasma membrane bound Ca (McDonald et al., 1976), and to alter Ca homeostasis in plasma membranes. (Pershadsingh & McDonald, 1979).
Pershadsingh & McDonald (1984) proposed that insulin prevents efflux of Ca from the adipocytes through the inhibition of Ca-Mg-ATPase. They proposed this initial increase in cytosolic Ca may lead to augmented cycling of K+ across the plasma membrane with a net increase in K influx.

Draznin et al (1987) using fluorescence spectra observed the ability of insulin to increase cytosolic free Ca in isolated rat adipocytes. The Ca influx was inhibited by a voltage-dependent Ca channel blocker - nifedipine, and potentiated by higher glucose concentrations in the presence of insulin. High concentrations of glucose alone were ineffective. Thus an inward movement of Ca is triggered by insulin either directly or by insulin stimulated glucose transport or metabolism. Studies by Clausen et al. (1974) and Kissebah et al. (1975a) have shown that insulin and calcium decreased efflux of [45Ca] from preloaded fat pads and adipocytes.

A23187 which increases influx of calcium has also been shown to increase glucose transport in adipocytes (Sorenson et al., 1980), although Taylor et al. (1979b) did not detect this in their study. Verapamil, however inhibited glucose transport in their study. Similarly Bonne et al. (1977) in their work did not detect stimulatory effect on glucose oxidation by A23187. The concentrations used by these workers were comparable to those used in our study and it is surprising that they did not detect responses with A23187 since they concluded that the magnitude of metabolic stimulation in the adipocyte depends upon calcium concentrations. They also showed that cobalt, an antagonist of calcium action, inhibits the action of insulin. These studies taken together with our results demonstrating the additive effect of calcium ionophore on the stimulatory effect of insulin on adipocyte lipogenesis and the antagonism with
verapamil of both insulin-stimulated lipogenesis and the additive effect of A23187 demonstrate that insulin-stimulated lipogenesis is dependent on the influx of extracellular calcium. In our experiments, action of phorbol esters was also synergized by A23187 and antagonized by verapamil. It is relevant that the synergistic mechanisms between phorbol esters and A23187 are not confined to the adipocyte. For example, phorbol ester stimulated release reactions by mast cells (Nishizuka et al., 1984c), neutrophils (Kajikawa et al., 1983), and platelets (Kajikiawa et al., 1983; Kaibuchi et al., 1982; Kaibuchi et al., 1983; Yamanishi et al., 1983) are synergized markedly by concentrations of A23187 which have no effect by themselves. These data suggest that activated protein kinase C may itself initiate the influx of activator calcium. In this context it has recently been proposed that protein kinase C activates both voltage-dependent and receptor-linked calcium channels in chromaffin cells (Wakade et al., 1986) and smooth muscle (Jeremy & Dandona, 1987), possibly through hyperphosphorylation of these portals (Jacobs et al., 1983; May et al., 1984). Wolf et al. (1985) described that increases of intracellular Ca2+ cause a translocation of protein kinase C from a soluble to a membrane bound compartment in human erythrocyte vesicles. The subcellular compartment to which the enzyme binds may depend on the precise site of calcium mobilization and the resulting local intracellular Ca levels can lead to membrane binding of protein kinase C, thus exposing it to inner leaflet phospholipids and to the potential generation of DAG. This may be viewed as a 'priming' of the protein kinase C system, allowing it to participate in transmembrane signalling. Interestingly Wolf et al. suggested a dual effect of Ca on PKC; the binding of PKC to the membrane is maximal at 500nM Ca while
enzyme activation occurs mostly between 5µM and 50µM Ca in the absence of DAG or phorbol esters. This discrepancy between the Ca dependent enzyme binding to membrane and enzyme activation by Ca may arise from the existence of multiple Ca binding sites on the enzyme. At intracellular Ca levels (low ionic concentration), the Ca dependent membrane binding reaction is likely to be the more relevant process. Thus Ca induced PKC binding to membranes may not be a sufficient condition for enzyme activation. These workers also discuss the synergistic action of Ca and phorbol esters and suggest that the increase in intracellular Ca levels will permit a much faster response to external stimuli which activate PKC. IgG, another lipogenic substance was not potentiated by A23187 in its effect on rat adipocytes, although all other probes (PKC inhibitors and calmodulin inhibitors) effected the IgG response in a similar way to the insulin response. This may imply that IgG itself could behave as an ionophore or a calcium channel facilitator through its binding to the putative Fc receptor on the adipocyte membrane.

Another reason why A23187 does not stimulate basal lipogenesis to the extent that verapamil causes inhibition may be because calcium availability for basal lipogenesis is adequate. Thus, further increases in intracellular calcium do not alter lipogenesis significantly while diminished availability causes inhibition. However, when PMA or insulin stimulate protein kinase C and lipogenesis, an increased calcium availability becomes necessary; A23187 is able to provide this extra calcium and thus it exerts a synergistic effect with insulin and PMA.

The fact that PMA causes the insulin dose response curve to shift to the left without changing the maximal response of adipocyte lipogenesis to insulin suggests that PMA and insulin probably affect the same
metabolic mechanisms. Since it is established that PMA exerts its effect through the activation of protein kinase C, it is likely that insulin exerts its action through PKC activation. This concept is further supported by the data on the inhibitory effect of H7, an inhibitor of PKC and insulin action. This aspect is discussed in greater detail further on.

A consideration of the buffer glucose concentration in our assays is necessary. We have used 0.3mM glucose in our system which is below the physiological concentration and therefore may be considered rate limiting. In this context if we consider the possibility that PKC activation may phosphorylate the glucose transporter permitting glucose entry, it may be this very aspect alone which would permit glucose entry. Witters et al. (1985) recently demonstrated that the purified glucose transporter from human erythrocytes is a substrate for protein kinase C. Furthermore these workers found that PMA, which activates protein kinase C, stimulates the in vivo phosphorylation of glucose transporter in erythrocytes. PMA is known to stimulate glucose transport in a variety of cell types (O'Brien & Saladik, 1982; Kirsch et al., 1985). If this was the case one would not expect A23187, which allows Ca influx (and calmodulin activation) alone to cause stimulation of basal lipogenesis because of the limited glucose entry. Thus if glucose is a limiting factor and lipogenesis is proceeding, an increase in [Ca]i via an A23187 effect would not result in increased lipogenesis because of the limited glucose uptake. The stimulation observed due to PMA (on basal lipogenesis) is in our experiments more apparent than that resulting from A23187 alone. This again may be due to increased glucose entry due to PKC stimulation.
Verapamil, in our work inhibits both insulin and PMA stimulated lipogenesis. The effect of this antagonist on glucose metabolism is not always apparent in the work of other investigators. Bonne et al. (1977) for example did not detect an inhibitory effect of verapamil on glucose oxidation. Verapamil has been shown to cause no detectable change in the initial rapid influx of calcium into adipocytes but clearly decreases the subsequent rise suggesting that it affects the transport of calcium across the plasma membrane. D-600 (5μM), an analogue of verapamil has been used by Bihler & Sawh (1980) to demonstrate the dependence of sugar transport on the influx of extracellular calcium in isolated rat atria. Direct measurements of [Ca] in fat cells presents with two main problems. Firstly the use of spectroscopic indicators (murexide, arsenazo III, antipyrylazo III) are not sensitive enough to measure basal levels of [Ca] (Miledi et al., 1982; Stinkare, 1980). The use of these indicators is limited to detection of [Ca] when the level of cation rises dramatically (up to 8μM). Insulin may effect [Mg2+]i (Hall et al., 1982) and/or [H+]i (Moore, 1983) and this would in turn affect absorbance of dyes. Secondly intracellular Ca2+ microelectrodes, although highly specific for this cation, can only be used in large cells which withstand puncture. Fat tissue does not fulfill this requirement. The mechanisms linking receptors to calcium channels is unknown but recent interest has focussed on signal transduction by intracellular PKC and the G proteins (Berridge, 1986;...Gilman, 1983). Mechanistically it has been proposed that agonist activated receptors initiate the binding of guanosine-5'-triphosphate (GTP) to and activation of G proteins which in turn activate the phosphodiesterase, phospholipase C (Guillon et al., 1986) an enzyme which hydrolyses membrane phosphoinositides to DAG and inositol
triphosphate (IP3) (Berridge, 1986; Nishizuka, 1984a). Fluoride is known to activate G proteins but it also is an activator of adenyl cyclase (Gilman, 1984). In the rat adipocyte lipogenesis system, it is this latter property of sodium fluoride which predominates.

The data obtained from experiments with verapamil are supported by those obtained with the use of calmodulin inhibitors. Since calmodulin activation is considered to be the final step in mediating the intracellular effect of calcium, the inhibition of a metabolic process by calmodulin inhibitors constitutes further evidence that calcium is cardinal in this process. However the concentration of inhibitor required should not be such that it inhibits enzymes/processes other than those mediated by calmodulin. The use of trifluoperazine as a calmodulin inhibitor is problematic since it has been shown to have a strong affinity for dopamine, 5 hydroxytryptamine (5HT2) (Landry et al., 1980) and histamine receptors, as well as being a calmodulin inhibitor (Tanaka et al., 1982).

Trifluoperazine has been used as a calmodulin antagonist by Shechter (1984) to study the possibility that $[\text{Ca}^{2+}]_i$ mediates insulin stimulation of hexose transport in adipocytes. However the concentration of inhibitor required was 0.15mM, over an order of magnitude higher than that needed to inhibit calmodulin-mediated events. Trifluoperazine was used in concentrations (>1.0x10^{-5}M) which are several orders of magnitude greater than the drug's affinity for dopaminergic or serotonergic receptors (Landry et al., 1980). Thus there are two pathways apparently involved in the lipogenic response in rat adipocytes, the PKC pathway and the Ca dependent calmodulin pathway. In our work the antagonism of lipogenesis by a calmodulin inhibitor or by a PKC inhibitor suggests that both paths
are necessary for agonist stimulated lipogenesis to occur. The relative importance of each pathway may vary with time, in that calcium may be responsible for initiation, whereas diacylglycerol may be more important in maintaining the response.

