ENHANCEMENT OF INSULIN SIGNALLING IN ADIPOSE TISSUE BY MALARIAL EXTRACTS

by

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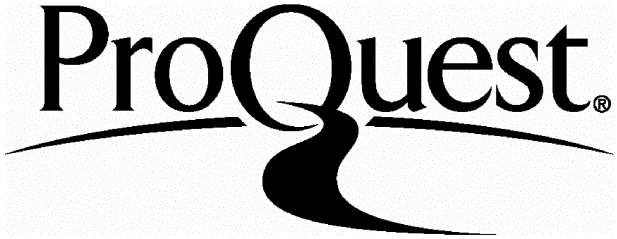

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ABSTRACT

Malarial extracts (MEs) derived from the erythrocytic stages of *Plasmodium* infection in mice have been shown to synergise with insulin in both stimulating lipogenesis and inhibiting lipolysis in rat adipocytes *in vitro*. In this study, rat adipocytes were used as a test system in an attempt to determine where in the insulin signalling network these malarial extracts were exerting their effects.

Initial studies demonstrated that the MEs had no effect on insulin receptor binding or internalisation, nor did they enhance insulin stimulated 3-O-methylglucose uptake. Investigation of their effects on the activity of the downstream enzymes involved in lipogenesis showed that they had no effect on the stimulation of pyruvate dehydrogenase by insulin. They did however potentiate insulin's stimulation of the committal enzyme in fatty acid synthesis, acetyl-CoA carboxylase (ACC), without changing its total activity.

The MEs were also shown to synergise with insulin in the activation of glycogen synthesis. They appeared to exert this effect by enhancing the insulin stimulation of glycogen synthase, having no effect on the inactivation of glycogen phosphorylase activity by insulin. Neither epidermal growth factor nor isoproterenol had any effect on the ability of the MEs to synergise with insulin and enhance glycogen synthesis and the MEs did not induce EGF to stimulate glycogen synthesis. Isoproterenol did however block any insulin-mimetic effect of the MEs.

The MEs synergistically enhanced the effect of insulin to increase anti-phosphotyrosine immunoprecipitatable phosphatidylinositol 3-kinase (PI3-kinase) activity. Since both ACC and glycogen synthase have been shown to be stimulated by insulin through a PI3-kinase dependent pathway, these results suggest that the ME's effects on PI3-kinase may be a key step in their synergistic enhancement of insulin signalling.

As well as synergising with insulin, some of the ME preparations were also able to stimulate the production of tumour necrosis factor (TNF) by isolated macrophages. However there was no correlation between these two properties of the MEs. There was also no correlation between the synergistic effects of the MEs and their ability to mimic insulin.

While these results were very interesting it should be noted that the malarial extracts used in this project were specifically chosen for their ability to synergise with insulin. Of the total number of malarial extracts prepared only approximately 40% actually demonstrated this ability.

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THIS THESIS IS DEDICATED TO MY MUM AND DAD

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ABBREVIATIONS

Ab	Antibody
ACC	Acetyl-Coenzyme A carboxylase
AICAR	5-amino-4-imidazolecarboxamine ribonucloeside
αMu	Anti-murine
AMPK	AMP-activated protein kinase
ARDS	Adult respiratory distress syndrome
BCA	Bicichinoic acid
BSS	Buffered salt solution
CaMK	Calmodulin-dependent multiprotein kinase
cAMP-PK	cyclic AMP-dependent protein kinase
cGI-PDE	cyclic GMP-inhibited cyclic AMP phosphodiesterase
cGI-PDE IK	cGI-PDE Insulin-stimulated serine kinase
CHO	Chinese Hamster Ovary
CK-I/II	Casein kinase I/II
CPT	Carnitine palmitoyl transferase
DTNB	5-5'dithiobis(2-)nitrobenzoic Acid
DTT	Dithiothreitol
EGF	Epidermal Growth Factor
eIF-2B/4E	eukaryotic Initiation Factor-2B/4E
4E-BP	eIF-4E Binding Protein
Erk 1/2	Extracellular regulated kinase 1/2
FCS	Foetal calf serum

FKBP12	FK506 Binding Protein 12
GAP	GTPase Activating Protein
GH	Growth Hormone
G1P	Glucose 1-phosphate
G6P	Glucose 6-phosphate
GPI	Glycosylphosphatidylinositol
GSK-3	Glycogen synthase kinase-3
GTPase	Guanosine triphosphatase
HMG-CoA	β-hydroxy-β-methylglutaryl-Coenzyme A
HSL	Hormone sensitive lipase
ICAM	Intracellular Adhesion Molecule
IEF	Insulin enhancing factor
IFN	Interferon
IGF-1	Insulin-like Growth Factor-1
IL	Interleukin
INT	p-Iodonitrotetrazolium violet die
i.p.	Intra-peritoneal
IRS-1/2	Insulin Receptor Substrate-1/2
i.v.	Intravenous
JNK	*jun* N-terminal kinase
KRB	Krebs Ringer Bicarbonate
LAR	Leukocyte common Antigen Related
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide

LPY	Lethal *Plasmodium yoelii*
Mab	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MAPKAPK	MAPK-activated protein kinase
ME	Malarial extract
MeOH	Methanol
Nrbc	Normal red blood cell
P.	*Plasmodium*
PbA	*Plasmoduim berghei Anka*
PBS	Phosphate buffered saline
P. chab	*Plasmodium chabaudi*
PDGF	Platelet Derived Growth Factor
PDH	Pyruvate dehydrogenase
PDK-1	PI-3,4,5-P_3-dependent protein kinase-1
PH	Pleckstrin Homology
PHAS-1/2	Phosphorylated Heat and Acid Stable protein-1/2
PI	Phosphatidylinositol
PI3-K	Phosphatidylinositol 3-kinase
PIP	Phosphatidylinositol phosphate
PKA/B/C	Protein kinase A/B/C
PP-1/2A/2B/2C	Protein phosphatase-1/2A/2B/2C
Prbc	Parasitized red blood cell

$p70^{S6K}$	70 kDa S6 kinase
PTPase	Protein tyrosine phosphatase
Rsk 1/2/3	Ribosomal S6 kinase 1/2/3
SD	Sprague-dawley
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error mean
SH2/3	Src Homology 2/3
SHPS-1	Src Homology containing Phosphatase Substrate-1
SH-PTP 2	Src Homology containing Protein Tyrosine Phosphatase
TAG	Triacylglycerol
TCA	Tricarboxylic acid cycle
TNF	Tumour necrosis factor
Tris.	Tris (hydroxymethyl) aminomethane
UDPG	Uridine 5'diphosphoglucose

All other abbreviations used are standard abbreviations according to the Biochemical Journal

CHAPTER ONE :

INTRODUCTION

1.1 BACKGROUND TO PROJECT

Hypoglycaemia has, in the past 15 to 20 years, become recognised as a common and serious complication of *falciparum* malaria (Phillips, 1989), particularly in young children (White, N. *et al.*, 1987; Taylor *et al.*, 1988; Grau *et al.*, 1989) and pregnant women (White *et al.,* 1983; Looareeswuwan *et al.*, 1985). There is a correlation between hypoglycaemia and mortality from malaria in many parts of the world. This is seen in both children, (White *et al.*, 1987; Taylor *et al.*, 1988) and adults, particularly those who have severe disease (White *et al.*, 1983). Hypoglycaemia is also a risk factor for residual neurological sequelae in patients who have been treated for the disease (White *et al.*, 1983; Taylor *et al.*, 1988; Bond, 1992). Several hypothesis have been proposed as the cause of hypoglycaemia in malarial infections. These include increased glucose consumption by both the host (White *et al.*, 1983) and the parasite (Sherman, 1979), impairment of gluconeogenesis (White *et al.*, 1983; White, N. *et al.*, 1987; Taylor *et al.*, 1988), depletion of liver glycogen (Kawo *et al.*, 1990) and overproduction of cytokines such as TNF, Il-1α and IL-6 (Grau *et al.*, 1989; Kwiatkowski *et al.*, 1990; Clark *et al.*, 1992). Hyperinsulinaemia due to quinine treatment has also been cited as the cause of hypoglycaemia in some studies, (White *et al.*, 1983; Looareesuwan *et al.*, 1985) however hypoglycaemia has also been noted before the onset of this treatment (Taylor *et al.*, 1988) . Hypoglycaemia can also be seen in murine models of malaria. Rats infected with *Plasmodium berghei* (*P. berghei*) (Holloway *et al.*, 1991) and mice infected with lethal *P. Yoelii* (Elased & Playfair, 1994) exhibit hypoglycaemia in the terminal stages of infection. The non-lethal *P. chabaudi* AS strain has been shown to cause transient hypoglycaemia during

the crisis stage of the infection in mice when parasite levels are high, the blood glucose levels returning to normal as the parasite levels fall (Elased & Playair, 1994).

In 1992, a group who had been working on the ability of soluble malarial antigens to induce TNF production by macrophages (Bate *et al.*, 1988, 1992a,b,c), found that these same malarial antigen preparations could induce hypoglycaemia when injected into normal mice (Taylor *et al.*, 1992a). The hypoglycaemic agent(s) from these malarial antigens, which were derived from mouse erythrocytes infected with *P. yoelii*, have been shown to contain an inositol phosphate motif (Taylor *et al.*, 1992a). A number of studies have shown that inositol phosphate glycans from both mammalian and trypanosome origin can mimic insulin effects in liver and adipose tissue cells (Kelly *et al.*, 1986; Saltiel & Cuatrecasas, 1986; Alemany *et al.*, 1987; Alvarez *et al.*, 1987; Kelly *et al.*, 1987; Mato *et al.*, 1987; Saltiel & Sorbara-Cazan, 1987; Machicao *et al.*, 1990; Misek & Saltiel, 1992; Field, 1997). Consequently it was hypothesised that these inositol phosphate containing malarial antigens may be inducing hypoglycaemia by mimicking insulin. To test this theory a collaboration was set up with Prof. E.D. Saggerson to determine if the malarial antigens could mimic insulin's effects on isolated rat adipocytes. They found however that the malarial antigens alone had little or no ability to stimulate lipogenesis or inhibit lipolysis in these cells. They did however find that the antigens were able to act synergistically with insulin and potentiate its effects both *in vivo* and *in vitro*, even at maximally effective insulin concentrations (Taylor *et al.*, 1992b)

It was this novel finding which led directly to the establishment of this project, the aim of which was to try and determine where in the insulin signalling network these insulin

synergising malarial extracts are exerting their effects.

1.2 MALARIA

1.2.1 History

Malaria is an ancient disease which has been infecting humans since prehistoric times. Mosquitoes, which are responsible for spreading the disease, have been found in a fossilised state which are more than thirty million years old. The fever caused by malaria and its association with marshes was first described in detail in the writings of the Greek philosopher Hippocrates in the 5th century BC. People believed that the disease was caused by the putrid air above stagnant water. Even in these classical times the Greeks and Romans practised malaria prevention by draining marshy land and building their houses on elevated positions. It was in the 18th century that the term 'Malaria' was coined, derived from the Italian 'Mal'aria', meaning 'bad-air' (Phillips, 1983; Wellcome Trust, 1991; Giles, 1993).

Despite being such an ancient disease, it was not until the late 19th century that the most important events in the history of malaria took place. In 1880, a French army surgeon working in Algeria, called Laverain, first saw and described malarial parasites in the red blood cells of human beings. Then in 1897, Ronald Ross , a surgeon major in the Indian army found malaria parasites in the stomach of a small mosquito which had previously fed on a patient with malaria. This small mosquito turned out to be a female *Anopholes* mosquito which was confirmed as the vector for malaria by Patrick Mason and his colleagues in 1900 (Giles, 1993). Mosquitoes lay their eggs into still water such as ponds and marshes which explains the association the early victims of

the disease made between the fever and marshy ground. The female can lay between 50 and 150 eggs at a time hence the potential for breeding, and consequently infection, is massive (Phillips, 1983).

As recently as 300 years ago most of Europe and Northern America were considered endemic areas for malaria. In 1982 the WHO published figures which showed that up until a few decades ago 72% of the world population still lived within malaria zones. This figure has now reduced to 40%. However if the theories of global warming are correct, the spread of the *Anopholes* mosquito's habitat and hence the malarial parasites, could endanger the mainly immmunologically naive population of Europe (Anderson *et al.*, 1996).