A comment about the specificity of the PKC and calmodulin inhibitors used in our experiments is relevant, since these compounds are known to exert actions on other systems. Firstly, H7 is a relatively specific inhibitor of PKC (Hidaka et al., 1984) but is also known to inhibit cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (Hidaka et al., 1984). However, since cAMP antagonizes lipogenesis, inhibiting protein kinase A would enhance lipogenesis rather than inhibit it. The Ki value of H7 for PKC obtained from rabbit brain was shown to be 6μmol.l⁻¹ (Hidaka et al 1984), which is consistent with the concentrations at which H7 inhibited lipogenesis in the present study. Kawamoto and Hidaka (1984) also demonstrated that H7 antagonized PMA-stimulated phosphorylation of platelet proteins but did not block Ca-calmodulin dependent phosphorylation in Ca ionophore stimulated cells, consolidating the specificity of H7 for PKC and not other crucial systems involved in lipogenesis (viz. calmodulin). These observations may also be relevant to other cellular systems. There is always an aspect of any probe used which may cause concern. For example Cherqui et al. (1986) investigated the implication of protein kinase C in insulin stimulated glucose metabolism in rat adipocytes using mellitin as a PKC inhibitor (Couturier et al., 1984; Kuo et al., 1983). Mellitin however is also a phospholipase A2 inhibitor (Metz, 1986) and it can release lipoxygenase mediated metabolites of arachidonic acid in rat islet preparations. Furthermore as a cationic, membrane active peptide mellitin (like many drugs such as
48/80, mepacrine, tetracaine or trifluoperazine) may directly or indirectly release via electrostatic forces a membrane pool of calcium (Low et al., 1979; Smolen & Weissmann, 1982).

Polymyxin on the other hand has effects other than that on PKC. Polymyxins, at molar ratios 5/1 and 7/1 are known to cause a rapid permeability change of the cytoplasmic membrane (Ranck & Tocanne, 1982), and also result in a release of cellular materials (Storm et al., 1977). Thus the recent conclusion of Amir et al (1987) stating that polymyxin B (100μg.ml⁻¹ in vitro, 12/1 molar ratio of polymyxin B to insulin in vivo) is an inhibitor of insulin-induced hypoglycemia in the whole animal model and in vitro due to a specific inhibition of hexose transport (and subsequent glucose metabolism) is suspect.

The use of calmodulin inhibitors is more problematic, since they have been shown to inhibit other kinases, including C kinase (Kawamoto & Hidaka, 1984). However calmidazolium is considerably more specific than trifluoperazine (Van Belle, 1981) and as such was used in the present experiments. Caution should be exercised when interpreting the effects of such agents.

Thus we have used inhibitors/stimulators whose action is fairly specific in the light of today's knowledge. It is also important to mention that these agents are not 'toxic' at the concentrations used since they have been used at similar concentrations in other biological systems. Furthermore adipocytes were intact at the end of the incubation periods in our experiments and excluded trypan blue.

H7 the PKC inhibitor inhibited basal lipogenesis only at concentrations greater than 25μg.ml⁻¹, whilst 12.5μg.ml⁻¹ H7 was very significantly inhibitory to PMA stimulated lipogenesis. Similarly only
the highest polymyxin B dose (30μg.ml⁻¹) was significantly inhibitory to basal lipogenesis whilst 7.5μg.ml⁻¹ PB (not effecting basal lipogenesis) was sufficient to inhibit PMA and insulin stimulated lipogenesis. Verapamil and calmidazolium similarly are inhibitory to basal lipogenesis in higher concentrations than are required to inhibit PMA or insulin stimulated lipogenesis. The inhibitory effect of these inhibitory agents on basal lipogenesis is not surprising since the processes (enzymatic or otherwise) involved in basal lipogenesis are probably the same as those involved following stimulation. The only difference probably lies in the fact that these processes are accelerated following stimulation. The inhibitory probes which we have used act at various post-receptor sites of insulin action. Not unnaturally these sites of action are responsible for the metabolic activity of adipocytes even in the basal state.

From our experiments in the rat adipocyte it can be concluded that insulin receptor activation, calcium, protein kinase C and calmodulin activation are all necessary for lipogenesis to occur, if any one of these aspects is missing lipogenesis is markedly inhibited.

A precis of other studies when collated is as follows: insulin receptor activation stimulates the phosphodiesterase, phospholipase C, which hydrolyses membrane phosphoinositides to generate diacyl glycerol (Koepfer Hobelsberger & Wieland, 1984a; Farese et al., 1986). Insulin receptor-associated protein (tyrosine) kinase can also act as a phosphoinositol-kinase generating diacyl glycerol (Machicao & Wieland, 1984) and inositol triphosphate. Stimuli which raise intracellular Ca²⁺ levels can lead to membrane binding of PKC, thus exposing it to inner leaflet phospholipids and to the potential generation of DAG. This may be the 'priming' of the PKC system, allowing it to participate in
transmembrane signalling (Wolf et al., 1985). Diacyl glycerol activates PKC (Berridge, 1986).

Protein kinase C activates calcium channels, possibly through hyperphosphorylation of these portals (May et al., 1984; Jacobs et al., 1983), eliciting influx of extracellular calcium. Ca\(^{2+}\) seems to have a dual effect on PKC; membrane binding of PKC has been shown to be maximum at nanomolar calcium concentrations, whilst enzyme activation occurs at higher calcium concentrations. The Ca\(^{2+}\) - induced PKC binding to membranes may not be a sufficient condition for enzyme activation.

Insulin stimulates phosphorylation of calmodulin, a Ca\(^{2+}\) dependent process (Graves et al., 1986). Increased concentrations of intracellular calcium activates calmodulin, thereby initiating adipocyte lipogenesis. The synergism of Ca\(^{2+}\) and PKC activation is usually attributed to the requirement for Ca\(^{2+}\)-calmodulin, as well as PKC-mediated phosphorylation (Nishizuka, 1984b). A diagramatic representation of the possible intracellular post-receptor pathways involved in eliciting the lipogenic response are shown in Figure 6:1.

It must be noted that other studies have opposed each of the above and some of our observations. This aspect of insulin's second messengers seems to be divided by/into those workers who do not believe that calcium and PKC play a role in the post-receptor cascade (Denton, 1986). Denton also quotes work by Witters et al. (1986) stating that phorbol esters do not stimulate fatty acid synthesis. Klip & Ramlal (1987) have made it quite clear that a Ca\(^{2+}\) controversy exists also in the proposed relationship between hexose uptake and changes in cytoplasmic Ca\(^{2+}\) in fat cells and in other cells.
Figure 6.1

Hypothetical model of insulin receptor activation and second messenger system in rat adipocytes.
We used the A23187 potentiated insulin and PMA pathways to determine the site of action of the inhibitors prepared by ammonium sulphate precipitation. Inhibition of both these pathways by the 10 - 30kDa and 30 - 50kDa inhibitory fractions indicates that these inhibitors inhibit PKC and calcium mediated stimulation of adipocyte lipogenesis. Unfortunately, since there is no available method for stimulating calmodulin directly, it is difficult to assess the specific point of antagonism by these inhibitors. It is necessary to permeabilize cells to permit effective entry of calmodulin (MW 16,680). Koepfer-Hobelsberger & Wieland (1984b) have permeabilized adipocytes but state that the insulin effect is not demonstrable on permeabilized adipocytes. Furthermore, these workers should have demonstrated no changes in gross morphology of the adipocytes after permeabilization. Thus, although we have used a PKC agonist and antagonist, a calcium channel agonist and antagonist, only a calmodulin antagonist has been used in this work.

Another point at which the serum insulin inhibitor fractions could exert their effect was at the level of the glucose transporter. After the brain, the primary tissues with the greatest abundance of glucose transporter appear to be fat and muscle. Both these tissues are important for in vivo glucose homeostasis and their glucose transport systems are well known to be insulin sensitive. The erythrocyte (where transport is insulin insensitive) has a highly homologous transporter, as assessed by distribution of glucose transporter messenger RNA transcripts, to that present in fat tissue (Flier et al., 1987) suggesting a similar if not an identical transporter allowing its use in our studies. A further support of this observation is that antibodies against the erythrocyte glucose transporter recognize putative transporters in adipocytes (Cushman -
personal communication to Flier et al., 1987) and there is considerable homology between the rat and human glucose transporters in several tissues (Flier et al., 1987). The erythrocyte is therefore a useful tool in a study such as ours. Since it has no sensitivity to insulin, it is a useful model for examining the glucose transporter alone. The 0-1kDa, 10-30kDa and 30-50kDa serum inhibitory fractions did not affect cytochalasin B binding to erythrocyte membranes. The bound/free values achieved by these fractions being similar to the bound/free value of the buffer effect. The unfractionated 'crude' ammonium sulphate inhibitor, however, showed high cytochalasin B binding activity as did the >50kDa fraction. This possibly represents binding to human albumin. Albumin has hydrophobic sites for the binding of fatty acids, and cytochalasin B is a small hydrophobic molecule.

Chronic hyperinsulinaemia in humans is usually associated with systemic insulin resistance (Rabinowitz & Zierler, 1962; Salans et al., 1968; Olefsky et al., 1982; Kolterman et al., 1980). In several rat models however it is accompanied by insulin hyperresponsiveness at the cellular level (Kobayashi & Olefsky, 1979; Guerre-Millo et al., 1985) and is associated with increased insulin-stimulated glucose transport (Kobayashi & Olefsky, 1979). Kahn et al. (1987) suggest that experimental hyperinsulinaemia in the rat may be in part due to an increase in glucose transporter translocation in response to insulin. States of insulin resistance could be explained by depletion of intracellular glucose transporters in the basal state resulting in fewer being translocated to the plasma membrane in response to insulin. Insulin probably shifts transporters from the interior to the surface of the membrane. After this translocation an activation of the transporter at the surface probably
occurs. Witters et al. (1985) have recently demonstrated that the purified glucose transporter from human erythrocytes is a substrate for protein kinase C. Furthermore, these workers found that PMA stimulates the in vivo phosphorylation of the glucose transporter in erythrocytes. There is also evidence that the stimulation of glucose transport by PMA is due to translocation of intracellular transporters to the plasma membrane. (Kitagawa et al., 1985). Gibbs et al. (1986) however, have demonstrated that insulin does not lead to phosphorylation of the glucose transporter and since the stimulation of transport by insulin is much greater and more rapid than by PMA they claim it is unlikely that the stimulation of transport by insulin proceeds by the same mechanism as that for PMA, which involves PKC activation. However, Kahn et al. (1987) have demonstrated that there is an increase in glucose transporter translocation in response to insulin. The insulin-like growth factor II receptor is another integral membrane protein that is translocated to the plasma membrane in adipocytes in response to insulin (Wardzala et al., 1984; Oka et al., 1984). Collating this information it is quite likely that PMA induced activation of PKC and the consequent phosphorylation/translocation of the glucose transporter represents only one part of the stimulatory effect of insulin. Insulin may have other effects independent of protein kinase C. In this context, it is of interest that Olefsky (1976) has shown an insulin stimulated effect on glucose oxidation independent of glucose transport i.e. an effect on intracellular mechanisms is implied.

The inhibitors did not inhibit cytochalasin B binding to erythrocyte membranes in our experiments. We therefore conclude that the inhibitors do not affect the glucose transporter. We can also conclude that the
inhibitors have a post-receptor effect, therefore they are not insulin receptor antagonists i.e. stimulation of lipogenesis via PKC alone and PKC+A23187 is blocked by these inhibitors. Another aspect of this same point is that the nominal molecular weight classification of 10 - 50kDa also precludes the possibility of entry into cells. Very few proteins cross membranes. Substances that are known to do so are the steroids and the toxins such as cholera toxin. This leaves the possibility that these inhibitors block Ca\textsuperscript{2+} channels.