Malaria is one of the worlds most common tropical diseases. There are approximately one hundred million cases reported annually, of which between 1.5 and 2.7 million are fatal (Anderson *et al.*, 1996). The severity of the disease and outcome of the infection are dependent upon many factors, including the immune status and the drug treatment given to the host. Fatalities generally occur amongst immigrants from non-endemic areas and young children who have not yet developed immunity to the disease (Marsh *et al.*, 1987).

Attempts to control malaria in the past have included a combination of removal of the *Anopheles* vector by reducing its habitat and using insecticides, and by drug prophylaxis against the parasite. In the late 1950s the WHO programme to eradicate malaria managed to relieve many temperate regions, such as Europe and North America, of the disease but were less successful in tropical countries. The emergence

of resistant strains of both *Anopholes* and the parasite has led to a resurgence of the disease in some areas. As a result scientists are now developing new drugs and investigating the possibility of vaccination as a way of controlling malaria. (Phillips, 1983; Gilles, 1993)

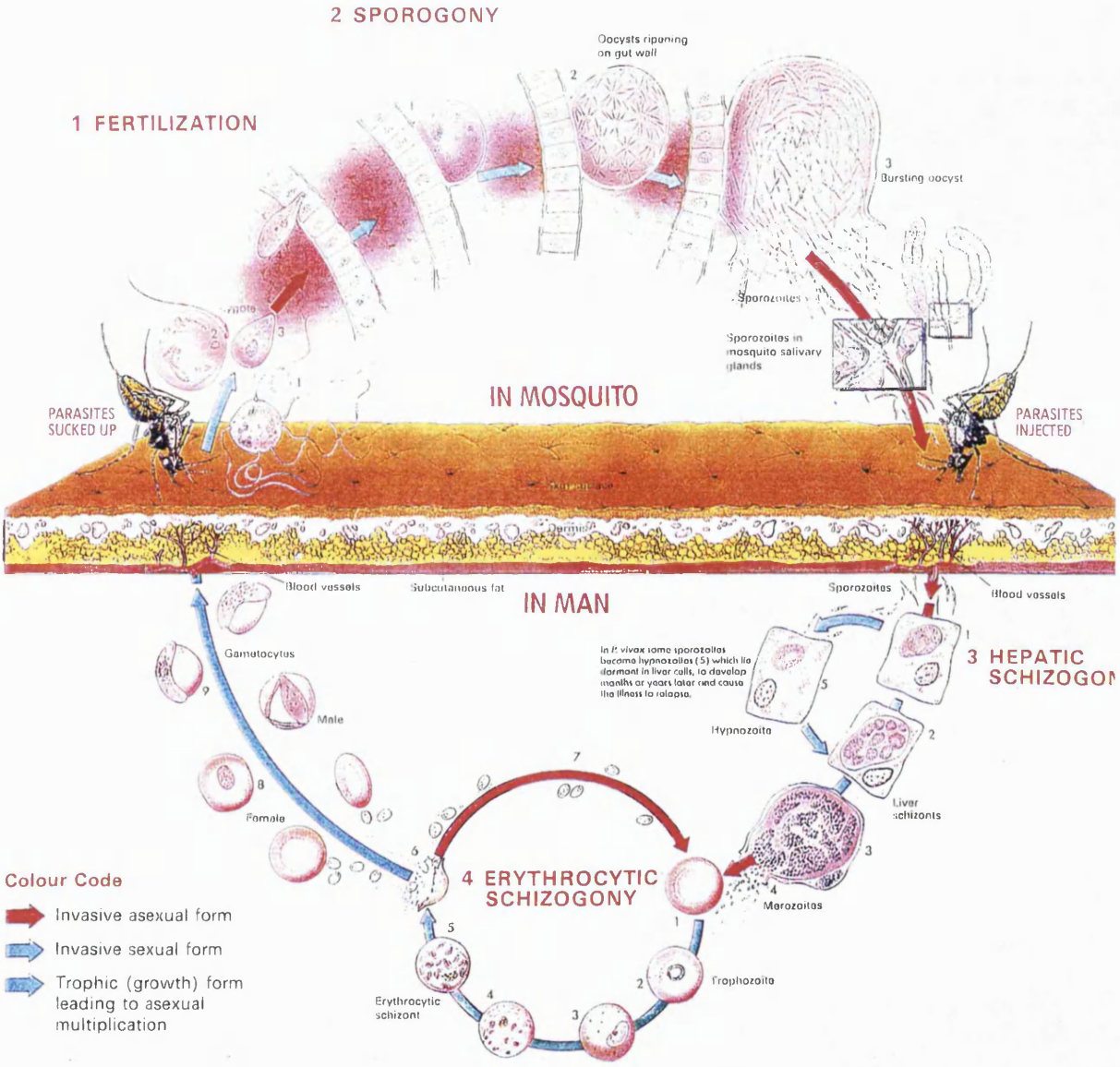
1.2.2 The Parasite

The parasite which is actually spread by the *Anopholes* mosquito, causing malaria, is the protozoan parasite from the genus *Plasmodium.* There are over 100 strains of *Plasmodium* which are found in a wide range of vertebrates including birds, reptiles and apes. All the strains are species specific, except for those found in some monkeys which can be transmitted to humans (Giles, 1993). This is due to the parasites recognising and binding to specific receptors on the surface of red blood cell membranes. These receptors differ from species to species, hence there is little chance of cross infection occurring (Sherman, 1979). This species specificity is another indicator of the long evolutionary history of *Plasmodia.*

1.2.3 Life-Cycle of Human Malaria Parasites

The life cycle of human malaria does not change greatly from one strain to another. They all involve development stages both in man and in the mosquito (fig. 1.1). When an infected female *Anopheles* mosquito takes a blood meal it can transmit sporozoites from its salivary gland into the capillary bed of its victim's skin. The sporozoites are then conveyed in the victims blood to the liver where they invade hepatocytes and undergo exo-erythrocytic schizogony, or nuclear division. This process of multiplication and development produces merozoites within a schizont. Rupture of the

Fig. 1.1 The Life Cycle of *Plasmodium*



Shows the life cycle of *Plasmodium* both in man and in the mosquito

[taken from Wellcome Trust, 1991]

hepatocyte releases the merozoites into the bloodstream where they invade erythrocytes. If the malaria strain is one which causes relapse, instead of undergoing pre-erythrocytic schizogony some of the sporozoites from the mosquito will become hypnozoites (Krotoski *et al.*, 1982). These hypnozoites remain dormant in the hepatocytes until a predetermined time when they undergo exo-erythrocytic schizogony releasing merozoites into the blood which causes a clinical relapse.

The intra-erythrocytic stage involves the parasites multiplying asexually and undergoing maturation through the ring, trophozoite and finally the schizont stage. When the schizont-filled erythrocyte ruptures it releases merozoites which can invade other erythrocytes close by. This process can be repeated many times and is responsible for the periodic fevers which are associated with malaria. The actual fever pattern depends on the strain of the parasite involved.

Some of the merozoites differentiate into the sexual forms, micro (male) and macro (female) gametocytes within the red cell. Transmission of the parasite relies on the uptake of mature gametocytes by a mosquito in a blood meal. Within the mid-gut of the mosquito the gametocytes undergo further development. The female gametocyte becomes a macrogamete, the male becomes a microgamete and fertilisation takes place producing a zygote. Within a few hours the zygote becomes a motile ookinete which penetrates the gut wall and matures into an oocyst. Within the oocyst sporozoites develop which emerge and migrate to the mosquito's salivary glands, ready to initiate the cycle all over again. (Zaman & Keong, 1990; Giles, 1993).

1.2.4 Human Malaria

Once a person becomes infected with malaria, the length of time before their symptoms start to show, i.e. the incubation period, depends upon the strain of the parasite involved. Diagnosis depends upon the presence of symptoms and the presence of parasites in the blood. Clinical symptoms vary between parasite strains. The most common symptoms are periodic fevers which are associated with the release of merozoites from rupturing erythrocytes. As the disease progresses headaches, pains, fever, gastro-intestinal symptoms such as nausea, vomiting and diarrhoea, and rigors will develop. If the malaria is not treated at this stage life threatening complications can arise, particularly in *P. falciparum* .

Table 1.1 Characteristics of *Plasmodium* species infecting humans

	Species			
	P. falciparum	*P. vivax*	*P. ovale*	*P. malariae*
Average incubation period (Days)	12	13-17	14	28
Pre-erythrocytic stage (Days)	5-7	6-8	9	14-16
Erythrocytic cycle (hrs)	24-48	48	50	72
Hypnozoite production	None	Yes	Yes	None
Mosquito Cycle (Days)	9	8	12	16

1.2.4.1 Human *Plasmodia* Strains

1.2.4.1.i P. vivax (Benign Tertian malaria) The classic periodic fevers associated with malaria only develop if the patient remains untreated until the infection becomes synchronised. In the case of *P. vivax* paroxysms occur every other day (or days 1 and 3, hence 'tertian') due to the rupture of erythrocytes every 48 hours. Fatalities are rare with *P. vivax.* Mild anaemia, which can be dangerous in children, is a common symptom, as is splenic rupture due to enlargement of the spleen which can be fatal if not treated quickly. Relapses, due to the differentiation of parasites into hypnozoites in the liver, are common with this strain of malaria. The period of quiescence depends upon the strain of *P. vivax* involved and the climatic zone (Warrell, 1993). *P. vivax* has the largest geographical range of all the human malarias, but is rarer than *P. falciparum.* At one time it was common in many temperate countries but now is found mainly in Southern Asia and Central America. Vivax malaria is very rare among West African people as many of them lack the Duffy blood group antigen alleles which *P. vivax* requires as a receptor for erythrocyte invasion.

1.2.4.1.ii P. ovale (Tertian malaria) Ovale malaria very closely resembles vivax malaria. It has the same tertian periodicity of fever and also produces hypnozoites, but relapses tend to be less frequent than with *P. vivax.* Spontaneous recovery is quite common in ovale malaria and the clinical symptoms seen in vivax malaria such as anaemia and splenic enlargement are less severe, hence it is not normally fatal (Warrell, 1993). *P.ovale* does not have a wide distribution, it is largely restricted to the African continent although a few cases in Asia have been reported (Zaman & Keong, 1990).

1.2.4.1.iii P. malariae (Quartan malaria) In synchronous infections, *P. malariae* schizonts rupture every 72 hours causing febrile paroxysms on days 1 and 4 (hence quartan). Again the clinical symptoms resemble those of vivax malaria and the outcome is rarely lethal. The fatalities which do occur are usually due to a nephrotic syndrome which *P.malariae* appears to cause in a small number of patients who are exposed to repeated infections. *P. malariae* can become chronic and can last in the body for up to fifty years. This is not due to hypnozoites, it is caused by recrudence of persistent erythrocytic forms of the parasite in small numbers. Malariae malaria is restricted mainly to Africa.

1.2.4.1.iv P. falciparum (Malignant tertian malaria) This is the most deadly of the human malarias and is responsible for the majority of the 2 million, mainly infant, deaths each year. Of the human malarias it has the shortest duration of infection, the shortest incubation period, the greatest number of merozoites per exo-erythrocytic schizont and results in the highest parasitaemias. The fevers associated with this strain of malaria tend not to be periodic, they are either continuous, remittent or irregular. When periodic fever does occur it is either daily (quotidian), every third day (tertian) or twice every 3 days (bi-tertian). In non-immune and susceptible people such as children the disease can develop rapidly into a life-threatening condition unless treatment is started. The main complications which can lead to death in falciparum malaria are cerebral malaria, severe anaemia, renal failure, pulmonary oedema and hypoglycaemia

1.2.4.2 Complications of Severe Falciparum Malaria

1.2.4.2.i Cerebral Malaria Cerebral malaria is the most severe clinical complication of *P. falciparum* and is defined as a state of unrousable coma attributable solely to falciparum malaria (Warrell, 1989). In reality any impairment of consciousness or convulsions could suggest cerebral malaria. It is invariably fatal if left untreated and is still associated with 20% mortality in treated patients. Cerebral malaria normally starts with several days (less than two days in children) of fever and non-specific symptoms such as headaches and drowsiness. This can lead to impaired consciousness, delirium and convulsions followed by persistent coma if it is not treated quickly (Warrell, 1993; W.H.O., 1990). A number of hypotheses have been put forward to explain the pathophysiology of cerebral malaria. Increased permeability of the blood-brain barrier, cerebrovascular thrombosis and immunological mechanisms have all been suggested but no convincing evidence has been found for any of them (Phillips & Warrell, 1986). Blockage of the cerebral microvasculature leading to a reduction in the supply of oxygen and other nutrients to the brain is now thought to be the major cause of the comatose state which is associated with cerebral malaria.

Lining of the cerebral capillaries and venules with infected erythrocytes (sequestration) is a common pathophysiological feature of severe falciparum malaria (MacPherson *et al.*, 1985). It is caused by the cytoadherence of infected erythrocytes to certain host molecules on the vascular endothelium, through 'knobs' on the surface of the parasitized red blood cell (prbc) which contain parasite specific antigens (Fujioka & Aikawa, 1996). As a result of cytoadherence there is a reduction in cerebral blood flow which leads to anaerobic glycolysis and an increase in lactate production (anaerobic parasite metabolism can also contribute to high lactate

concentrations) (White *et al.*, 1985; Warrell *et al.*, 1988). An increase in the concentration of circulating TNF has also been associated with cerebral malaria (Grau *et al.*, 1989). This and other cytokines may enhance cytoadherence by up-regulating the expression of endothelial receptors such as ICAM-1 which bind to the parasitized erythrocytes (Miller *et al.*, 1994).

1.2.4.2.ii Severe Anaemia Severe anaemia is an almost inevitable complication of severe malaria and results in a reduction of the oxygen carrying capacity of the blood. It is a big killer of young children, particularly those under 2 years old, and is common in pregnant women. Although the malaria infection is a major contributor to the anaemia, it has also been associated with secondary bacterial infections and hepatic and renal dysfunction. Falciparum malaria related anaemia is thought to be caused by a combination of the increased haemolysis of parasitized erythrocytes, an accelerated clearance of non-parasitized erythrocytes by the spleen and ineffective erythropoiesis by the bone marrow (Phillips & Warrell, 1986; Clark and Chaudhri, 1988; W.H.O., 1990; White & Ho, 1992; Frances & Warrell, 1993). When parasitized erythrocytes have undergone schizogony they burst releasing merozoites, hence haemolysis must contribute to a low blood count. Surprisingly severe anaemia is not always associated with a high parasitaemia, however this may be due to the parasitaemia being counted after the majority of the parasitized cells have burst (Miller *et al.*, 1994). The reason for the increased splenic removal of non-parasitized erythrocytes, which can last for up to three weeks after the infection has gone, is not clear. Immune-mediated haemolysis has been suggested as the cause, but the evidence for this has been conflicting (Phillips & Warrell, 1986; W.H.O., 1990; Francis & Warrell, 1993), and recent studies in mice suggest that this mechanism is unlikely (Salmon *et al.*, 1997).

Alterations in the cell membranes of both parasitized and non-parasitized erythrocytes may play a role. These changes are analogous to accelerated cell ageing and may provide targets for autoantibodies which would accelerate the removal of the cells (White & Ho, 1992). The response of the bone marrow to acute anaemia is delayed in falciparum malaria, with reticulocytosis not beginning for many days. When it does begin it is often insufficient. This may be due to the release of cytokines in the bone marrow. TNF and IFNγ have both been shown to suppress haematopoiesis (Young & Alter, 1994) and in rodent malaria TNF appears to contribute to bone marrow dysfunction (Miller *et al.*, 1989). In humans however, definitive proof of a cytokine-mediated mechanism is lacking. Another possible explanation is that sequestration of parasitized erythrocytes in the bone marrow causes local hypoxia (White & Ho, 1992).

1.2.4.2.iii Renal Failure Renal failure is common among adults with severe falciparum malaria but not children. It is associated with high parasitaemia, haemoglobinuria, jaundice and cerebral malaria. Approximately half of all adults with cerebral malaria show biochemical signs of renal impairment such as raised blood urea and serum creatine. Histopathological studies have shown that falciparum malaria patients with renal dysfunction develop acute tubular necrosis. This is thought to result from a reduction in renal microvasculatory blood flow. Cytoadherence has been seen in the glomerular capillaries but to a lesser extent than in the brain or heart. Whether this is responsible for the reduced blood flow is unknown. The outcome of malaria associated renal failure depends on the overall severity of the infection and the availability of dialysis. If there are no other complications and dialysis is available,

renal function can return to normal (Phillips & Warrell, 1986; W.H.O., 1990; White & Ho, 1992; Francis & Warrell, 1993)

1.2.4.2.iv Pulmonary Odema Pulmonary odema is a grave complication of severe falciparum malaria. It kills approximately 80% of the people affected, despite treatment. Like renal failure it is commonly associated with dysfunction of other vital organs and is rarely seen in children but is a particular problem of severe malaria in pregnant women. The manifestations of this complication are similar to those of Adult Respiratory Distress Syndrome (ARDS) which is thought to be due to increased pulmonary capillary permeability caused by leukocyte products released through complement/cytokine stimulation. Pathological reports have shown that the alveoli of malaria patients with pulmonary odema are congested with macrophages, neutrophils and other cells. Hence a similar mechanism to that which causes ARDS may be responsible for the pulmonary odema associated with severe falciparum malaria (Phillips & Warrell, 1986; W.H.O., 1990; White & Ho, 1992; Francis & Warrell, 1993).

1.2.4.2.v Hypoglycaemia In adults hypoglycaemia is defined as a blood glucose concentration of less than 2.2 mmol/l (Gregory & Aynsley-Green, 1993). It causes anxiety, breathlessness, feelings of coldness, light-headedness, impaired consciousness, extensor posturing, convulsions, shock and coma. All these symptoms can also of be attributed to cerebral malaria and as a result hypoglycaemia has only recently been recognised as a complication of severe malaria (Phillips, 1989). Previously it was assumed that the symptoms caused by hypoglycaemia were due to cerebral malaria, and blood glucose was never measured. Now however, blood

glucose concentrations are measured routinely in malaria patients, particularly pregnant women as they can develop asymptotic hypoglycaemia (White *et al.*, 1983; Looareesuwan *et al.*, 1985) which may cause foetal distress or death in utero. Hypoglycaemia in both adults and children is commonly associated with other manifestations of severe falciparum malaria such as cerebral malaria, renal failure and hyperparasitaemia and carries a poor prognosis (White *et al.*, 1983; White, N. *et al.*, 1987; Taylor *et al.*, 1988).

As was stated previously, there are a number of factors which can contribute to malarial hypoglycaemia. The symptoms of malaria, such as fever, can increase the rate of glucose consumption by the host. Consumption of glucose by the parasite itself increases proportionally with the stage of development. Erythrocytes containing mature parasites require 70 times more glucose than non-infected erythrocytes (Sherman, 1979). Malaria parasites metabolise glucose anaerobically producing lactate (Sherman, 1979), which partially explains the high levels of lactate associated with hypoglycaemia in malaria (White *et al.*, 1983; White, N. *et al.*, 1987; Taylor *et al.*, 1988). Anaerobic glycolysis by the host may also contribute to the high lactate concentration. In microcirculatory beds where parasites are sequestered in high numbers there is strong competition for the glucose available (Macpherson *et al.*, 1985) These high plasma lactate levels are normally accompanied by high plasma alanine levels, suggesting that impairment of hepatic gluconeogenesis may also contribute to the hypoglycaemia (White *et al.*, 1983, White *et al.*, 1987, Taylor *et al.*, 1988). This inhibition of gluconeogenesis may be caused by cytokines such as TNF (Grau *et al.*, 1989). There is a strong correlation between hypoglycaemia and the release of cytokines, particularly TNF, in both humans (Grau *et al.*, 1989;

Kwiatkowski *et al.*, 1990) and rodents (Clark *et al.*, 1987; Clark *et al.*, 1992). Hypoglycaemia due to starvation and glycogen depletion is a particular problem of young children with falciparum malaria. They require between 2 and 4 times more glucose than adults and without food their glycogen stores only last for 12 hours. They are then dependent on gluconeogenesis to maintain their glucose levels, but as this is impaired in falciparum malaria they can quickly become hypoglycaemic(White, N. *et al.*, 1987; Kawo *et al.*, 1990).

In some studies patients have developed hypoglycaemia together with hyperinsulinaemia following the initiation of quinine treatment (White *et al.*, 1983; Looareesuwan *et al.*, 1985). This effect is thought to be caused by the quinine stimulation of insulin release from pancreatic islet cells, a phenomenon which has been demonstrated *in vitro* (Henquin *et al.*, 1975). Pregnant women have hyper-reactive beta cells (Gilmer *et al.*, 1975), which may explain why they are more susceptible to hypoglycaemia and hyperinsulinaemia when treated with quinine (White *et al.*, 1983; Looareesuwan *et al.*, 1985). Hypoglycaemia and hyperinsulinaemia can also occur in the absence of quinine treatment. This may be due to the inhibition of lipoprotein lipase by TNF triggering the compensatory release of insulin; insulin normally up-regulates this enzyme but cannot do so when TNF is present, therefore extra insulin may be produced in an attempt to overcome the inhibition (Clark *et al.*, 1987).

1.2.4.3 Treatment of Human Malaria

In the early 17th century it was discovered that bark from the Cinchona tree could be used to control the fevers associated with malaria. The use of this remedy, known as 'Jesuit's powder' spread rapidly throughout Europe, but it was not until 1820 that

Pelletier and Caventou isolated the two main alkaloids in the bark, quinine and cinchonine (Wellcome trust, 1991). Quinine and its diastereomer quinindine are arylaminoalcohols which act on the blood stages of the malaria parasite and as a result have been termed blood schizontocides. They work rapidly and hence are commonly used to treat acute disease. Quinine however is rather toxic so new, less toxic substances have been synthesised to replace it. The most widely used of these substances is chloroquinine which is a 4-aminoquinoline. Like quinine it is a blood schizontocide, but it can also be used for suppressive prophylaxis i.e. prevention of symptoms. Unfortunately overuse of this and other synthetic antimalarial drugs has led to the emergence of resistant strains of the parasite. As a result, quinine is once again the treatment of choice for severe falciparum malaria (Warrell, 1993).

1.2.5 Animal Models

Up until the 1950's only avian and simian malarias were available as animal models for malaria. Due to their similarity to human malarias, non-human primate malarias make very good laboratory models for the disease but are very expensive. Avian malarias develop in a slightly different way to mammalian malarias and as a result are not very good models for the human disease. The discovery of malaria in rodents by Vincke & Lips in 1948 however has provided a cheap and well characterised host for the infection, the laboratory mouse.

1.2.6 Rodent Malaria

Since the discovery of the first rodent malaria in thicket rats in 1948 a total of eleven rodent strains have been identified. Of these eleven strains, four from murine rodents have been shown to infect laboratory mice, they are:

(1) *P. berghei*

(2) *P. yoelii*

(3) *P. chabaudi*

(4) *P. vinckei*

Table 1.2 Characteristics of *Plasmodium* species infecting rodents

	Species			
	P. berghei	*P. vinckei*	*P. yoelii*	*P.chabaudi*
Pre-erythrocytic stage (hrs)	50	60-68	50	53-58
No. of blood merozoites per schizont	6-18	6-16	6-18	4-10
Erythrocytic cycle (hrs)	24	24	24	24
	asynchronous	asynchronous	asynchronous	synchronous
Red cell preference	reticulocytes	mature	reticulocytes	mature
Mosquito cycle (Days)	14	9-10	10-13	10-11

Unfortunately none of the rodent malarias represent an exact model for human malaria. However certain aspects of the pathology and immunology of rodent malarias do resemble the situation in man and hence may provide some insight into what is happening in the human disease.

Laboratory models of cerebral malaria are particularly difficult to achieve as rodent malaria parasites do not sequester in the microvasculature in the same way as *P. falciparum* does (Cox, 1988). However inoculation of susceptible mice with the

parasite strain *P. berghei* Anka (PbA) can cause acute neurological manifestations which resemble some features of human cerebral malaria (Finley *et al.*, 1983). Both human and rodent cerebral malaria are characterised by the cytoadherence of cells in small blood vessels, but with PbA it is monocytes which are sequestered rather than parasitized erythrocytes as is the case with the human disease (Macpherson *et al.*, 1985). Rodent cerebral malaria is accompanied by an elevation in the level of TNF in the blood (Grau et al., 1987). TNF appears to be required for the up-regulation of endothelial ICAM-1 (which allows the binding of monocytes to the blood vessels) and the subsequent microvascular pathology which results in fatal cerebral malaria (Rudin, *et al.*, 1997). A similar mechanism is thought to be responsible for the sequestration of parasitized erythrocytes in human malaria (Miller *et al.*, 1994)

Severe anaemia is another pathophysiological characteristic which is shared by human and rodent malarias. As with the human disease the anaemia associated with rodent malaria is thought to be caused by a combination of the haemolysis of parasitized erythrocytes, increased erythrophogacytosis and dyserythropoiesis (Cox, 1988). It appears that high levels of TNF may, in part, be responsible for the inhibition of erythropoeisis (Miller et al., 1989) and the increase in erythrophagocytosis (Clark & Chaudhri, 1988) seen in rodent malaria, possibly through an indirect mechanism. Whether TNF is responsible for these effects in humans has yet to be established. Evidence does show that mice infected with malaria can develop anaemia in the absence of TNF (Rudin *et al.*, 1997), but this could be due to haemolysis of the parasitized red cells as rodents can achieve parasitaemias of up to 80%.