We investigated another serum insulin inhibitor recently found by acid-ethanol extraction and discussed by Dean et al. (1984). These workers collected their blood samples in heparin, presumably 2U.ml\textsuperscript{-1}. Kriauciunas et al. (1987) have demonstrated in adipocytes incubated with heparin (10 - 100U.ml\textsuperscript{-1}) a dose dependent inhibition of insulin stimulated glucose oxidation and to a lesser extent, of basal glucose oxidation. The effect of heparin was greater in insulin-stimulated glucose oxidation suggesting a possible effect on receptor signal transduction or a direct effect on the glucose transport protein. To avoid any artefactual possibility we chose to use serum instead of plasma in our study. Another effect of heparinized blood collection is an elevation of non-esterified fatty acids due to the lipolytic effect (activation of lipoprotein lipase) of heparin in vivo (Mikhailidis et al., 1987). Unsaturated NEFAs are known to potentiate insulin action on adipocytes (Lomeo et al., 1986). Thus, in the study of insulin inhibitors it is more appropriate to use a heparin free environment.

Furthermore, Dean et al., (1984) in their preparation of the acid-ethanol extract adjusted the pH of the supernatant to 7.4 but did not discard the resultant precipitate. This precipitate consists mainly of
sodium chloride and although some proteins may also be precipitated it is not correct to freeze dry such a suspension to be used as an inhibitory preparation. Furthermore Dean et al. (1984) do not specify whether they used buffer, saline or water to reconstitute their freeze dried extract. We found that the freeze drying of the extract after adjustment of the pH to 7.4 (and without removal of the resultant precipitate) resulted in a turbid suspension on reconstitution with water.

Removal of both glucose and insulin in preparations which are to be assayed in a system dependent on these factors is also necessary. This aspect has not been attended to in the work of Dean et al. (1984). The procedure for glucose removal by PD10 column chromatography was chosen for its simplicity and reproducibility. This procedure did, however, exclude all <5kDa molecular weight substances which were then assayed after ultrafiltration into a <1kDa and a 1-5kDa fraction. Some insulin (MW: 6000) contained in the acid-ethanol extracts is eluted with the low molecular weight fraction, thus there is decreased insulin concentrations in the Preparations B (glucose free acid-ethanol extracts).

If one was conducting a study in the control and NIDD populations, the differences in these factors (glucose and insulin) would be minimal on account of the dilutional effect of various assay procedures. Therefore, one could assume that the interference in assays was similar throughout the study. However, if the IDD population was to be included these assumptions would not hold. For this reason we found acid-ethanol extracts from the control and NIDD populations had similar activities, having no effect on basal lipogenesis but inhibiting insulin (10mU.l⁻¹) stimulated lipogenesis. The acid-ethanol extracts from the IDD population
showed a different pattern of effect in the lipogenesis bioassays, being stimulatory to basal lipogenesis due to their high insulin content but significantly inhibitory and not additive in their effect to insulin-stimulated lipogenesis. The stimulation of basal lipogenesis by these preparations, although significant, was not commensurate with the extremely high immunoreactive insulin concentrations (>160mU.l⁻¹ in Preparations A and >16mU.l⁻¹ in incubates containing 0.1ml of Preparations A with 0.9ml of adipocyte suspensions in buffer).

We have previously shown that IgG stimulates adipocyte lipogenesis and that this effect is mediated by the Fc portion of the molecule. An IgG-mediated effect would also enhance the effect of insulin stimulation in the lipogenesis bioassay system. Therefore, the relative inability of the acid-ethanol extracts to stimulate adipocyte lipogenesis and in fact to inhibit insulin-stimulated lipogenesis is probably indicates that this effect is not mediated by IgG. Furthermore, acid-ethanol treatment of serum precipitates all proteins of high molecular weight, including IgG. This was confirmed by the fact that IgG was not detectable in these preparations.

Thus, the removal of insulin and glucose from acid-ethanol extracts reduced the inhibitory activity of extracts prepared from controls and NIDDs and 'unmasked' the inhibitory activity of extracts from IDDs. When assayed in the cartilage-based somatomedin bioassay the acid ethanol extracts (Preparations A) from the three groups were inhibitory to [³⁵S]-sulphate uptake but there was no difference between the control, NIDD and IDD responses. These extracts are therefore inhibitory to the peripheral action of both insulin and somatomedin. Thus the inhibitor prepared by
acid-ethanol extracts of plasma is similar to the inhibitor(s) prepared by ammonium sulphate precipitation of sera in its biological activity.

Our current work emphasises the importance of excluding unlabeled (cold) glucose from test substances in incubates as this reduces the uptake of radiolabelled glucose by adipocytes giving the impression of an inhibitory activity. In our experiments Preparations A (containing glucose and insulin) were consistently more inhibitory than Preparations C (with insulin and glucose removed) from controls and NIDDS. It is also relevant to mention that chromatography of the acid-ethanol extract, on Sep-pak columns, led to the elution of glucose amongst the fractions which were inhibitory to insulin. This method of purification of acid-ethanol fractions has been used by Dean et al. (1984) in their work. The ultrafiltration of the low molecular weight (<5kDa) obtained after filtration on PD10 columns resulted in a 0-1kDa fraction which was consistently inhibitory. Some of this inhibition was probably due to its glucose content. The 1-5kDa fractions consistently stimulated basal lipogenesis and synergized insulin-stimulated lipogenesis; this was probably due to their intrinsic insulin content. Whether the presence of glucose and insulin in acid-ethanol extracts prepared by Dean et al. (1984) could have qualitatively altered their results is difficult to comment on. It is nevertheless clear from our experiments that (a) the markedly elevated serum glucose concentration in some NIDDS would lead to higher glucose–concentrations in the acid-ethanol extracts and to a greater likelihood of interference in the adipocyte lipogenesis assay; and (b) acid-ethanol extracts from IDDs may have their intrinsic inhibitory activity masked by insulin. This latter fact may explain why Dean et al. (1984) found no inhibitory activity in extracts from IDDs.
More recently Dean et al. (1987) have reported that further characterization of the inhibitor from the sera of NIDDs has revealed an inhibitor with molecular weight of 300-400Da.

Insulin inhibitory activity in rat sera has been described in hypoinsulinaemic states: malnutrition (Salmon, 1972), hypophysectomy (Salmon, 1974; Salmon, 1975), and diabetes (Phillips et al., 1979; Phillips & Scholz, 1982). Their nature is poorly understood, but they appear to be proteins with the major species of molecular weight of approximately 25kDa. Our work shows that diabetic rats have three major somatomedin-inhibiting fractions, <1kDa, 10-30kDa and >300kDa. Of these fractions, >300kDa is the most potent and is also found in the serum of normal rats.

Phillips et al. (1982) detected increased somatomedin inhibitors in renal failure. Uraemic sera contained increased levels of inhibitors of molecular weight about 900. These appear to be peptides, and were found in normal urine as well. Minuto et al. (1982) have also reported the presence of a circulating inhibitor in uraemic subjects, but did not characterize it. Folli et al. (1986) demonstrated that human uraemic serum renders normal rat hepatocytes resistant to insulin. These workers did not pretreat the uraemic serum, nor did they preselect any molecular weight, although previous workers (Bergstrom et al., 1979) assume the molecular weight range of 1-5kDa for the 'uraemic toxin'.

Thus, acid-ethanol extracts of sera from control and diabetic patients have inhibitory activity which antagonizes the action of both insulin and somatomedin. The activity of this inhibitor in NIDDs and IDDs is not greater than that in controls. It cannot, therefore, explain the pathogenesis of insulin resistance in diabetics. We also conclude that
any future work with anti-insulin or insulin-like factors must exclude glucose and insulin from interfering with the bioassay systems used.

In conclusion, we have identified insulin inhibitory activity in the sera of control and diabetic subjects of molecular weight ranges <1kDa, 10-30kDa and 30-50kDa. This inhibitory activity appears to have a post receptor effect both in the rat adipocyte lipogenesis assay and the $[^{35}S]$-sulphate uptake by cartilage. The protein kinase C and calcium-calmodulin pathways are essential for lipogenesis to occur and inhibition of protein kinase C and calcium mediated stimulation of lipogenesis by the 10-30kDa and 30-50kDa inhibitory fractions indicates that these inhibitors indeed have an effect on post-receptor pathways. Whereas the inhibitory activity of these fractions is not increased in sera of diabetic or obese patients, it is significantly increased in obese non insulin-dependent diabetics. Finally, this work indicates the necessity of removing interfering substances such as glucose and insulin from human sera fractions when testing for their insulin inhibitory/stimulatory activity.
FUTURE WORK

1. Further purification of the inhibitors identified in this work may be carried out by gel filtration, electrophoresis on polyacrylamide gels, isoelectric focusing, high pressure liquid chromatography.

2. As new and more specific agonists and antagonists of the protein kinase C and calcium-calmodulin pathways are identified, further investigation of the pathways involved in eliciting the insulin response may be investigated. The identification of these pathways would help in the determination of the point of action of the serum inhibitors of insulin.

3. Chronic renal failure, chronic liver disease, systemic infections and pregnancy are some of the states associated with insulin resistance. Measurement of insulin inhibitory activity in serum fractions described in this work may provide an insight into the pathogenic mechanisms involved.


J Lab Clin Med 7: 251-266


Bergstrom J, Furst P & Zimmerman L. (1979) Uremic middle molecules exist and are biologically active. 
Clin Nephrol 11: 229-238.


Br Med J 2: 1541-1544


Acta Endocrinol (Kbh) 75: 233-242

J Biol Chem 234: 2540-2543


Science 207: 19-29.

Acta Endocrinol (Copenhagen) 77 Suppl 191: 137-143.

Biochem J 164: 2511-2555.

Diabetes 29: 665-667

Biochem Biophys Res Commun 121: 448-455.


Czech M P. (1981) Insulin action
Am J Med 70 (1) 142-150


Farese R V, Davis J S, Barnes D E (1985) The de novo phospholipid effect of insulin is associated with increases in diacylglycerol but not inositol phosphates or cytosolic Ca\(^{2+}\).
Biochem J 231: 269-278.


Diabetes 5: 391-396.


J Clin Invest 73: 1-4

Cell 36: 577-579.


Diabetes 23: 674-678.

Gomperts B D. (1983) Involvement of guanine nucleotide-binding protein in the gating of Ca$^{2+}$ by receptors.
Nature 306: 64-66


Lancet I: 127-130.


Phosphatidic acid and phosphatidylinositol labelling in adipose tissue.
Relationship to the metabolic effects of insulin and insulin-like agents.