Mice infected with *P. berghei* and *P. yoelii* have been shown to develop glomerulonephritis which is comparable to the transient renal dysfunction caused by *P. falciparum*, but not the chronic nephrotic syndrome associated with *P. malariae*. This glomerulonephritis is thought to be initiated by immune complexes. Whether these complexes are due to malarial or non-malarial antigens is not clear (Cox, 1988).

Hypoglycaemia has been noted as a complication of *P. yoelii* and *P. chabaudi* (Elased & Playfair, 1994) in mice and of *P. berghei* in rats (Holloway *et al.*, 1991). As is the case with falciparum malaria, the exact cause of hypoglycaemia in rodent malaria is not clear. Studies using *P. vinckei* have indicated that cytokines, including TNF, may be responsible for the hypoglycaemia (Clark *et al.*, 1987; Clark *et al.*, 1992). Elased and co-workers on the other hand have shown that the hypoglycaemia induced by non-lethal *P. chabaudi* and lethal *P. yoelii* is related to hyperinsulinaemia (Elased & Playfair, 1994) and that diazoxide, a drug which inhibits the secretion of insulin, can block the hypoglycaemia associated with the *P. chabaudi* infection. They also demonstrated that neither pentoxyffilline, which blocks TNF production, nor antibodies against TNF could prevent the induction of hypoglycaemia by either *P. chabaudi* or *P. yoelii* which suggests that TNF itself is not the cause of hypoglycaemia in rodent malaria (Elased *et al.*, 1996). TNF could however be working with other cytokines such as IL-1 and IL-6, perhaps synergistically, to produce this response.

1.3 INSULIN

Insulin is a small protein of molecular weight 5700. It is composed of two peptide chains joined by disulphide bonds, the A chain which has 21 amino acid (aa) residues and the B chain which has 30 residues. These peptides are synthesised in the β-cells of pancreatic islets from a single poplypeptide precursor known as proinsulin. Proinsulin has 84 aa residues and forms a characteristic tertiary configuration that is stabilised by disulphide bonds. In the golgi it is cleaved by trypsin-like enzymes releasing a 33 aa peptide known as the C-peptide, leaving the A and B insulin chains linked by disulphide bonds. The 5% of the proinsulin which remains unconverted is packaged into granules along with insulin and the C-peptide. Also present in the granules is zinc which forms an insoluble complex with insulin. Upon stimulation the granules fuse with the β-cell membrane and release their contents into the precapillary space. Insulin can then enter the circulation in solution (Espinal., 1989).

Insulin has a half-life in the plasma of only a few minutes, yet its biological effects do not reach a maximum for 2-4 hours. This is a result of insulin interacting with specific high affinity receptors on the plasma membranes of target cells. This interaction in turn leads to the broad array of anabolic intracellular events which are associated with insulin's actions.

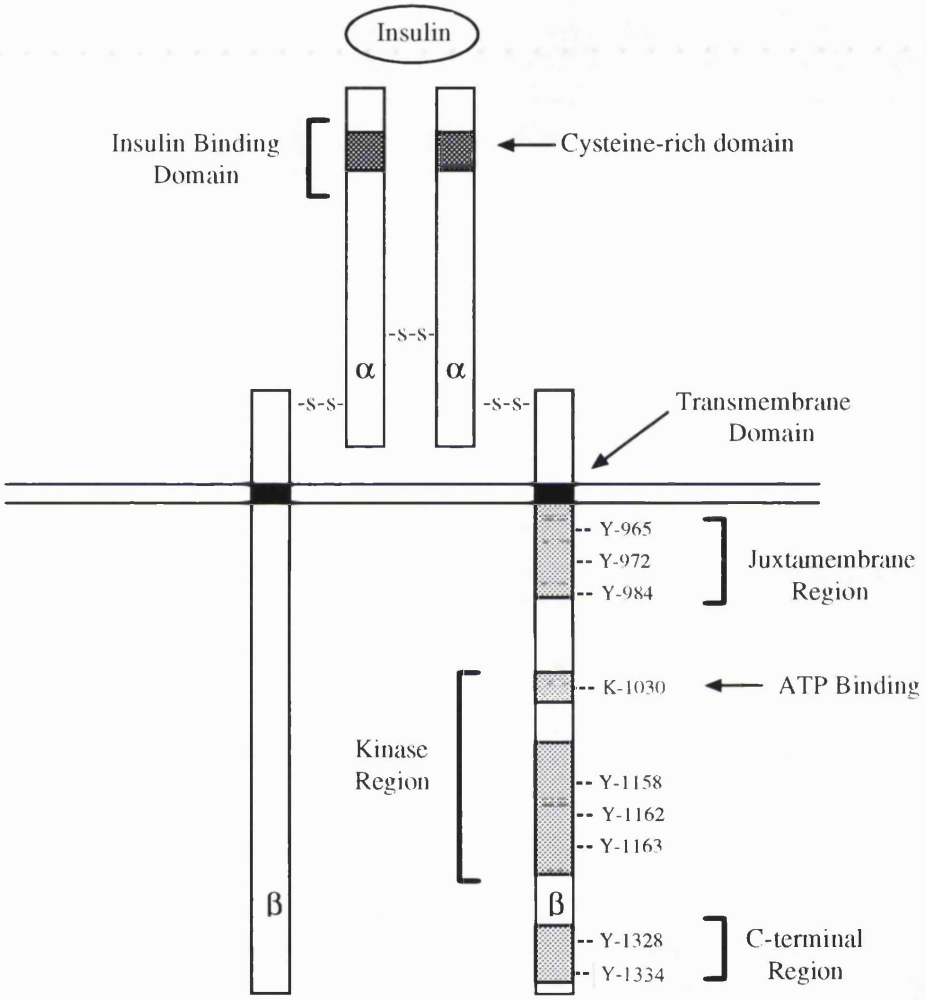
1.3.1 The Insulin Receptor

The insulin receptor is a heterotetrameric glycoprotein complex consisting of two α and two β subunits in the arrangement β–α–α–β and has a molecular weight of 300-

400 kDa by SDS-PAGE. The α-subunits are each linked to a β-subunit and to each other by disulphide bonds (fig.1.2) (for review see Lee & Pilch, 1994). Photoaffinity labelling studies have shown that insulin binds to its receptor through the 135 kDa α–subunits (Jacobs *et al.*, 1979). These are located entirely on the external surface of the cell, but are anchored to the cell membrane by disulphide linkage to the β-subunits which span the membrane (Ebina *et al.*, 1985; Ullrich *et al.*, 1985). Under physiological conditions one molecule of insulin binding per receptor is thought to be the normal situation. For high affinity binding both αβ receptor halves have to be present (Boni-Schnetzler *et al.*, 1987). This suggests that the insulin molecule makes contact with both α-subunits, similar to the interaction of growth hormone (GH) with its receptor (Cunningham *et al.*, 1991). Although the exact site(s) of hormone-receptor contact have yet to be clearly defined, various studies have identified three regions in the first 400 N-terminal amino acids of the α-subunit as potential binding determinants (Yip *et al.*, 1988; Waugh *et al.*, 1989; Wedekind *et al.*, 1989; Fabry *et al.*, 1992; Yip, 1992). This area encompasses a cysteine-rich region containing intrachain disulphide bonds (Boni-Schnetzler *et al.*, 1987).

1.3.1.i Receptor Tyrosine Kinase When insulin binds to its receptor there is an increase in the phosphorylation of the 95 kDa β-subunit of the receptor (Kasuga *et al.*, 1982a,b). Addition of insulin to partially purified receptors, in the presence ATP, Mg^{2+} and Mn^{2+}, has shown that this increase in phosphorylation is due to rapid autophosphorylation of the β-subunit on tyrosine residues (Petruzzelli *et al.*, 1984; White *et al.*, 1984), by a kinase activity intrinsic to these subunits (Roth & Cassell, 1983). This intrinsic tyrosine kinase activity has been demonstrated by a number of

Fig. 1.2 The Insulin Receptor



Shows a diagram of the insulin receptor illustrating the relative position of the autophosphorylation sites and the other functional regions.

groups working on intact cells and partially purified receptors from a variety of tissues (Machicao *et al.*, 1982; Petruzzelli *et al.*, 1982; Van Obberghen & Kowalski, 1982; Kasuga *et al.*, 1983; Zick *et al.*, 1983a,b; Haring *et al.*, 1984) Labelling with ATP affinity analogues (Roth & Cassell, 1983; Van Obberghen *et al.*, 1983) and sequencing of the receptor cDNA has confirmed that the β-subunit contains a consensus ATP binding site and has extensive homology to other protein tyrosine kinases (Ebina *et al.*, 1985; Ullrich *et al.*, 1985).

1.3.1.ii Autophosphorylation Autophosphorylation is the earliest detectable event in insulin action and leads to an insulin-independent increase in the activity of the receptor kinase towards intracellular substrates (for reviews see Lee & Pilch, 1994; White & Kahn, 1994). Phophopeptide mapping has revealed tyrosine residues in three regions of the intracellular domain of the β-subunit which can be phosphorylated in response to insulin *in vitro*. These are tyrosines 965, 972 and 984 in the juxtamembrane region, 1158, 1162 and 1163 in the kinase region and 1328 and 1334 in the carboxy-terminal region (Taverá & Denton, 1988; Tornqvist *et al.*, 1988; White *et al.*, 1988). In cell lines transfected with high levels of insulin receptors there appears to be no phosphorylation of the juxtamembrane region (Taverá & Denton, 1988), and in cells isolated from rat livers only the three tyrosines in the kinase domain appeared to be phosphorylated in response to insulin (Issad *et al.*, 1991). Thus the relevance of each of these phosphorylation sites to insulin signalling has yet to be fully determined. But, the evidence does suggest that phosphorylation of tyrosines 1158, 1162 and 1163 in the kinase domain is required for amplification of the kinase activity (White *et al.*, 1988). Substitution of 1,2 or 3 of the tyrosine residues in this region with phenylalanine progressively reduces insulin-stimulated

kinase activity and results in loss of biological activity (Ellis *et al.*, 1986; Wilden *et al.*, 1992a,b).

Although the exact mechanism is unclear, it appears that insulin may stimulate autophosphorylation by inducing conformational changes in the receptor. Insulin binding to the receptor alters the conformation of the α-subunits, changing the relative positions of the two α-subunits within the tetramer (Pilch & Czech, 1980; Donner & Yonkers, 1983). These changes appear to be transmitted to the β-subunit which also undergoes conformational change(s) allowing autophosphorylation to take place (Baron *et al.*, 1992; Lee *et al.*, 1997). Whether autophosphorylation is controlled by a single conformational change throughout the insulin receptor, or by a series of conformational changes is not yet clear. Autophosphorylation of the receptor causes a further conformational change in the β-subunit cytoplasmic domain (Baron *et al.*, 1992). This may increase the exogenous kinase activity of the insulin receptor.

1.3.1.iii Serine/Threonine Phosphorylation The insulin receptor is phosphorylated on serine and threonine residues in the basal state. This basal phosphorylation is increased in response to insulin stimulation (Gazzano *et al.*, 1983; Pang *et al.*, 1985; Stadtmauer & Rosen, 1986; Tavaré *et al.*, 1988) and activators of protein kinase C or cAMP dependent protein kinase (Jacobs & Cuatrecasas, 1986; Stadtmauer & Rosen, 1986, Takayama *et al.*, 1988; Isaad *et al.*, 1992) in intact cells. The role of insulin receptor serine/threonine phosphorylation is not fully understood. It is thought that it may be involved in regulation of the insulin signal in response to insulin and/or counter-regulatory hormones (Chin *et al.*, 1993; Coghlan & Siddle, 1993; Liu &

Roth, 1994). Recently an insulin-stimulated insulin receptor serine kinase has been isolated both from human placenta and rat liver which can phosphorylate serine 1078 of the insulin receptor β-subunit (Carter *et al.*, 1996). There is also some evidence that the insulin receptor kinase has intrinsic serine kinase activity *in vitro* (Al-Hasani *et al.*, 1997).