Hope-Gill H, Kissebah A, Clark P, Vydelingum N, Tulloch B & Fraser T R.
(1976) Effects of insulin and procaine hydrochloride on glycogen synthetase activation and adipocyte calcium flux: evidence for a role of calcium in insulin activation of glycogen synthetase.
Horm Metab Res 8: 184-190.

Diabetes 30: 562-567.


Jacobs S, Sahyoun N E, Saltiel A R & Cuatrecasas P. (1983) Phorbol esters stimulate the phosphorylation of receptors for insulin and somatomedin C.
Proc Natl Acad Sci USA 80: 6211-6213.


Science 206: 1047-1048.


Europ J Pharmacol 136: 311-316.


Kahn C R & Rosenthal A. (1979)
Immunologic reactions to insulin, insulin allergy, insulin resistance and the auto immune insulin syndrome.
Diabetes Care 2: 283-295.


J Clin Invest 79: 853-858.


Biochem Biophys Res Commun 128: 824-832.


Eur J Clin Invest. 5: 339-349.

Lancet 1, 7899: 144-147.

Horm Metab Res 6: 247-255.


Lowell F C. (1944) Immunologic studies in insulin resistance. II. The presence of a neutralizing factor in the blood exhibiting some characteristics of an antibody. J Clin Invest 23: 233-240


Arch Exp Pathol Pharmakol 26: 271-287.

Metz S A. (1986) Lack of specificity of mellitin as a probe for insulin release mediated by endogenous phospholipase A2 or lipoxygenase.

Diabetologia 14: 255-259.


J Physiol (Lond) 333: 655-679.
Life Sci 34: 1205-1221.


Horm Metab Res 6: 12-16.

Biochim Biophys Acta 737: 1-49.


J Clin Endocrin Metab 51: 739-743.

Biochem Biophys Res Comm 128: 1364-1372.


Nature (Lond) 287: 863-865

Mishikawa M, Uemura Y, Hidaka H & Shirakawa S. (1986) 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7). a potent inhibitor of protein kinases, inhibits the differentiation of HL-60 cells induced by phorbol diester.


Science 225: 1365-1370.

Phospholipid turnover in hormone action.
Recent Prog Horm Res 40: 301-345.


Proc Natl Acad Sci USA 81: 4028-4032.


Biochem Biophys Res Comm 71: No 1 106-113.


Cell Calcium 5: 111-130.


Lancet 1: 1144-1145


Diabetes 32: 1117-1125.


Endocrinol 104: 1519-1524.


Clin Research 30: 781A.


Physiol Rev 64: 938-984.


Horm Metab Res 18: 244-246.

Diabetes 25: 250-255.


Diabetologia 28: 70-75.


Biochim Biophys Acta 755: 144-147.


Diabetologia 13: 243-249.


Siddle K & Hales C N. (1974) The action of local anaesthetics on lipolysis and on adenosine 3'5'-cyclic monophosphate content in isolated rat fat cells.
Biochem J 142: 345-351.

Tokyo McGraw-Hill Kogakusha Ltd.


Arch Biochem Biophys 211: 192-201.


- 301 -

Insulin-like growth factors, somatomedins, basic chemistry, biology, clinical importance: 619-628 (Ed) E M Spencer W de Gruyter

Inhibition of somatomedin-like activity by serum from streptozotocin-diabetic rats: prevention by insulin treatment and correlation with skeletal growth.
Endocrinol 121: 1360-1365.

Tuerkischer E B & Wertheimer E. (1946) Factors influencing deposition of glycogen in adipose tissue of rat.

Uhlenbeck O C. (1972) Complementary oligonucleotide binding to transfer RNA.
J Mol Biol 65: 25-41

Lancet 1: 1226-1228


J Lipid Res 2: No 4 293-316.


Am J Med 73: 461-464
Wertheimer E & Shapiro B. (1948) Physiology of adipose tissue.


Annals N Y Acad Sci 411: 135-140.

Arch Dis Child 54: 295-298.


Medicine, Baltimore, 42: 385-402.

Publications of Author

Diabetologia 27:296A.

Clinical Science 67:72P.

Diabetes 33:173A

Diabetes Research and Clinical Practice Suppl 1:S553.

Diabetes 34: Suppl 1:613

Clinical Science 70: Suppl 13:81P.


Human plasma fractions inhibit the action of insulin on adipocytes and somatomedin on cartilage

N. Avasthy, M. A. Khokher, A. M. Taylor and P. Dandona

Metabolic Unit, Department of Chemical Pathology and Human Metabolism, Royal Free Hospital and School of Medicine, London, UK

Summary. Since human immunoglobulins exert an insulin-like stimulatory effect on adipocyte lipogenesis at concentrations markedly lower than those found in vivo, and since human serum or plasma are only mildly stimulatory, we predicted that human serum probably contains an inhibitor of adipocyte lipogenesis. Supernatant preparations, obtained from the precipitation of immunoglobulins from plasma in 2.5 mol/l ammonium sulphate, were extensively dialysed and tested for their activity on bioassay systems commonly used for measuring insulin. The supernatants produced a marked inhibition of basal and insulin- or IgG-stimulated lipogenesis and glucose oxidation by adipocytes at protein concentrations of 10 mg/l. The supernatants were further purified through ultrafiltration to demonstrate two main inhibitory fractions, 10 to 30 K and 30 to 50 K, which again produced marked inhibition of basal and insulin- or IgG-stimulated adipocyte lipogenesis and glucose oxidation. These fractions were then tested for basal and serum somatomedin-stimulated 35S sulphate uptake by porcine cartilage: both basal and serum somatomedin-stimulated 35S uptake were significantly inhibited (p < 0.01). Therefore, normal human serum contains at least two peptides which are markedly inhibitory to glucose metabolism and insulin action on adipocytes and 35S transport and somatomedin action on cartilage.

Key words: Insulin inhibitors, somatomedin inhibitors.

We have recently demonstrated that human immunoglobulins IgG [1] and IgM [2] exert an insulin-like stimulatory effect upon lipogenesis and glucose oxidation by rat and human [3] adipocytes. This effect is mediated through the Fc moiety [3, 4] of the IgG molecule, and is not neutralised by anti-insulin antisera. This effect of Ig must therefore contribute to the non-suppressible insulin-like activity of serum/plasma. In fact, we have observed that serum non-suppressible insulin-like activity attributable to a high molecular weight fraction (NSILP) may be antigenically similar to IgG [5]. Since this potent stimulatory effect of immunoglobulins is exerted at very low concentrations when compared with those present in plasma, and since plasma itself has only a marginal stimulatory effect upon adipocyte metabolism, we postulated the presence in plasma of an inhibitor of immunoglobulin stimulated adipocyte lipogenesis [1, 3]. Our preliminary observations did, indeed, reveal the presence of such an inhibitor [1]. We have now expanded our investigations to attempt to define the nature of this inhibitor. The experiments described in this paper show that there are probably two inhibitors in human plasma which are active not only against the stimulatory effect of insulin and immunoglobulins on adipocytes, but also on that of serum somatomedin on 35S uptake by cartilage.

Materials and methods

Analytical grade sodium chloride, sodium hydroxide, sodium hydrogen carbonate, potassium chloride, calcium chloride, sodium dityrogen phosphate, glucose, triton X-100 (scintillation grade), toluene, hyamine-10x hydroxide (scintillation grade), methanol and sulphuric acid were obtained from British Drug House (BDH, Enfield, UK). D-[3,5-3H] glucose and D-[U-14C] glucose were obtained from Amersham International (Amersham, UK). Bovine serum albumin fraction V was obtained from Armour Pharmaceuticals (Eastbourne, UK). Collagenase from clostridium histolyticum (specific activity 131 U/mg) was obtained from Millipore Corporation (Bedford, NJ, USA). Epididymal fat pads were obtained from caesarian-delivered male Sprague-Dawley rats, weighing 100-180 g and kept on an ad libitum diet (Grain Harvesters Brand 41B). Amicon ultrafiltration membranes were obtained from Amicon Limited (Stonehouse, Glous., UK).

Cartilage was removed from the sternal ends of the lowest ribs of freshly killed pigs in an abattoir and transported in isotonic saline (4°C) to the laboratory. Discs were cut from the cartilage and used in the somatomedin bioassay the same afternoon.

Preparation of the inhibitory fraction from serum

Supernatants were obtained from normal human serum by precipitation of serum globulins in 2.5 mol/l ammonium sulphate followed by 10-min centrifugation at 1500 g. The supernatants were dialysed extensively for 72 h against water. The protein content of dialysed supernatants was then measured by the method of Lowry et al. [6]. These fractions were assayed for their inhibitory activity on adipocyte lipogenesis and [14C]glucose oxidation to CO2 as described below.

Further fractionation of these supernatant preparations was carried out through sequential filtration on ultrafiltration membranes (Amicon) with nominal molecular weight cut-offs: - 1, 5, 10, 30, 50, 100, and 500 K.

Each fraction thus obtained was assayed for its action on adipocytes and 35S uptake by cartilage. Initial preparatory experiments were carried out at room temperature, but later it was found that there was some loss of biological activity of these inhibitor fractions at room temperature. Further preparatory work was carried out at 4°C.

The biological activity of these supernatant preparations was tested in the following biological systems: (1) adipocyte lipogenesis; (2...
The effect of serum inhibitor on rat adipocyte lipogenesis in the presence of either insulin or IgG

Table 1.

<table>
<thead>
<tr>
<th>Insulin concentration (mU/l)</th>
<th>Insulin alone</th>
<th>Insulin + inhibitor (10 mg/l)</th>
<th>IgG concentration (mg/l)</th>
<th>IgG alone</th>
<th>IgG + inhibitor (10 mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>75 ± 8*</td>
<td>0</td>
<td>100</td>
<td>41 ± 5*</td>
</tr>
<tr>
<td>5</td>
<td>215 ± 44</td>
<td>196 ± 7</td>
<td>50</td>
<td>152 ± 8</td>
<td>44 ± 56</td>
</tr>
<tr>
<td>10</td>
<td>500 ± 28</td>
<td>200 ± 8*</td>
<td>100</td>
<td>182 ± 10</td>
<td>75 ± 8*</td>
</tr>
<tr>
<td>20</td>
<td>1054 ± 40</td>
<td>360 ± 12*</td>
<td>200</td>
<td>817 ± 13</td>
<td>163 ± 4*</td>
</tr>
</tbody>
</table>

Results are expressed as percentage stimulation over basal ±SEM. Basal counts: 257 cpm (100%); non-specific background counts: 87 cpm; n = 6. *p < 0.01 when compared with basal; b p < 0.01 when compared with the corresponding dose of insulin or IgG.

Studies on rat adipocytes

Rat adipocytes were prepared from epididymal fat pads by collagenase digestion in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 1% albumin [7]. The cells were gently but extensively washed and resuspended in the same buffer. Adipocyte lipogenesis was assayed by the method described by Moody et al. [8] as adapted by us [9]. [U-14C]-glucose oxidation to [14C]-CO2 was measured as described by us previously [10]. All incubations with insulin-inhibitory fractions, with and without insulin, were carried out by incubating the fractions with adipocytes for the entire incubation period of 120 min.

The effect of heat treatment on inhibitor preparations was assessed by incubating these preparations for 20 min at 60°C and 80°C respectively in a shaking water bath. These treated preparations were assayed by the rat lipogenesis bioassay.

Porcine cartilage 35S-sulphate uptake

The effect of the supernatants on 35S-sulphate uptake by porcine cartilage was tested in the presence and absence of serum somatomedin activity as described by Spencer and Taylor [11] and as used to test the effect of other agents on this system [12]. All incubations of cartilage with inhibitory fractions were carried out for the entire incubation period of 24 h, as previously described [11].

Statistical analysis and expression of results

Results were analysed using paired Student's t-tests (two-tailed), and are all expressed as % change over basal (100%). The absolute basal counts per minute are shown with each Table and Figure. A minimum of 4 experiments was carried out for each Table or Figure shown. Each Table or Figure shows the results obtained from one experiment only so that the values shown are directly comparable.

Results

Supernatants obtained following ammonium sulphate precipitation of serum and prolonged dialysis against water caused a marked inhibition of basal and insulin-stimulated lipogenesis. A similar inhibition of [U-14C]-glucose oxidation to [14C]-CO2 was observed. Heat treatment of the supernatant decreased the inhibitory activity observed over basal and insulin-stimulated lipogenesis. This preparation was not tested for its effect on 35S-sulphate uptake by cartilage.

Further purification of the inhibitor using ultrafiltration membranes showed that two major molecular fractions, 10–30 K and 30–50 K, were markedly inhibitory to adipocyte lipogenesis. Minimal inhibition was also observed with a fraction of <10 K (data not shown). Fractions of >50 K were consistently stimulatory in the adipocyte lipogenesis assay (data not shown).

Each of the fractions inhibitory in the adipocyte assays, 10–30 K and 30–50 K, were also assayed for their action on 35S-sulphate uptake by porcine cartilage. Both fractions inhibited the basal and serum stimulated 35S-uptake by cartilage markedly in a dose-related fashion (Figs. 1, 2).

Incubation of the crude inhibitory fraction at 60°C resulted in over 75% loss of inhibitory activity whilst that at 80°C resulted in its total loss.
Table 4. Inhibition of basal and insulin stimulated-lipogenesis in rat adipocytes by serum inhibitor fractions 10-30 kilodaltons (A) and 30-50 kilodaltons (B)

<table>
<thead>
<tr>
<th>Test</th>
<th>A Percentage stimulation + SEM (basal counts: 270 cpm = 100%) n = 6</th>
<th>B Percentage stimulation + SEM (basal counts: 250 cpm = 100%) n = 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>1.25 mU/l</td>
<td>226 ±28</td>
</tr>
<tr>
<td></td>
<td>2.50 mU/l</td>
<td>264 ±37</td>
</tr>
<tr>
<td></td>
<td>5.00 mU/l</td>
<td>338 ±38</td>
</tr>
<tr>
<td></td>
<td>10.00 mU/l</td>
<td>448 ±34</td>
</tr>
<tr>
<td></td>
<td>20.00 mU/l</td>
<td>531 ±27</td>
</tr>
<tr>
<td>Fractions</td>
<td>0.5 mg/l</td>
<td>88 ±15</td>
</tr>
<tr>
<td></td>
<td>1.0 mg/l</td>
<td>72 ±18*</td>
</tr>
<tr>
<td></td>
<td>2.0 mg/l</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td>3.0 mg/l</td>
<td>0*</td>
</tr>
<tr>
<td>Fractions + 2.5 mU/l insulin</td>
<td>0.5 mg/l</td>
<td>193 ±17b</td>
</tr>
<tr>
<td></td>
<td>1.0 mg/l</td>
<td>84 ±15b</td>
</tr>
<tr>
<td></td>
<td>2.0 mg/l</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3.0 mg/l</td>
<td>0</td>
</tr>
</tbody>
</table>

* p <0.01 when compared with basal;  
* p <0.01 when compared with corresponding dose of insulin

Discussion

The data presented show a marked antagonistic effect of the inhibitor preparations on basal and insulin- or IgG-stimulated lipogenesis. A similar potent inhibitory effect was observed on the oxidation of glucose to CO₂ by adipocyte suspensions. Clearly, therefore, plasma/serum contains potent inhibitors of adipocyte metabolism; these inhibitors may have a modulatory effect upon the metabolic actions of insulin and immunoglobulins in vivo. The two active inhibitory fractions, 10-30 K and 30-50 K also inhibited ³⁵ S uptake markedly. This inhibition was evident with and without the addition of the standard human serum which has a stimulatory effect on this preparation due to its intrinsic somatomedin content.

It is noteworthy that an inhibitor of somatomedin action has previously been recognised in sera of rats rendered experimentally diabetic [13]; this inhibitor is a protein which neutralises the biological effect of non-suppressible insulin-like activity-S (NSILA-S). It has been suggested that this protein inhibits plasma NSILA-S by binding to it. The large molecular weight of this NSILA-S binding protein complex prevents the transcapillary passage of NSILA-S and this is thought to protect the putative target tissues from an effect of NSILA-S in vivo. The inhibitory effect of this carrier protein was also observed when assessing the activity of NSILA-S in vitro; this was caused by inhibiting binding of NSILA-S to perfused rat heart. This carrier protein does not, however, inhibit the stimulatory effect of insulin on myocardial glucose uptake, and exerted no effect on the binding of insulin to perfused rat heart.

In contrast to these observations, our inhibitor preparations were equally effective in inhibiting: (1) basal
and insulin- and IgG-stimulated adipocyte metabolism and (2) basal and serum somatomedin-stimulated 35S sulphate uptake by cartilage. Therefore, it is not likely that these inhibitory fractions are specific binding proteins. No comment, however, is possible regarding the association/binding of these inhibitors to any of these stimulators in circulation. Ultrafiltration experiments show that either there are two molecular species (10-30 K and 30-50 K) of inhibitor. Whereas the two fractions are equipotent on the rat adipocyte lipogenesis assay, the higher molecular weight fraction (30-50 K) is more potent in the cartilage bioassay. This difference in inhibitory potency is evident against both basal and human serum-stimulated 35S uptake by cartilage (Figs. 1, 2). Phillips and co-workers [14] have recently isolated three molecular fractions from sera of diabetic rats which are inhibitory to 35S sulphate uptake by cartilage under basal conditions and following somatomedin stimulation. Whereas one of our inhibitors may be related to their 24 K inhibitor, their other two inhibitors of molecular weights of 250 K and 940 K are not likely to be related to the two main adipocyte/cartilage inhibitors described by us.

The inhibitors described by us inhibit both basal and insulin/IgG-stimulated adipocyte metabolism. Since insulin and IgG stimulate adipocyte metabolism through different receptors, our inhibitory fractions interfere with insulin/IgG effects on adipocytes at a post-receptor level. Furthermore, these inhibitory fractions have profound effects on two different targets organs, the adipose tissue and the cartilage. This suggests that the inhibitors may have a general toxic effect, or that they affect a key pathway involved in the mediation of hormonal effects on various tissues: more definite comments on the mechanism of action are not possible at the present stage. Our preparatory techniques indicate that these inhibitors are not fatty acids, since they were not dialysed out even after 72-96 h of continuous dialysis against water. The inhibitors appear to be heat labile proteins which lose over 75% of their biological activity at 60 °C and all of it at 80 °C. These inhibitors are not related to synalbumin antagonist of insulin described by Vallance-Owen [15, 16], since (1) they are not associated with albumin on ultrafiltration (molecular weight of inhibitors is 10-50 K, whereas that of albumin is >70 K); (2) synalbumin antagonist has no effect on adipose tissue; and (3) synalbumin antagonist is found only in sera of diabetic patients.

The biological significance of these inhibitors is not clear at present and requires further elucidation. Whether the deficiency of such inhibitors may result in hypersensitivity or hyper-responsiveness to insulin in vivo, or that their excess would result in insulin resistance, is an area which requires critical evaluation. Similarly, these inhibitors may modulate sensitivity to somatomedins and thus affect growth processes. Meuli et al. [13] have suggested that their carrier protein may be responsible for 'protecting tissues' from uncontrolled activity of NSILA; our inhibitor may be responsible for a similar effect.

References


Received: 29 November 1985
and in revised form: 3 April 1986
Inhibition of Somatomedin-Like Activity by Serum from Streptozotocin-Diabetic Rats: Prevention by Insulin Treatment and Correlation with Skeletal Growth*


Department of Chemical Pathology and Human Metabolism (A.M.T., P.D.), and Department of Neurology (P.K.T.), Royal Free Hospital and School of Medicine, London NW3 2QG, United Kingdom; Department of Growth and Development (A.M.T.), Institute of Child Health, London, United Kingdom; and Department of Anatomy (A.K.S., I.G.M.D., D.S.B.), Marischal College, University of Aberdeen, Aberdeen, United Kingdom

ABSTRACT. Diabetes mellitus was induced in rats by streptozotocin. This gave rise to a loss of somatomedin activity in serum. The loss of somatomedin activity was due to the presence of inhibitors associated with serum proteins having mol wts of less than 1, 1-10, 30-50, and 300 K. Whereas less than 1, 1-10, and 30-50 kilodalton fractions were not inhibitory in control and insulin-treated animals, greater than 300 kilodalton fraction was inhibitory in control and insulin-treated animals; the inhibitory activity of this fraction in diabetic animals was significantly greater than that in controls and insulin-treated animals. The appearance of these inhibitors in diabetic animals was accompanied by reduced skeletal growth. Treatment of diabetic animals with insulin abolished the somatomedin-inhibitory activity of serum and corrected the skeletal growth deficit. Serum inhibitors of somatomedin may, therefore, be involved in the causation of some of the complications of diabetes, including impaired skeletal growth. (Endocrinology 121: 1360-1365, 1987)

SKELETAL growth is impaired in rats with streptozotocin-induced diabetes (1). It is known that serum from diabetic rats has significantly lower somatomedin activity (2) and immunoreactive somatomedin C concentrations (3) than serum from normal rats. It has also been shown that serum from diabetic rats contains inhibitors of somatomedins (4). These are inhibitory both to somatomedins in [35S]Sulfate uptake-based bioassays and to insulin in assays based on adipocyte lipogenesis. Three mol wt fractions have so far been identified: less than 1, 23, and 250 k (5). We have also recently identified two fractions in normal human sera which are inhibitory to the stimulatory effect of somatomedin on porcine cartilage and to that of insulin on rat adipocyte lipogenesis (5) and glycogen synthesis by the rat diaphragm (6).