1.3.1.iv Protein Tyrosine Phosphatases Protein tyrosine phosphatases (PTPases) also appear to be involved in the regulation of insulin receptor activity. For instance, dephosphorylation of tyrosines 1158, 1162 and 1163 in the kinase domain has been shown to closely correlate with deactivation of receptor kinase activity (King *et al.*, 1991). Also, vanadate, a potent inhibitor of PTPases, is able to potentiate insulin's action (Fantus *et al.*, 1989; Shechter, 1990; Meyerovitch *et al.*, 1991). A number of PTPases have now been identified which may play a role in insulin signalling (for review see Golstein, 1992). One of these PTPases, LAR (for leukocyte common antigen-related) was recently shown to be functionally associated with the insulin receptor in intact cells (Ahmad & Golstein, 1997). Also, overexpression of PTPase1B in cultured cells has been shown to inhibit insulin-stimulated receptor autophosphorylation, phosphorylation of insulin receptor substrate proteins and glucose incorporation into glycogen (Kenner *et al.*, 1996). This enzyme complexes with the insulin receptor *in vivo* and is tyrosine-phosphorylated in the presence of insulin (Bandyopadhyay *et al.*, 1997).

1.3.1.v Insulin Receptor Internalisation As well as mediating signal transduction, the insulin receptor also mediates insulin internalisation and degradation. This involves a process known as receptor-mediated endocytosis, the first two steps of which

depend on different regions of the β-subunit. Movement of the insulin-receptor complex to coated-pits depends on kinase activation and autophosphorylation of the three tyrosines in the kinase region and association with the coated-pits involves specific signal sequences which are present in the juxtamembrane region (Carpentier *et al.*, 1992, 1993b). Once internalised the receptor complex is delivered to endosomes. From here insulin is targeted to lysosomes to be degraded and the receptors are recycled to the cell membrane (for review see Carpentier, 1993a). When the insulin receptor is internalised it is still in its activated, phosphorylated form (Khan *et al.*, 1986, 1989) and hence may be involved in the phosphorylation of intracellular substrates which are inaccessible to the cell membrane. Therefore, as well as modulating insulin's actions by regulating the number of receptors at the cell surface and degrading the hormone, insulin-receptor internalisation may also be directly involved in insulin signalling.

1.3.2 Insulin Receptor Substrates

Insulin binding causes autophosphorylation of tyrosine residues within the kinase domain of the receptor β-subunit, this in turn increases the catalytic activity of the receptor tyrosine kinase and results in tyrosine phosphorylation of cellular substrates (for review see Saltiel, 1994; Cheatham & Kahn, 1995).

1.3.2.i Insulin Receptor Substrate-1 (IRS-1) A protein of relative molecular mass 185 kDa, known as pp185, was the first insulin receptor substrate to be identified. It was originally detected in Fao hepatoma cells using anti-phosphotyrosine antibodies (White *et al.*, 1985) and has subsequently been found in many other insulin-stimulated tissues (Kadowaki *et al.*, 1987; Shemer *et al.*, 1987; White *et al.*, 1987; Maegawa *et*

al., 1988; Momomura *et al.*, 1988; Tobe *et al.*, 1990). Purification of this protein from insulin stimulated rat liver (Rothenberg *et al.*, 1991) and partial sequencing led to the cloning of IRS-1 (Insulin Receptor Substrate-1) (Sun *et al.*, 1991). This has now been purified and cloned from several other sources (Keller *et al.*, 1991; Nishiyama & Wands, 1992; Araki *et al.*, 1993; Keller *et al.*, 1993). IRS-1 contains 35 potential Ser/Thr phosphorylation sites and approximately 20 potential tyrosine phosphorylation sites, at least 8 of which are phosphorylated in response to insulin (Sun *et al.*, 1991; Shoelson *et al.*, 1992). A number of these tyrosine residues lie within conserved motifs which are potential binding sites for proteins with SH2 (src homology 2) domains (Sun *et al.*, 1991; Shoelson *et al.*, 1992). Indeed IRS-1 has been shown to bind several SH2-containing proteins (for review of SH2 domains, see Pawson & Gish, 1992)

PI3-kinase (phosphatidylinositol 3-kinase) was the first SH2-containing protein found to associate with IRS-1 (Sun *et al.*, 1991). It binds to phosphorylated IRS-1 through the SH2 domains in its p85 regulatory subunit (Myers *et al.*, 1992). PI3-kinase binds to IRS-1 and is activated in an insulin-dependent fashion in a number of cell types (Ruderman *et al.*, 1990; Hadari *et al.*, 1992; Backer *et al.*, 1993; Kelly & Ruderman, 1993; Okamoto *et al.*, 1993). The significance of PI3-kinase in insulin signalling will be discussed later.

Syp (or SH-PTP2) is a protein tyrosine phosphatase with two SH2 domains which binds IRS-1 upon insulin-stimulated tyrosine phosphorylaton (Kuhne *et al.*, 1993; Sun *et al.*, 1993). Occupation of the SH2 domains of Syp by phosphorylated peptides increases the catalytic activity of the enzyme (Leichleider *et al.*, 1993), hence IRS-

1:Syp binding may result in activation of the enzyme (Sugimoto *et al.*, 1994). The expression of catalytically inactive Syp mutants and the use of antibodies against Syp have shown that this enzyme plays a positive regulatory role in insulin-stimulated mitogenesis in a number of cell types (Milarski & Saltiel, 1994; Noguchi *et al.*, 1994; Hausdorff *et al.*, 1995; Sawada *et al.*, 1995; Yamauchi *et al.*, 1995a; Tanaka *et al.*, 1996; Ugi *et al.*, 1996).

Syp has also been shown to bind, through its SH2 domains, a 115 kDa protein which is tyrosine phosphorylated upon insulin stimulation (Milarski & Saltiel, 1994; Yamauchi *et al.*, 1995b; Noguchi *et al.*, 1996). This protein, termed SHPS-1 (Src homology containing Phosphatase Substrate-1) has now been purified and cloned from rat fibroblasts and is also thought to be involved in cell signalling (Fujioka *et al.*, 1996). It is possible that this receptor-like protein may be another insulin receptor kinase substrate (Noguchi *et al.*, 1996).

Grb2 is a small cytoplasmic protein which has two SH3 domains and one SH2 domain, through which it binds IRS-1 (Skolnik *et al.*, 1993a,b). This has no enzymatic activity but is thought to act as an 'adapter molecule' linking IRS-1 (and other tyrosine phosphorylated proteins) to the guanine nucleotide exchange factor for $p21^{ras}$, mSos (mammalian Son-of -sevenless) (Chardin *et al.*, 1993; Egan *et al.*, 1993; Simon *et al.*, 1993). Thus binding of Grb2:Sos to IRS-1 may be involved in the insulin stimulation of $p21^{ras}$ and hence the MAP kinases (Mitogen Activated Protein kinases) (Scholnik *et al.*, 1993a,b; Byrne et al., 1996).

Nck is a 47 kDa widely expressed cytoplasmic protein which has no catalytic activity (Li *et al.*, 1992). It is made up of one SH2 domain and three SH3 domains in the arrangement SH3-SH3-SH3-SH2 (Lehmann *et al.*, 1990). Nck has been shown to bind via its SH2 domain to intracellular tyrosine phosphorylated proteins such as IRS-1 (Lee *et al.*, 1993) and to the PDGF and EGF receptors (Li *et al.*, 1992). Like Grb2, Nck acts as an adapter protein, binding to effector proteins via its SH3 domains. It is thought that binding of these effector proteins may in turn trigger cellular signalling events, as Grb2 binding to Sos does. However, the effector molecules which Nck interacts with are poorly characterised and its biological function in insulin signalling is uncertain. Nck has been shown to associate with casein kinase I in transformed rat hepatocytes overexpressing the insulin receptor (HTC-IR), which could implicate this enzyme in insulin signalling (Lussier & Larose, 1997). Indeed casein kinase I has been shown to phosphorylate the β-subunits of the insulin receptor *in vitro* (Tuazon *et al.*, 1985), and IRS-1 contains consensus motifs for phosphorylation by casein kinase I (Kemp & Pearson, 1990). This suggests that *in vivo* this enzyme may be involved in the regulation of the insulin receptor and IRS-1. However no proof for this has been found.

IRS-1 therefore appears to acts as a multisite docking protein which binds a number of signal-transducing molecules involved in insulin signalling (Sun *et al.*, 1993).

1.3.2.ii Shc Proteins The protein products of the *shc* gene are also substrates for the insulin receptor tyrosine kinase (Pronk *et al.*, 1993) and other tyrosine kinases (Pelicci *et al.*, 1992; Rozakis-Adcock *et al.*, 1992; Egan *et al.*, 1993). This gene codes for three mRNAs of different sizes which translate into three proteins of molecular weight

46, 52 and 66 kDa. Each of these proteins has a carboxy-terminal SH2 domain and amino-terminal glycine/proline rich region (Pelicci *et al.*, 1992). As is the case for IRS-1, tyrosine phosphorylation of the Shc proteins (particularly p46 ^{shc} and p52 ^{shc}) provides binding sites for Grb2. This results in the formation of the Grb2:Sos complex (Pronk *et al.*, 1993; Skolnik *et al.*, 1993a,b; Sasaoka *et al.*, 1994). Evidence suggests that binding of Grb2 to Shc, not IRS-1, is the major pathway for insulin stimulation of the Ras signalling pathway (Ohmichi *et al.*, 1994; Myers *et al.*, 1994b; Pronk *et al.*, 1994; Sasaoka *et al.*, 1994). However, IRS-1 is still required for insulin stimulated DNA synthesis, even if formation of the Grb2:IRS-1 complex is not (Myers *et al.*, 1994b).

1.3.2.iii Insulin Receptor Substrate-2 (IRS-2) A 170 kDa protein has now been identified which shares key structural features with IRS-1 and has been termed IRS-2 (Sun *et al.*, 1995). Of the 20 potential tyrosine phosphorylation sites in IRS-1, 14 are conserved in IRS-2, some of which have SH2 binding motifs for known signalling proteins (Sun *et al.*, 1995; Pawson, 1995). This protein was originally shown to be tyrosine phosphorylated upon IL-4 stimulation which led to rapid associated with the p85 subunit of PI3-kinase (Wang *et al.*, 1992). This finding suggested that this protein might act via a similar mechanism to insulin stimulation of IRS-1. And indeed, upon insulin and IL-4 stimulation in FD-5 cells IRS-2 was able to bind the p85 subunit of PI3-kinase (Wang *et al.*, 1993; Welham *et al.*, 1997), Grb2 and Syp (Welham *et al.*, 1997) as can IRS-1 when stimulated by insulin. This suggests that IRS-1 and IRS-2 mediate similar pathways. However IL-4 does not stimulate the tyrosine phosphorylation of Shc, activation of p21^{ras} or activation of MAP kinase in these cells (Welham *et al.*, 1994a,b) which suggests that the association of IRS-2 with Grb2 is

not enough to stimulate the $p21^{ras}$ pathway. Also IRS-1 knockout mice are insulin resistant and show growth retardation (Araki *et al.*, 1994; Tamemoto *et al.*, 1994; Tobe *et al.*, 1995; Kadowaki *et al.*, 1996) which indicates that IRS-2, or other tyrosine-phosphorylated insulin receptor substrates, cannot entirely substitute for IRS-1.

Insulin and IL-4 have now been shown to tyrosine phosphorylate IRS-2 in primary cultures of murine T and B lymphocytes and macrophages (Welham *et al.*, 1997) as well as murine myeloid cell lines (Wang *et al.*, 1993). These cells express only IRS-2, unlike 3T3 cells which express both IRS-1 and IRS-2. In a T lymphocyte cell line and primary bone marrow mast cells neither IRS-1 nor IRS-2 were expressed (Welham *et al.*, 1997). This suggests that within cell lineages the expression of IRS-1 and IRS-2 may be differentially regulated. IRS-2 is not however limited to haematopoietic cells, IRS-2 mRNA transcripts can be found in murine skeletal muscle, lung, brain, liver, kidney and spleen (Sun *et al.*, 1995).