In view of these observations, we set out to investigate whether we could confirm the presence of insulin-somatomedin inhibitors in sera from normal rats and from rats with experimental diabetes, to establish whether the activity of these inhibitors would be altered after treatment with insulin, and to correlate the findings with changes in skeletal growth.

Materials and Methods

Three groups each of nine male Sprague-Dawley rats, aged 11 weeks and weighing between 357 and 515 g (mean: 456 g) were studied over 16 weeks: controls, untreated diabetics, and diabetics treated with a daily sc injection of ultralente insulin (Novo Alle, Denmark). Diabetes was induced by the ip injection of a buffered solution of streptozotocin at a dosage of approximately 55 mg/kg BW. Blood glucose was estimated daily at midday, using a Glucometer (Ames Corporation, UK) for each individual animal in the insulin-treated group. Insulin dosage was adjusted to maintain blood glucose levels within the normal range (4-7 mmol/liter). Every effort was made to keep the dosage as low as possible, to avoid the occurrence of hypoglycemia. Blood glucose concentrations were also estimated in the control and untreated diabetic rats at weekly intervals. Additionally, plasma glucose and glycosylated hemoglobin (HbA1c) levels were measured for the three groups at four weekly intervals. Plasma glucose measurements were made using a Beckman glucose analyzer and HbA1c was determined according to the method of Ross and Gibson (7). Body weights were recorded once weekly. The animals were reared in plastic cages and maintained on Oxoid diet with water ad libitum.

1360
Skeletal growth was assessed in the control group at the beginning of the study and for all three groups in the final week. One hind limb was radiographed in a standard position with the animal under ether anesthesia, and tibial length measured on x-ray plates using a magnifying eyepiece containing a graticule.

At the end of the study, animals were anesthetized with ether and 7–10 ml blood obtained by cardiac puncture, allowed to coagulate, and centrifuged for 10 min at 1000 rpm. The serum was removed and stored at −20°C.

In order to confirm the consistency of our data related to the appearance of an inhibitory activity in serum of diabetic rats and its disappearance after insulin therapy, we repeated the entire experiment three times. Detailed growth measurements, however, were carried out only once.

Preparation of plasma fractions

Pooled plasma from diabetic treated, diabetic untreated, and control rats was separated into size-graded macromolecular mixtures by fractionation through Amicon's Diaflo (5) ultrafiltration membranes using a stirred cell. The plasma was sequentially ultrafiltered through membranes with the following nominal mol wt cut-offs: 1,000; 10,000; 30,000; 50,000; 100,000; 300,000. Each preparation of the fractions was reconstituted in saline to the volume of the original serum source.

Assay of sera and serum fractions

Serum samples and the serum fractions prepared as above, from the three experimental groups, namely end controls, untreated diabetics, and diabetics treated with insulin, were then assayed for: 1) somatomedin activity, in a system using [*S] sulfate uptake as an index of stimulation (8, 9) (each fraction was assayed at serial concentrations of 50%, 25%, 12%, and 6% in Ham's F12 medium); and 2) insulin-like activity, in a system based on rat adipocyte lipogenesis (10, 11). Similar bioassays were carried out on the fractions prepared from serum.

Statistical analysis

Comparisons were made on mean values (±SE) by Student's t test.

Results

Blood glucose, HbA1c, and BW

The blood glucose concentrations in the control rats throughout the study ranged between 3.9 and 6.9 mmol/liter with little interanimal variation. The values for the insulin-treated diabetic group ranged from 2.0–19.8 mmol/liter during the first week of insulin therapy; however, the extremes of this range rarely occurred, and never on 2 consecutive days. After the first week of therapy, when the dose of insulin had been adjusted, blood glucose levels were generally between 3.0 and 9.0 mmol/liter throughout the period of the study. The plasma glucose concentrations in the untreated diabetic rats ranged between 18.8 and 32.7 mmol/liter, with a mean of 25.5 mmol/liter. The HbA1c levels, measured for all groups at 4-weekly intervals, are shown in Fig. 1. This fraction in the untreated diabetic rats was slightly but significantly increased at 4 weeks as compared with controls (P < 0.005) but the increase was more marked from 8 weeks onwards (P < 0.001). The HbA1c levels in the insulin-treated group were normalized throughout the entire period of the study, except at 8 weeks, when the mean fraction was slightly but significantly less in the insulin-treated group when compared with the controls (P < 0.05), suggesting a degree of overcorrection of the blood glucose with insulin. The mean insulin dosage required to obtain control for each individual animal was initially 8–14 daily, but the dosage had to be increased progressively, presumably because of the increasing body weights of the animals. Body weight increased progressively in the insulin-treated diabetic animals in parallel with the controls but declined significantly from 445 ± 13.3 to 388 ± 18.2 g in the untreated diabetic animals (P < 0.02). Thus satisfactory metabolic control was achieved in the insulin-treated diabetic animals as assessed by measurements of body weight, blood, and plasma glucose levels and HbA1c concentrations.

Skeletal length

Measurements of tibial length for all groups are shown in Table 1. The mean value was significantly greater for the end control as compared with the onset control stage (P < 0.001), and thus growth occurred over the period of observation. It was significantly less in the untreated diabetic rats as compared with the end control value (P < 0.001), but was not significantly different from the onset control value. Tibial length in the insulin-treated group did not differ from that of the end control, and the values for both these groups were significantly greater than for the untreated diabetics and the onset controls.
INHIBITION OF SOMATOMEDIN-LIKE ACTIVITY IN DIABETIC RATS

Skeletal length was therefore normalized by insulin treatment.

Assays of sera from animals

There was a remarkable consistency in the data obtained from three separate experiments. Basal and somatomedin-stimulated uptake of $[^35]S$ sulfate by porcine cartilage was markedly inhibited by sera from diabetic animals (see Figs. 2 and 3).

Control sera and sera from diabetic animals treated with insulin had an intrinsic stimulatory activity. Sera from control and insulin-treated diabetic animals had no effect on the action of standard human serum.

Assay of various molecular fractions revealed that there were four inhibitors in the diabetic rat serum: less than 1, 1–10, 30–50 kilodaltons (kDa) and greater than

<table>
<thead>
<tr>
<th>TABLE 1. Effect of diabetes and insulin treatment of diabetes on tibial length in rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tibial length (mm)</td>
</tr>
<tr>
<td>Onset controls (9)*</td>
</tr>
<tr>
<td>End controls (9)</td>
</tr>
<tr>
<td>Diabetics (10)</td>
</tr>
<tr>
<td>Insulin-treated diabetics (9)</td>
</tr>
</tbody>
</table>

Note that untreated diabetics do not differ from controls, and that insulin-treated diabetic rats do not differ from end controls.

* Number of animals.

* Mean ± SEM.

* Significantly different from onset controls (P < 0.001).

* Significantly different from end controls (P < 0.001).

* Significantly different from diabetics (P < 0.001).

FIG. 2. Somatomedin activity ($[^35]S$-uptake by cartilage) of sera from control (●), diabetic (O), and insulin-treated diabetic (A) rats. The figure shows the results of three separate experiments with nine animals in each group. Horizontal bars show SD.
The inhibitory activity of greater than 300 kDa fraction was the greatest (Fig. 6). Less than 1, 1-10, and 30-50 kDa fractions from control and insulin-treated diabetic animals had no intrinsic inhibitory activity. Greater than 300 kDa fraction exhibited inhibitory activity in control, diabetic, and insulin-treated animals. The inhibitory activity of the greater than 300 kDa fraction from diabetic rats was significantly greater than that seen in controls or in insulin-treated diabetic rats. Insulin treatment reduced the inhibitory activity of this fraction but the inhibitory activity was still significantly lower than that in controls. The inhibitory activity of all three inhibitory fractions was observed against both basal and somatomedin-stimulated [³⁵S]sulfate uptake (Fig. 6). Thirty to 50 kDa fraction in control and insulin-treated animals exhibited stimulatory activity consistent with the presence of somatomedin bound to its carrier protein.

In the rat adipocyte lipogenesis bioassay, sera from all three animal groups were stimulatory. No evidence of inhibition or of differences in stimulation between the end controls, untreated diabetic, and insulin-treated diabetic groups was detected.

There was no correlation between the degree of impairment of growth (weight gain or increase in tibial length) with the overall inhibitory activity of diabetic sera. Correlations between the inhibitory activities of various fractions and growth could not be assessed since fractions could be prepared only from pooled specimens.

Discussion

Rats continue to grow throughout a large proportion of their lifespan. Animals rendered diabetic with strepto...
activity in the sera of the untreated diabetic rat, and that this is related to the development of inhibitory factors against somatomedin. Treatment with insulin prevented the appearance of this inhibitory activity. These observations confirm the findings of Phillips et al. (2, 4), who also described a diminution in somatomedin activity in the serum of diabetic rats and the prevention of this diminution after treatment with insulin. They have likewise attributed this to the emergence of inhibitory activity. Neither the study by Phillips et al. (2, 4) nor our own distinguishes between the effect of malnutrition on the increase in serum inhibitory activity in diabetic rats. Whether malnutrition results in the formation of inhibitory fractions in the same molecular weight range as described in this paper is the subject of further investigation in our laboratory.

Our preliminary experiments aimed at elucidating the molecular species of the inhibitors in diabetic rat plasma demonstrate four different types: the fractions with mol wt of 1) less than 1 K and 2) 1-10 K; 3) a fraction with a mol wt between 30-50 K; and 4) a fraction with a mol wt greater than 300 K. Phillips and his co-workers (4) have also described three molecular species of inhibitors in the diabetic rat: 250, 24, and 0.91 K. All three are similar to the mol wts of the fractions that we have detected. It is important, however, that the most potent inhibitor in our experiments on streptozotocin-diabetic rats was one with a mol wt greater than 300 K in all three groups of animals, being markedly more inhibitory in sera from diabetic rats than in that from controls. Phillips et al. (2, 4) reported that the high mol wt inhibitor (250 K) had no effect on basal $^{35}$S-uptake, and that the inhibitory activity of this fraction was weak. Our high mol wt inhibitor (>300 K) was extremely potent and inhibited both basal and somatomedin-stimulated $^{35}$S-uptake. This difference may be due to the difference in the rat strains used. The emergence of an inhibitory activity in the 30-50 kDa fractions which are stimulatory in controls and insulin-treated diabetic rats is also of interest. It signifies the production of a potent inhibitor in this fraction which normally has somatomedins bound to their carrier proteins. The inhibitory activity of this fraction was totally suppressed (or prevented) by adequate insulin treatment. This observation is also new. Further characterization of these inhibitors is important, since at least one of them (>300 K) exists in normal animals. They may thus have a role in the regulation of anabolic processes in general and growth in particular.