1.3.2.iv pp60 Receptor Substrates Recently it has been shown that at least two distinct 60 kDa proteins can be tyrosine phosphorylated in response to insulin (Hosomi *et al.*, 1994). One of these pp60 proteins has been shown to be directly phosphorylated by the insulin receptor in vitro (Hosomi *et al.*, 1994) and associates with the GTPase-activating protein (GAP) in an insulin dependent fashion (Porras *et al.*, 1992; Zhang & Roth, 1992; Hosomi *et al.*, 1994). The other has only been found in rat adipose tissue and HTC-IR cells (rat hepatoma cells overexpressing human insulin receptors). This pp60 was originally found to associate with the p85 subunit of PI3-kinase in insulin treated cells (Lavan & Leinhard, 1992; Kelly & Ruderman, 1993)

and has now also been shown to associate with Grb2 and Syp upon insulin stimulation (Zhang-Sun *et al.*, 1996). As is the case with IRS-1, these associations appear to take place through SH2 domains (Zhang-Sun *et al.*, 1996).

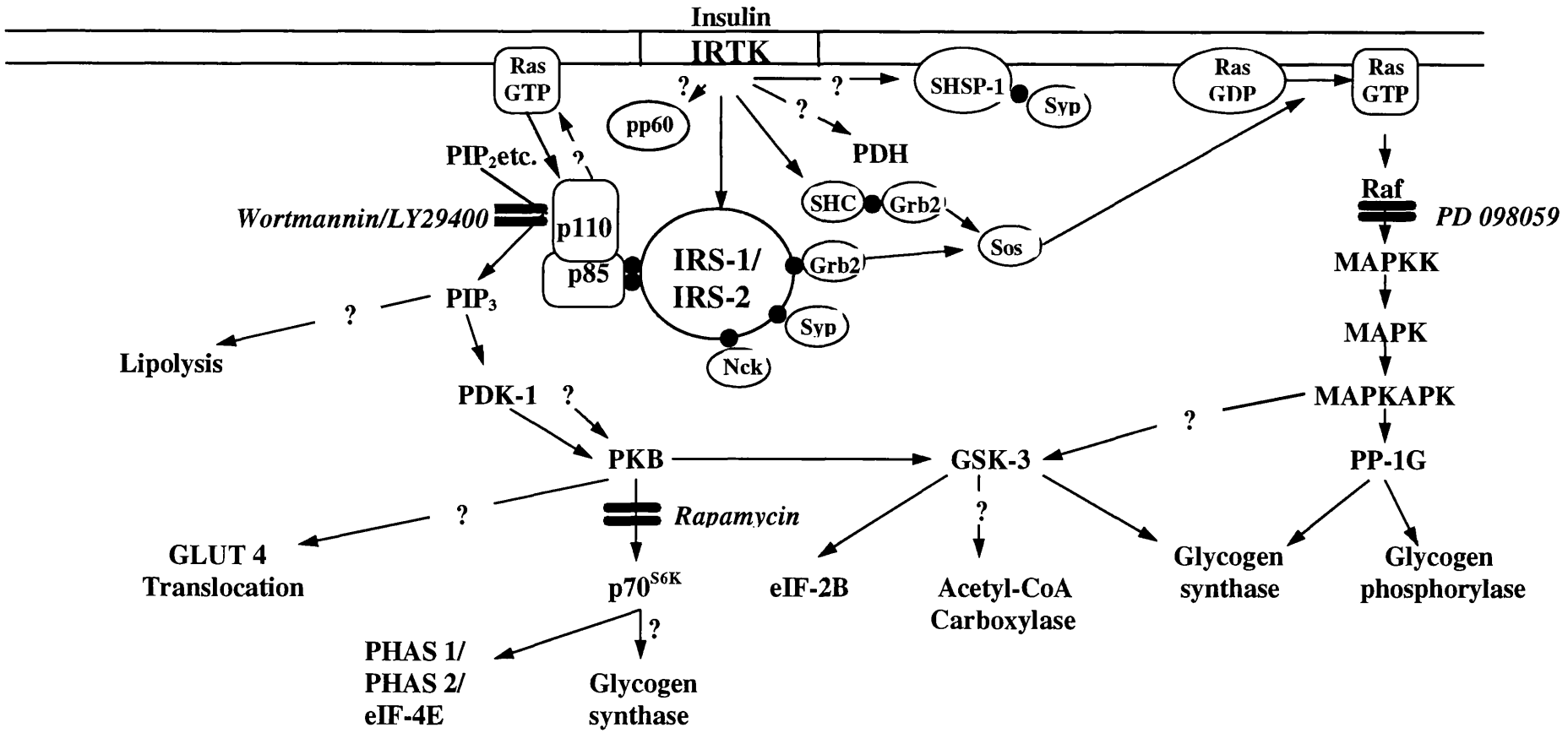
1.3.3 Insulin Signalling Pathways

When insulin binds to its receptor it causes autophosphorylation of the receptor β-subunit which leads to enhanced tyrosine kinase activity towards cellular substrates. Tyrosine phosphorylation of these cellular substrates allows 'docking' and activation of a number of intermediates through their SH2 domains. These intermediates in turn activate the signalling pathways which lead to the downstream actions of insulin (Fig. 1.3) (for reviews see Lee & Pilch, 1994; White & Kahn, 1994; Cheatham & Kahn, 1995; Shepherd *et al.*, 1996). There are two main pathways involved in the propagation of the insulin signal, the MAP kinase pathway and the PI3-kinase pathway.

1.3.3.1 The MAP Kinase Pathway

1.3.3.1.i MAP kinase MAP kinase (Mitogen Activated Protein Kinase) can be activated by a number of growth factors and mitogens, including insulin. Activation by insulin was first demonstrated in 3T3-L1 adipocytes by Ray and Sturgill (1987) and has since been observed in a variety of cultured cell lines (Dickens *et al.*, 1992; Peraldi & Van Obberghen, 1993; Cross *et al.*, 1994; Pang *et al.*, 1994) and in mammalian cells (Haystead *et al.*, 1990b, 1994; Sevetson *et al.*, 1993; Lin & Lawrence, 1994). A number of isoforms of this enzyme have been found in mammalian cells, all of which are members of the serine/threonine protein kinase superfamily (Boulton *et al.*, 1991; Kyriakis *et al.*, 1994). The two main isoforms which appear to be involved in insulin

Fig 1.3 The Insulin Signalling Network



signalling are $p42^{MAPK}$ and $p44^{MAPK}$ or Erk-1 and Erk-2 (extracellular signalling regulated kinases 1 and 2) as they are also known (Haystead *et al.*, 1990, Sevetson *et al.*, 1993; Lin & Lawrence, 1994). In order to fully activate MAP kinase it must be phosphorylated on both threonine and tyrosine residues, dephosphorylation of either residue results in reduced activity of the kinase (Anderson *et al.*, 1990).

1.3.3.1.ii MAPKK Phosphorylation of the MAP kinases is brought about by MAP kinase kinases (MAPKKs) which have dual specificity for tyrosine and threonine (Ahn *et al.*, 1992; Zheng & Guan, 1993). This phosphorylation is thought to cause a conformational change in the enzyme, which may allow substrate binding (Zhang *et al.*, 1994a). Activation of the MAPKKs is in turn the result of phosphorylation on serine and threonine residues by MAP kinase kinase kinases (MAPKKKs). There are a number of these enzymes but only Raf-1 and a distinct MAPKKK found in fat cells have been shown to be stimulated by insulin (Kovacina *et al.*, 1990; Cross *et al.*, 1994; Haystead *et al.*, 1994; Porras *et al.*, 1994).

1.3.3.1.iii Raf and Ras Activation of 74kDa Raf-1 by insulin involves p21 Ras which is a member of a large family of low molecular weight (20-29kDa) G-proteins (GTP-binding protein) (for review see Denhardt, 1996). Raf-1 interacts with the GTP-bound form of Ras via two distinct regions (Brtva *et al.*, 1995; Hu *et al.*, 1995; Drugan *et al.*, 1996). This interaction allows the relocation of Raf-1, which is normally cytosolic (Schulte *et al.*, 1995), to the plasma membrane where Ras is located (Leevers *et al.*, 1994; Wartmann & Davis, 1994). For Raf-1 to be fully activated it must then be phosphorylated on serine, threonine and tyrosine residues (Dent *et al.*, 1995). Although Ras-induced activation is thought to be dependent on tyrosine

phosphorylation (Jelinek *et al.*, 1996) The kinase(s) responsible for this phosphorylation have yet to be determined. Activation of Raf-1's serine/threonine kinase activity towards MAPKK is thought to be a result of conformational change(s) induced by such phosphorylation (Zheng & Guan, 1994).

As was mentioned previously, the guanine nucleotide exchange factor Sos promotes the conversion of Ras from its inactive GDP-bound form to its active GTP-bound form. Sos in turn is activated through its association with the adapter molecule Grb2 (Chardin *et al.*, 1993). Thus insulin may be able to stimulate Ras and hence MAP kinase activation through interaction of the tyrosine phosphorylated insulin receptor substrates with Grb2:Sos (Skolnik *et al.*, 1993a,b).

1.3.3.1.iv MAPKKK MAP kinase can also be stimulated by insulin via a Ras-independent pathway. Carel *et al.* (1996) have shown that metabolically active 3T3-L1 adipocytes can activate MAP kinase in the absence of Ras or Raf activation, MAPKK activation however is required. This suggests that MAPKK is activated by a MAPKKK other than Raf-1. One possible candidate is the insulin-responsive MAPKKK found in rat adipocytes (Haystead *et al.*, 1994). However it is not known if this enzyme is activated by Ras.

1.3.3.1.v MAP kinase Substrates The MAP kinases can phosphorylate a variety of proteins *in vivo* including other serine/threonine kinases such as the MAP kinase-activated protein (MAPKAP) kinases 1α and 1β which are also known as the p90 S6 kinases Rsk-1 and Rsk-2 (Seger & Krebs, 1995). These enzymes were originally recognised for their ability to phosphorylate the 40S ribosomal subunit protein S6.

Phosphorylation of this protein is thought to result in more efficient initiation/ translation of mRNAs and/or to permit the selective translation of certain mRNAs (Duncan & McConkey, 1982; Thomas *et al.*, 1982). However a second class of S6 kinase, $p70^{S6K}$, which is not a substrate for MAP kinase, has now been identified as the major physiological S6 kinase in mammalian cells (Kozma & Thomas, 1994). MAPKAP kinase-1/p90Rsk may still be involved in mRNA translation through its effects on eIF-2B via GSK-3 (Glycogen Synthase Kinase-3). It is also thought to be involved in the activation of glycogen synthesis by insulin which will be discussed in section 1.3.4.2.

As well as the downstream substrates just mentioned, MAP kinase also has a number of potential upstream substrates. These include IRS-1 (Myers *et al.*, 1994a), Sos (Cherniack *et al.*, 1994; Ueki *et al.*, 1994; Waters *et al.*, 1995), Raf (Ueki *et al.*, 1994; Wartmann *et al.*, 1997) and MAPKK- 1 (Gardner *et al.*, 1994; Saito *et al.*, 1994; Ueki *et al.*, 1994) which may all be involved in a complicated feedback inhibition system (Alessi *et al.*, 1995). Protein phosphatase 2C can also be phosphorylated and activated by MAP kinase (Peraldi *et al.*, 1994) and may be involved in its regulation.

1.3.3.2 Phosphatidylinositol 3-Kinase (PI3-kinase) Pathway

1.3.3.2.i Phosphatidylinositol 3-kinase PI3-kinase appears to be involved in the signal transduction pathways of a number of growth factors, oncogenic proteins and cytokines (For Reviews see Cantley *et al.*, 1991; Kapeller & Cantley, 1994; Carpenter & Cantley, 1996; Vanhaesebroeck et al., 1997). Inhibitor and mutational studies have implicated PI3-kinase in a number of cellular functions including inhibition of

apoptosis (Franke *et al.*, 1997a), mitogenesis, regulation of actin and integrin function, intracellular vesicle trafficking/secretion (Carpenter & Cantley, 1996) and in the case of insulin-stimulated PI3-kinase, regulation of metabolism (Shepherd *et al.*, 1996).

The first PI3-kinase to be purified and characterised was the mammalian p85-p110 dimeric enzyme which associates with protein tyrosine kinases (Carpenter *et al.*, 1990). Since the cloning of the p110 subunit of this isoform (Hiles *et al.*, 1992) a number of molecules with homology to this catalytic domain have been identified in a variety of eukaryotic cells (Zvelebil *et al.*, 1996). However only the heterodimeric p85-p110 isoform is known to be stimulated by insulin.