Our data not only confirm the presence of somatomedin inhibitory fractions as described by Phillips et al. (2, 4), they also demonstrate the presence of the most inhibitory of these fractions in normal rat serum. Whether this inhibitor has a role in the anabolic and growth processes of normal rats is worth further investigation. Two of the smaller fractions arise de novo in diabetic rats at weaning show a retardation of growth (1). The rats used in the present study had passed the period of most rapid postnatal growth (1). Nevertheless, significant skeletal elongation was evident from the measurements of tibial length between the onset and end control stages, made at an interval of 16 weeks. Our data clearly demonstrate that the induction of diabetes still gives rise to an inhibition of skeletal growth at this age. Furthermore, body weight was reduced in the diabetic group; this is presumably partly related to an inhibition in growth, but also to the catabolic effects of uncontrolled diabetes.

The present results have established that the induction of diabetes is associated with a loss of somatomedin activity in the sera of the untreated diabetic rat, and that this is related to the development of inhibitory factors against somatomedin. Treatment with insulin prevented the appearance of this inhibitory activity. These observations confirm the findings of Phillips et al. (2, 4), who also described a diminution in somatomedin activity in the serum of diabetic rats and the prevention of this diminution after treatment with insulin. They have likewise attributed this to the emergence of inhibitory activity. Neither the study by Phillips et al. (2, 4) nor our own distinguishes between the effect of malnutrition on the increase in serum inhibitory activity in diabetic rats. Whether malnutrition results in the formation of inhibitory fractions in the same molecular weight range as described in this paper is the subject of further investigation in our laboratory.

Our preliminary experiments aimed at elucidating the molecular species of the inhibitors in diabetic rat plasma demonstrate four different types: the fractions with mol wt of 1) less than 1 K and 2) 1-10 K; 3) a fraction with a mol wt between 30-50 K; and 4) a fraction with a mol wt greater than 300 K. Phillips and his co-workers (4) have also described three molecular species of inhibitors in the diabetic rat: 250, 24, and 0.91 K. All three are similar to the mol wts of the fractions that we have detected. It is important, however, that the most potent inhibitor in our experiments on streptozotocin-diabetic rats was one with a mol wt greater than 300 K in all three groups of animals, being markedly more inhibitory in sera from diabetic rats than in that from controls. Phillips et al. (2, 4) reported that the high mol wt inhibitor (250 K) had no effect on basal $^{35}$S-uptake, and that the inhibitory activity of this fraction was weak. Our high mol wt inhibitor (>300 K) was extremely potent and inhibited both basal and somatomedin-stimulated $^{35}$S-uptake. This difference may be due to the difference in the rat strains used. The emergence of an inhibitory activity in the 30-50 kDa fractions which are stimulatory in controls and insulin-treated diabetic rats is also of interest. It signifies the production of a potent inhibitor in this fraction which normally has somatomedins bound to their carrier proteins. The inhibitory activity of this fraction was totally suppressed (or prevented) by adequate insulin treatment. This observation is also new. Further characterization of these inhibitors is important, since at least one of them (>300 K) exists in normal animals. They may thus have a role in the regulation of anabolic processes in general and growth in particular.

Our data not only confirm the presence of somatomedin inhibitory fractions as described by Phillips et al. (2, 4), they also demonstrate the presence of the most inhibitory of these fractions in normal rat serum. Whether this inhibitor has a role in the anabolic and growth processes of normal rats is worth further investigation. Two of the smaller fractions arise de novo in diabetic rats at weaning show a retardation of growth (1). The rats used in the present study had passed the period of most rapid postnatal growth (1). Nevertheless, significant skeletal elongation was evident from the measurements of tibial length between the onset and end control stages, made at an interval of 16 weeks. Our data clearly demonstrate that the induction of diabetes still gives rise to an inhibition of skeletal growth at this age. Furthermore, body weight was reduced in the diabetic group; this is presumably partly related to an inhibition in growth, but also to the catabolic effects of uncontrolled diabetes.

The present results have established that the induction of diabetes is associated with a loss of somatomedin activity in the sera of the untreated diabetic rat, and that this is related to the development of inhibitory factors against somatomedin. Treatment with insulin prevented the appearance of this inhibitory activity. These observations confirm the findings of Phillips et al. (2, 4), who also described a diminution in somatomedin activity in the serum of diabetic rats and the prevention of this diminution after treatment with insulin. They have likewise attributed this to the emergence of inhibitory activity. Neither the study by Phillips et al. (2, 4) nor our own distinguishes between the effect of malnutrition on the increase in serum inhibitory activity in diabetic rats. Whether malnutrition results in the formation of inhibitory fractions in the same molecular weight range as described in this paper is the subject of further investigation in our laboratory.

Our preliminary experiments aimed at elucidating the molecular species of the inhibitors in diabetic rat plasma demonstrate four different types: the fractions with mol wt of 1) less than 1 K and 2) 1-10 K; 3) a fraction with a mol wt between 30-50 K; and 4) a fraction with a mol wt greater than 300 K. Phillips and his co-workers (4) have also described three molecular species of inhibitors in the diabetic rat: 250, 24, and 0.91 K. All three are similar to the mol wts of the fractions that we have detected. It is important, however, that the most potent inhibitor in our experiments on streptozotocin-diabetic rats was one with a mol wt greater than 300 K in all three groups of animals, being markedly more inhibitory in sera from diabetic rats than in that from controls. Phillips et al. (2, 4) reported that the high mol wt inhibitor (250 K) had no effect on basal $^{35}$S-uptake, and that the inhibitory activity of this fraction was weak. Our high mol wt inhibitor (>300 K) was extremely potent and inhibited both basal and somatomedin-stimulated $^{35}$S-uptake. This difference may be due to the difference in the rat strains used. The emergence of an inhibitory activity in the 30-50 kDa fractions which are stimulatory in controls and insulin-treated diabetic rats is also of interest. It signifies the production of a potent inhibitor in this fraction which normally has somatomedins bound to their carrier proteins. The inhibitory activity of this fraction was totally suppressed (or prevented) by adequate insulin treatment. This observation is also new. Further characterization of these inhibitors is important, since at least one of them (>300 K) exists in normal animals. They may thus have a role in the regulation of anabolic processes in general and growth in particular.

Our data not only confirm the presence of somatomedin inhibitory fractions as described by Phillips et al. (2, 4), they also demonstrate the presence of the most inhibitory of these fractions in normal rat serum. Whether this inhibitor has a role in the anabolic and growth processes of normal rats is worth further investigation. Two of the smaller fractions arise de novo in diabetic
INHIBITION OF SOMATOMEDIN-LIKE ACTIVITY IN DIABETIC RATS

1365

rats; the emergence of both inhibitors in diabetic animals was prevented by insulin administration.

We have recently demonstrated at least two proteins with mol wts between 10–30 K and 30–50 K, respectively, separated from human serum, that are markedly inhibitory to basal and serum somatomedin-stimulated [³⁵S] sulfate uptake by porcine cartilage and to basal and insulin-stimulated adipocyte lipogenesis (5) and insulin-stimulated glycogen synthesis by the rat diaphragm (6). Our preliminary observations show that these inhibitors do not affect the binding of insulin to its receptors on adipocytes. This inhibitory activity is thus likely to be exerted at postreceptor level. We are at present investigating the possibility that these inhibitory fractions may be elevated in the serum of human diabetics. It is of interest that Dean et al. (12) have recently shown insulin inhibitory activity in sera from maturity onset diabetic patients which is found in supernatants prepared from the acid-ethanol precipitation of large mol wt proteins from these sera. Our own experiments show that these fractions are also inhibitory to somatomedin activity, and that this inhibitory activity also occurs in fractions prepared from sera of insulin-dependent diabetics and normal subjects (13).

The present results have demonstrated that the changes in somatomedin activity in the diabetic rat parallel those in skeletal length. Tibial length is reduced in the untreated diabetic animals, but is normalized in the insulin-treated rats in which the somatomedin activity is restored. The serum inhibitory fractions responsible for the changes in somatomedin activity may therefore have a role in the pathogenesis of some of the complications of diabetes, including impairment of growth. The elucidation of the nature, the biological effects, and the clinical role of these fractions requires further elucidation.

Acknowledgment

We thank Dr. I. S. Ross, Department of Chemical Pathology, University of Aberdeen, for plasma glucose and glycosylated hemoglobin estimations.

References

THE ROLE OF CALCIUM IN MEDIATING PHORBOL ESTER- AND INSULIN-STIMULATED ADIPOCYTE LIPOGENESIS

N. AVASTHY, J. Y. JEREMY and P. DANDONA

Metabolic Unit, Department of Chemical Pathology and Human Metabolism, Royal Free Hospital and School of Medicine, London, UK.
(Received 25 February 1988)

SUMMARY The roles of protein kinase C, calcium and calmodulin in mediating insulin-stimulated lipogenesis by rat adipocytes were investigated using the protein kinase C activator, phorbol myristate acetate (PMA); the protein kinase C inhibitors, H7 and polymixin B; the calcium ionophore, A23187; the calcium channel blocker, verapamil; and the calmodulin inhibitor, calmidazolium. PMA caused a concentration-dependent, parallel left shift of the insulin-lipogenesis dose response curve. Both PMA- and insulin-stimulated lipogenesis were inhibited by H7 and polymixin B. A23187 enhanced the stimulatory action of both insulin and PMA was not inhibited by H7. The stimulatory effects of insulin and PMA were inhibited by verapamil and calmidazolium. These data indicate that insulin receptor-lipogenesis coupling in rat adipocytes is mediated by protein kinase C-elicited calcium influx and activation of calmodulin.

Key words: Adipocyte lipogenesis, protein kinase C, insulin

INTRODUCTION

It is well established that insulin elicits changes in intracellular calcium concentrations in target tissues, and it has been proposed that calcium and calcium-activated calmodulin are key mediators of the insulin-induced metabolic signal (1, 2). The mechanisms linking insulin receptor to calcium mobilization are unknown but it has been proposed that insulin action is mediated by protein kinase C (PKC) (3-5). Evidence for this proposal comes from studies which demonstrated that in adipocytes insulin-stimulated lipogenesis was mimicked by the PKC activators, phorbol esters (3-5), and antagonized by the PKC inhibitor, mellitin (6). Furthermore, a recent study has demonstrated that adrenoceptor-calcium influx coupling linked to prostacyclin synthesis in vascular tissue is elicited by PKC activation (7). It is thus possible that insulin action is mediated by a similar protein kinase C-Ca^{2+} channel system.

In order to investigate the relationship between calcium and PKC in mediating insulin-stimulated lipogenesis by isolated rat adipocytes, the following experiments were carried out: (a) the effects of the PKC activator phorbol myristate acetate (PMA) (8) and interactions of PMA with insulin; (b) the effects of calcium ionophore A23187 and interactions of A23187 with PMA and insulin; (c) the effects of the PKC inhibitors, 1-(5-isoquinolinylsulphonyl)-2-methylpiperizine (H7) (9) and polymixin B (10) on insulin and PMA action; (d) the effect of the calcium channel blocker, verapamil (11), and the calmodulin inhibitor calmidazolium (R24571) (12) on PMA- and insulin-stimulated lipogenesis.