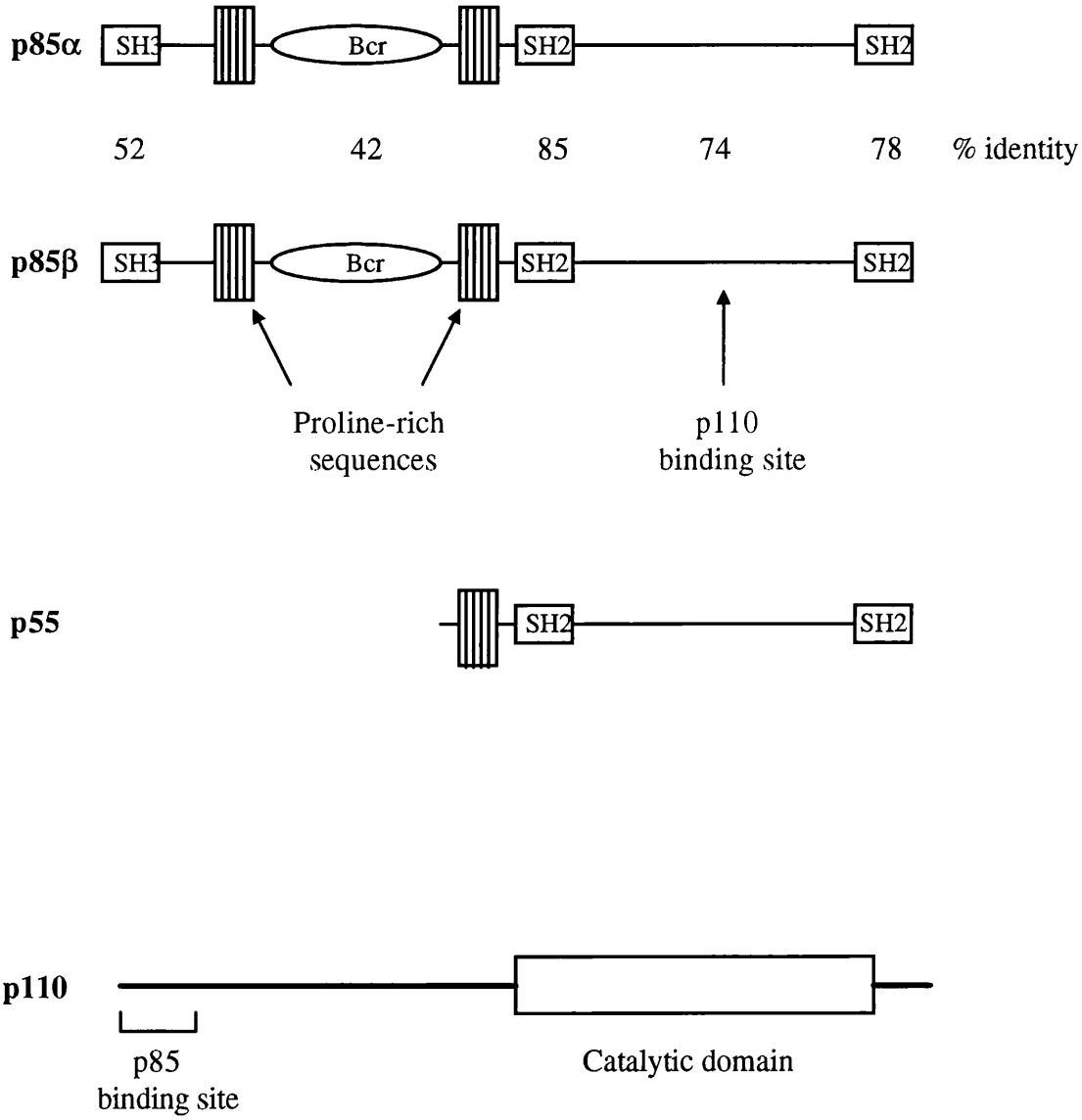
The p85 subunit of PI3-kinase acts as an 'adapter' linking the catalytic p110 subunit to specific phosphoproteins. This in turn activates the PI3-kinase activity endogenous to the p110 subunit. Several forms of this regulatory subunit have now been identified (Escobedo *et al.*, 1991; Otsu *et al.*, 1991; Skolnik *et al.*, 1991; Pons *et al.*, 1995; Antonetti *et al.*, 1996), each of which contains two SH2 domains. As was mentioned previously, it is through these SH2 domains that the p85 subunit associates with tyrosine phosphorylated motifs on the insulin receptor substrates IRS-1, IRS-2 and pp60 and on growth factor receptors (Myers *et al.*, 1992; Tobe *et al.*, 1995: Zhang-Sun *et al.*, 1996). The region between the two SH2 domains (the inter-SH2 domain) contains a sequence motif through which the p85 subunit binds the N-terminal region of the p110 catalytic subunit (Klippel *et al.*, 1993; Hu & Schlessinger, 1994). The gene products of p85α and p85β also contain an N-terminal SH3 domain and a bcr/rac GAP homology domain, located between the SH3 domain and the N-terminal

SH2 domain (Gout *et al.*, 1992). A role for these domains in insulin signalling has yet to be determined. Of the three other forms of p85 identified, two are splice variants of the p85α gene (Antonetti *et al.*, 1996) and the third, a 55 kDa protein, is encoded by a third PI3-kinase regulatory subunit gene found mainly in neuronal tissues (Pons *et al.*, 1995).

The p110 subunit is responsible for the catalytic activity of PI3-kinase. (Hiles *et al.*, 1992). Two isoforms of this subunit have been identified, p110α and p110β, which are encoded by two highly homologous genes (Hiles *et al.*, 1992; Hu *et al.*, 1993). Comparison of the amino acid sequence of p110 with conserved sequences in the catalytic domains of known protein kinases (Hanks *et al.*, 1988) has revealed that p110 has a DRHNSN motif which is homologous to the ATP binding domain of protein kinases (Taylor *et al.*, 1992c). This region appears to be important for the catalytic activity of PI3-kinase as a point mutation in this motif (Arg916-Pro) has been shown to abolish both its ability to phosphorylate the D-3 position on the inositol head group of phosphoinositides and serine residues (Dhand *et al.*, 1994). Fig. 1.4 shows a schematic diagram of p85 and p110 giving the relative posistions of their domain structures.

The endogenous serine/threonine kinase activity of p85:p110 PI3-kinase was only discovered upon purification of this enzyme to homogeneity (Carpenter *et al.*, 1993). This activity is not thought to be directly involved in downstream signalling, but may contribute to an autoregulatory feedback mechanism. The only substrates of this serine/threonine kinase activity identified so far are the p85 subunit of PI3-kinase itself (Dhand *et al.*, 1994) and IRS-1 (Lam *et al.*, 1994; Tanti *et al.*, 1994b).

Fig. 1.4 Phosphatidylinositol 3-Kinase Subunits



Shows schematic diagrams of the p85α, p85β and p55 regulatory subunits and p110 catalytic subunit of PI3-kinase showing their different domain structures and the percentage identity between p85α and p85β.

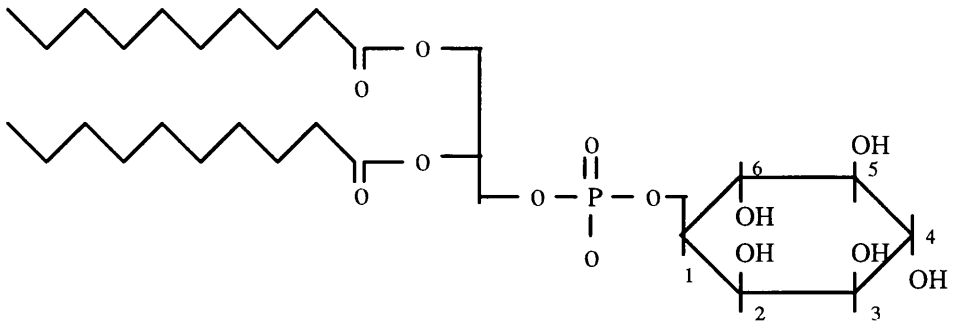
Phosphorylation of p85α on Ser-608 by p110 resulted in an 80% decrease in PI3-kinase activity. This could only occur when p85 was tightly bound to p110 (Dhand *et al.*, 1994). Upon insulin stimulation of 3T3-L1 adipocytes, the p110 serine kinase was able to phosphorylate both p85 and IRS-1 (Tanti *et al.*, 1994b). Phosphorylation of IRS-1 by the kinase, like the effect on p85, may play a role in modulating insulin signalling. Previous reports have shown that increased serine/threonine phosphorylation of IRS-1 can attenuate the effects of insulin (Rice *et al.*, 1992, 1993; Tanti *et al.*, 1994a).

The main effect of the PI3-kinases is to phosphorylate the D-3 position on the inositol ring of phosphatidylinositol (PI) (fig. 1.5A), phosphatidylinositol(4) phosphate (PI-4-P) and phosphatidylinositol(4,5)bisphosphate (PI-4,5-P_2), generating phosphatidyl-inositol(3) phosphate (PI-3-P), phosphatidyl-inositol(3,4)bisphosphate (PI-3,4-P_2) and phosphatidylinositol(3,4,5)triphosphate (PI-3,4,5-P_3) respectively (fig. 1.5B). The different forms of PI3-kinase in mammalian cells have distinct regulatory mechanisms and substrate specificitys (Carpenter & Cantley, 1996; Zvelebil *et al.*, 1996). *In vivo*, the major substrate of p110α and p110β seems to be PI-4,5-P_2 (Stephens *et al.*, 1993), which is also a substrate for phospholipase C. However no known phospholipase uses PI-3,4,5-P_3 as a substrate (Serunian *et al.*, 1989).

Activation of PI3-kinase by insulin appears to be stimulated by the interaction of the p85 subunit SH2 domains with specific tyrosine phosphorylated motifs (YMXM) on IRS-1 (and possibly other insulin receptor substrates) (Myers *et al.*, 1992; Giorgetti *et al.*, 1993; Herbst *et al.*, 1994). This interaction is also thought to bring the PI3-kinase complex into close proximity with its substrate which is found on membranes

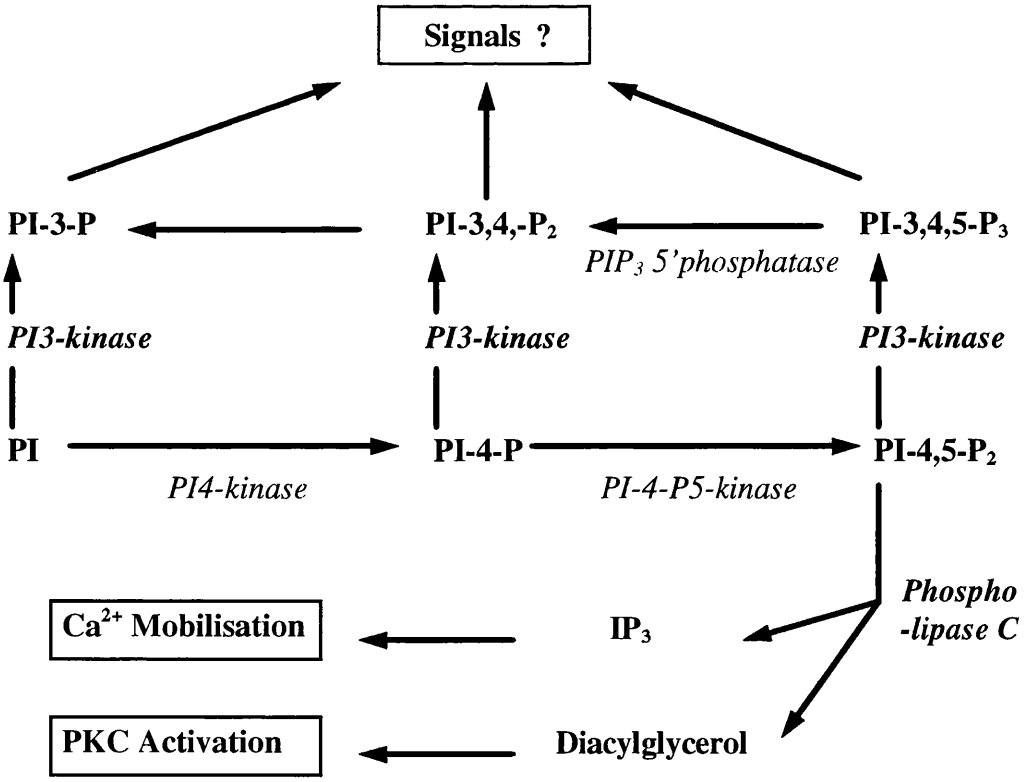
Fig. 1.5 Structure of Phosphatidylinositol and Reactions Catalysed by Phosphoinositide Kinases

A



Phosphadidylinositol (PI)

B



[Adapted from Shepherd *et al.*, 1996]

(Kelly & Ruderman, 1993). Insulin treatment leads to a rapid increase in the amount of PI-3,4-P_2 and PI-3,4,5-P_3 in cells (Ruderman *et al.*, 1990). The increase in PI-3,4-P_2 concentration may not be a direct result of PI-4-P phosphorylation by PI3-kinase. It may instead be due to hydrolysis of the D-5 phosphate from PI-3,4,5-P_3 by a 5'phosphatase (Stephens *et al.*, 1993) (Fig. 1.5). Indeed it has been shown that 5'phosphatases, which specifically use PI-3,4,5-P_3 as a substrate, can be recruited into signalling complexes upon growth factor stimulation (Jackson *et al.*, 1995; Damen *et al.*, 1996; Kavanaugh *et al.*, 1996). As these 3'phosphorylated phosphoinositides are not broken down by phospholipases to generate secondary messengers, it is thought that PI-3,4-P_2 and PI-3,4,5,-P_3 must themselves mediate the effects of PI3-kinase (Kelly & Ruderman, 1993). Evidence of this is now indeed beginning to emerge.