MATERIALS AND METHODS

Materials

Analytical grade sodium chloride, sodium hydroxide, sodium hydrogen carbonate, potassium chloride, sodium dihydrogen phosphate, sodium fluoride, verapamil, calcium ionophore A23187, phorbol myristate acetate (PMA), 1-(5-isoquinolinylsulphonyl)-2-methyl piperizine (H7), polymixin B sulfate, calmidazolium (R24571), and glucose were obtained from Sigma Chemical Company (Poole, Dorset, UK). Dimethyl sulphoxide (DMSO) was obtained from British Drug House (BDH; Enfield, Middlesex, UK). Extractive scintillation solution (EXSCINT) was obtained from National Diagnostics (Aylesbury, Bucks, UK). D[3-^3H]-glucose was obtained from
Figure 1. Effect of PMA on insulin-stimulated adipocyte lipogenesis. (□) insulin alone; (■) insulin + PMA (4 x 10^-8 M); (●) insulin + PMA (8 x 10^-8 M). Each point = mean ± S.E., n = 5.

Figure 2. Effect of H7 on PMA-stimulated lipogenesis. (1) basal; (2) PMA (1.6 x 10^-7 M); (3) H7 (4.3 x 10^-3 M); (4) H7 (8.6 x 10^-3 M); (5) H7 (1.7 x 10^-4 M); (6) PMA (1.6 x 10^-7 M) + H7 (4.3 x 10^-3 M). Each histogram = mean ± S.E., n = 5.

Figure 3. Effect of polymixin B (PB) on PMA-stimulated lipogenesis. (1) basal; (2) PMA (1.6 x 10^-7 M); (3) PB (7.5 mg.1^-1); (4) PB (15 mg.1^-1); (5) PB (30 mg.1^-1); (6) PMA (1.6 x 10^-7 M) + PB (7.5 mg.1^-1); (7) PMA (1.6 x 10^-7 M) + PB (15 mg.1^-1); (8) PMA (1.6 x 10^-7 M) + PB (30 mg.1^-1). Each histogram = mean ± S.E., n = 5.

Figure 4. Effect of polymixin B (PB) on insulin-stimulated lipogenesis. (1) basal; (2) insulin (5 mU.1^-1); (3) insulin (10 mU.1^-1); (4) H7 (4.3 x 10^-3 M); (5) H7 (4.3 x 10^-3 M) + insulin (5 mU.1^-1); (6) H7 (4.3 x 10^-3 M) + insulin (10 mU.1^-1); (7) PB (15 mg.1^-1); (8) PB (15 mg.1^-1) + insulin (5 mU.1^-1); (9) PB (15 mg.1^-1) + insulin (10 mU.1^-1). Each histogram = mean ± S.E., n = 5.

Methods

Rat adipocytes were prepared from epididymal fat pads by collagenase digestion in Krebs-Ringer bicarbonate buffer (KRB), pH 7.4, containing 1% albumin (13). The cells were gently but extensively washed and resuspended in the same buffer. Adipocyte lipogenesis was assayed by the method described by Moody et al. (14) and adapted by us (15).

Briefly, adipocytes were incubated with test material in KRB with [3-3H]-glucose (0.4 μCi per vial, overall final glucose concentrations 0.56 mmol.L^-1) for 2 h at 37°C under an atmosphere of 95% O_2, 5% CO_2, in a shaking water bath. The reaction was stopped at 2 h by the addition of Exscent (extractive scintillation solution), thorough shaking and then standing for 2 h. This procedure lyses adipocytes and extracts radio-labelled lipids. Lipid incorporated radioactivity was measured in a Philips Scintillation Spectrometer.

Stock test substances were prepared in KRB (polymixin B), DMSO (PMA and calcium ionophore) or ethanol (verapamil and H7), and diluted in KRB before assay. In pilot experiments the solvents used were found to be without effect on lipogenesis at the final concentrations of solvent in the assay buffer. In studies with antagonists (H7, polymixin B, verapamil and calmidazolium) adipocytes were preincubated for 30 min at 37°C with the antagonists prior to the addition of agonists (insulin, PMA and A23187) simultaneously with the addition of labelled glucose, and the adipocytes incubated for a further 2 h, as described above. These agents have been used at "non-toxic" concentrations, adipocytes being intact at the end of the 2 h incubations and excluding trypan blue. Other workers have used these probes at similar concentrations in other biological systems.

RESULTS

Phorbol ester (myristate acetate: PMA; 25 and 50 ng.ml^-1) caused a parallel left shift of the insulin-lipogenesis dose response curve (Figure 1). To demonstrate clearly the stimulation and inhibition of the lipogenesis assay in the bar diagrams, it must be noted that basal lipogenesis is taken as 100%. Synergism is
considered to occur when the stimulation is greater than the sum of the two separate responses above the basal value.

PMA (100 ng/ml)-stimulated lipogenesis was inhibited in dose-dependent manners by both the protein kinase C inhibitors, H7 (Figure 2) and polymixin B (Figure 3). In addition, insulin-stimulated lipogenesis was also inhibited by H7 (Figure 4) and polymixin B (Figure 4). These data indicate that insulin-induced lipogenesis is mediated by PKC.

The calcium ionophore A23187 (an agent which creates artificial calcium channels; 16) was a weak agonist of lipogenesis (Figure 5), but synergized the lipogenic action of both PMA (Figure 6) and insulin (Figure 5). The calcium channel blocker verapamil inhibited basal and PMA (Figure 7)- or insulin (Figure 8)-stimulated lipogenesis. These data indicate that both PMA- and insulin-stimulated lipogenesis involve the influx of extracellular calcium. Since phorbol ester-stimulated lipogenesis was blocked by verapamil, these data suggest that protein kinase C activation elicits this mobilization of activator calcium.

The calmodulin inhibitor calmidazolium inhibited both insulin- and A23187+insulin-stimulated lipogenesis (Figure 9), indicating that calmodulin mediates the lipogenic action of insulin. Trifluperazine also inhibited insulin- and A23187+insulin-stimulated lipogenesis (data not shown).

**DISCUSSION**

The stimulation of lipogenesis by the protein kinase C activator PMA, and the antagonism of PMA- and insulin-stimulated lipogenesis by protein kinase C inhibitors, reported here, consolidates the view that insulin action is mediated by PKC (3-5). The inhibition of insulin-stimulated lipogenesis by verapamil, a calcium
channel blocker (5), and the potentiation of insulin action by the calcium ionophore A23187, which alone had little stimulatory action, demonstrates that insulin-stimulated lipogenesis is mediated by the mobilization of extracellular and/or membrane-bound calcium. Furthermore, in the present study, PMA-stimulated lipogenesis was also inhibited by verapamil and potentiated by A23187, suggesting that the present phorbol ester action is also mediated by calcium mobilization. Synergistic interactions between phorbol esters and A23187 have been demonstrated in other tissues. For example, phorbol ester-stimulated release reactions by mast cells (17), neutrophils (18) and platelets (18-21) are markedly potentiated by concentrations of A23187 which alone have little or no effect (17-21). With respect to PKC-calcium interactions, it has recently been proposed that PKC activates both voltage-dependent and receptor-linked calcium channels in chromaffin cells and smooth muscle (7,10), possibly through hyperphosphorylation of these portals (22,23). In view of the present data, it is reasonable to propose that insulin receptor-lipogenesis coupling in rat adipocytes is mediated, in part, through a similar mobilization of activator calcium by PKC (activated by the diacyl glycerol generated from insulin receptor-initiated phosphoinositide hydrolysis).

The present study also demonstrates that calmodulin inhibition with calmidazolium antagonizes insulin-stimulated lipogenesis, confirming the findings of Shechter (24), who demonstrated that adipocyte lipogenesis was antagonized by the calmodulin inhibitor trifluperazine. We too have shown a similar inhibitory effect of trifluperazine on both basal and insulin-stimulated lipogenesis (Avasthy and Dandona, unpublished observations). Since calmodulin is activated by increases of intracellular calcium (25), it is possible that insulin/PKC initiation of calcium mobilization activates calmodulin, which in turn stimulates lipogenesis.

A comment about the specificity of the PKC and calmodulin inhibitors used in the present experiments is relevant, since these compounds are known to exert actions on other systems. Firstly, H7 is a relatively specific inhibitor of PKC (9) but is also known to inhibit cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (9). However, since cAMP antagonizes lipogenesis, inhibiting protein kinase A would tend to enhance lipogenesis rather than inhibit it. The Ki value of H7 for PKC obtained from rabbit brain was shown to be 6 μmol/l (9), which is consistent with the concentrations at which H7 inhibited lipogenesis in the present study. Kawamoto and Hidaka (26) also demonstrated that H7 antagonized PMA-stimulated phosphorylation of platelet proteins but did not block Ca²⁺-calmodulin-dependent phosphorylation in Ca²⁺ ionophore-stimulated cells, consolidating the specificity of H7 for PKC and not other systems involved in lipogenesis (viz. calmodulin). Polymixin B, on the other hand, may have effects other than that on PKC but this compound has not been fully investigated.

The use of calmodulin inhibitors such as calmidazolium and trifluperazine is more problematic, since they have been shown to inhibit other protein kinases, including C kinase (26) and to possess high affinity for other receptors (e.g., dopamine, serotonin and histamine; 27)). However, calmidazolium is considerably more specific than trifluperazine (12) and as such was used in the present experiments. Nevertheless, caution should be exercised when interpreting the effects of calmodulin inhibitors.

It is also important to mention that the inhibitory agents used inhibited both basal as well as PMA-stimulated adipocyte lipogenesis. This is not surprising, since the processes (enzymatic or otherwise) involved in basal lipogenesis are probably the same as those involved following stimulation, the only difference being that these processes are accelerated following stimulation. The inhibition of basal as well as agonist-stimulated adipocyte lipogenesis by inhibitors is well established (28,29).

From the results of the present study, a hypothetical scheme of PKC-calcium-calmodulin intracellular mediation of insulin receptor-lipogenesis coupling by adipocytes is postulated as:

(a) Insulin receptor activation stimulates the phosphodiesterase, phospholipase C, which hydrolyses membrane phosphoinositides to generate diacyl glycerol (and inositol phosphate; 30,31).
(b) Diacyl glycerol activates PKC (32).
(c) Activated PKC may then (i) stimulate phosphorylation of other calcium channels, possibly through hyperphosphorylation of these portals (7,22,23), eliciting influx of extracellular and/or membrane bound calcium.
(d) Increased concentrations of intracellular calcium...
activates calmodulin (25), thereby initiating adipocyte lipogenesis.

REFERENCES