1.3.3.2.vii Protein Kinase B PKBα (protein kinase Bα) (Coffer & Woodgett, 1991), also known as c-Akt (Bellacosa *et al.*, 1991) and RACα (Jones *et al.*, 1991) is the cellular homologue of the transforming v-Akt (Staal *et al.*, 1977). It is a serine/threonine kinase which is activated within one minute of stimulating cells with insulin (Cross *et al.*, 1995; Kohn *et al.*, 1995; Moule *et al.*, 1997). Activation of PKB by insulin or IGF -1 is a result of its phosphorylation at Thr-308 and Ser-473, phosphorylation of both residues being necessary for a high level of activity (Alessi *et al.*, 1996). Both the activation of PKBα (Burgering & Coffer, 1995; Franke *et al.*, 1995) and its phosphorylation (Alessi *et al.*, 1996) are prevented by the PI3-kinase inhibitor wortmannin, suggesting that this enzyme is downstream of PI3-kinase. Recently it has been shown by three different groups that the PI3-kinase product, PI-3,4-P_2, can bind to and activate PKBα via its N-terminal pleckstrin homology (PH) domain (Franke *et al.*, 1997b; Frech *et al.*, 1997; Klippel *et al.*, 1997). Yet others

have shown that although both PI-3,4-P_2 and PI-3,4,5-P_3 can bind to PKBα through its PH domain, they cannot activate it (James *et al.*, 1996; Alessi *et al.*, 1997). Also a mutant form of PKBα, lacking the PH domain was shown to be activated at least as well as wild-type PKBα (Kohn *et al.*, 1995, 1996b) indicating that binding of PI-3,4-P2 to this domain is not required for insulin-stimulated activation.

1.3.3.2.viii PDK-1 Alessi and colleagues have now purified and characterised a protein kinase which phosphorylates PKBα at Thr-308 and increases its activity over 30 fold (Alessi *et al.*, 1997). This 67 kDa enzyme was potently activated by low micromolar concentrations of PI-3,4-P_2 and PI-3,4,5-P_3 and hence was named PI-3,4,5-P_3 -dependent protein kinase-1 (PDK-1). Thus it would appear this kinase may be one of the 'missing links' in the PI3-kinase dependent activation of PKBα. The kinase which phosphorylates Ser-473, allowing high level activation of the enzyme, has not yet been found.

1.3.3.2.viii GSK-3 The only substrate of PKBα found to date is GSK-3 (glycogen synthase kinase -3) Cross *et al.*, 1995). This enzyme has two isoforms in mammalian cells, GSK-3α and GSK-3β (Woodgett, 1991). Insulin stimulation of cells results in an inhibition of GSK-3 (Welsh & Proud, 1993; Cross *et al.*, 1995) through phosphorylation on Ser-21 of GSK-3α and Ser-9 of GSK-3β by PKBα (Cross *et al.*, 1995). This effect of insulin can be inhibited both by wortmannin and the other PI3-kinase inhibitor LY 294002 (Welsh *et al.*, 1994; Moule *et al.*, 1997), suggesting that both PKBα and GSK-3 are downstream of PI3-kinase. Other protein kinases have also been shown to phosphorylate GSK-3 *in vitro*, including $p70^{S6K}$ and MAPKAP

kinase which can both be stimulated by insulin (Sutherland *et al.*, 1993b; Sutherland & Cohen, 1994). Studies using the $p70^{S6K}$ inhibitor rapamycin have ruled out this enzyme as a regulator of GSK-3 in L6 myocytes (Cross *et al.*, 1994), CHO cells (Welsh *et al.*, 1994) and rat adipocytes (Moule *et al.*, 1995). The evidence for MAPKAP kinase activation of GSK-3 is conflicting, some studies give evidence that the MAP kinase pathway does play a role (Stambolic & Woodgett, 1995; Eldar-Finkalman *et al.*, 1995) while others show that it doesn't (Cross *et al.*, 1995; Moule *et al.*, 1995). The relevance of this pathway in downstream events will be discussed later.

GSK-3 stimulates the phosphorylation and in most cases the inactivation of a number of substrates, many of which are involved in the downstream effects of insulin. These include glycogen synthase which catalyses glycogen synthesis, acetyl-CoA carboxylase and ATP citrate-lyase (Hughes *et al.*, 1992) which are involved in lipogenesis, and the protein synthesis initiation factor eIF-2B (Welsh & Proud, 1993) which mediates cycling of eukaryotic initiation factor-2 (eIF-2), the protein responsible for attachment of Met-tRNA to the ribosome during initiation of translation in eukaryotic cells. Inactivation of this enzyme by insulin would therefore seem to play a major role in insulin's metabolic effects.

1.3.3.2.ix $p70^{S6K}$ The enzyme $p70^{S6K}$, which is responsible for phosphorylating the S6 protein of the 40S subunit of eukaryotic ribosomes (Kozma & Thomas, 1994), is also acutely stimulated by insulin (Thomas, 1993). Activation of this serine/threonine kinase is accompanied by its phosphorylation on seven Ser/Thr residues (Ferrari *et al.*, 1992; Kozma *et al.*, 1993). Four of these residues lie immediately carboxy-terminal to

the catalytic domain (Ser-411, Ser -418, Thr-421 and Ser-424) and are each followed immediately by a proline residue (Ferrari *et al.*, 1992). This Ser/Thr-Pro motif is targeted by a range of kinases, including MAP kinase. It has been shown however that the pathway activating $p70^{S6K}$ is distinct from the MAP kinase pathway (Ballou *et al.*, 1991). These four carboxy-terminal residues, which are partially phosphorylated under basal conditions, undergo rapid phosphorylation in response to stimuli, as do Thr-229, Thr-389 and Ser-404 which are not basely phosphorylated (Han *et al.*, 1995). The kinase(s) responsible for the phosphorylation of $p70^{S6K}$ have yet to be identified, but the common Ser/Thr-Pro motif of the carboxy-terminal residues and the similarity in the sequence contexts of the other three residues suggest that at least two kinases may be involved. Rapamycin, an immunosuppressant which selectively inhibits activation of $p70^{S6K}$ (Calvo *et al.*, 1992; Chung *et al.*, 1992; Price *et al.*, 1992), prevents the phosphorylation of Ser-411 in the carboxy-terminal region and all three of the residues in the N-terminal region. The principle target of rapamycin-induced $p70^{S6K}$ inactivation appears to be Thr-389 (Pearson *et al.*, 1995).

As well as being inhibited by rapamycin, insulin-stimulated $p70^{S6K}$ activation can also be prevented by the PI3-kinase inhibitors wortmannin and LY294002 (Cheatham *et al.*, 1994; Chung *et al.*, 1994), suggesting that this Ser/Thr kinase lies downstream of PI3-kinase. Overexpression of PKB in Rat-1 cells led to the activation of $p70^{S6K}$ giving further evidence that stimulation of this enzyme is mediated by a PI3-kinase dependant pathway (Burgering & Coffer, 1995). Treatment of these cells with rapamycin ablated $p70^{S6K}$ activation but had no effect on PKB activation. This suggests that PKB is either upstream or in a parallel pathway to the target of rapamycin inhibition (Burgering & Coffer, 1995). Additional evidence, both from

inhibitor and mutational studies, seems to support the idea that $p70^{S6K}$ is activated via parallel pathways, one involving PI3-kinase and PKB which is wortmannin sensitive, and another which is rapamycin sensitive and involves mTOR (mammalian Target of Rapamycin) (Proud, 1996). Rapamycin inhibits mTOR by binding to a protein termed FKBP12 (FK506-binding protein 12, where FK506 is another immunosuppressant). Rapamycin-FKBP12 interacts with mTOR preventing its kinase activity which appears to be important in $p70^{S6K}$ activation (Brown *et al.*, 1995). However, mTOR is a homologue of the p110 subunit of PI3-kinase and can also be inhibited by wortmannin and LY294002, which suggests that the inhibition of $p70^{S6K}$ by these substances may, in part, be due to their effect on mTOR kinase (Brunn *et al.*, 1996).

1.3.3.2.x PHAS-I and -II PI3-kinase also appears to be involved in the insulin stimulated phosphorylation of the translation regulators PHAS-I and PHAS-II, (Phosphorylated Heat and Acid-Stable protein) also known as 4E-BP-1 and 4E-BP-2 (eIF-4E-Binding Proteins 1 and 2), via both rapamycin sensitive and insensitive pathways (Azipiazu *et al.*, 1996; Diggle *et al.*, 1996; Lin & Lawrence, 1996). Non-phosphorylated PHAS-I binds tightly to eIF-4E (eukaryotic initiation factor-4E) (Lin *et al.*, 1994; Pause *et al.*, 1994), a key component in the eIF-4F complex which catalyses 'melting' of secondary structure in the 5'-untranslated region of mRNAs. Melting of this secondary structure allows more efficient binding and/or scanning of the mRNA by the 40S ribosomal subunit (Rhoads, 1993). When eIF-4E is bound to PHAS-I it cannot become part of the eIF-4F complex, consequently PHAS-I inhibits cap-dependent mRNA translation (Pause *et al.*, 1994). Phosphorylation of PHAS-I in response to insulin and other growth factors promotes dissociation of PHAS-I:eIF-4E, increasing the amount of eIF-4E available to form the eIF-4F initiation complex

(Sonenberg, 1996). The amount of eIF-4E is thought to be rate limiting for initiation, which is in turn thought to be the rate limiting phase of protein synthesis (Hershey, 1991; Proud, 1992). The sites in PHAS-I which are phosphorylated in response to insulin in rat adipocytes have now been identified as Thr-36, Thr-45, Ser-64, Thr-69 and Ser82 (Fadden *et al.*, 1997). Certain sites appear to be more important than others in the dissociation of PHAS-I from eIF-4E as, for example, PHAS-I phosphorylated at Thr-36 remains bound to eIF-4E. All the phosphorylation sites in PHAS-I lie in Ser/Thr-Pro motifs, similar to the four sites in the carboxy-terminal domain of $p70^{S6K}$ (Ferrari *et al.*, 1992). This and the fact that that both PHAS-I and $p70^{S6K}$ are inhibited by rapamycin suggests that they may both be phosphorylated by the same protein kinase (Fadden *et al.*, 1997).

1.3.3.2.xi Ras As well as associating with tyrosine phosphorylated proteins such as IRS-1, PI3-kinase can also associate with the GTP-bound form of Ras (Kodaki *et al.*, 1994; Rodriguez-Viciana *et al.*, 1994). This association appears to take place between the effector binding region of Ras (Kodaki *et al.*, 1994; Rodriguez-Viciana *et al.*, 1994), and residues 133 to 314 in the p110 subunit of PI3-kinase which lie immediately C-terminus to the p85 binding site. (Rodriguez-Viciana *et al.*, 1996). Overexpression of Ras and p85/p110 in COS cells led to activation of PI3-kinase's lipid kinase activity, suggesting that association of Ras with PI3-kinase may activate the enzyme *in vivo* (Rodriguez-Viciana *et al.*, 1994). *In vitro* reconstitution assays have now shown that direct interaction of Ras with PI3-kinase leads to activation of PI3-kinase's lipid kinase activity. This effect was synergistic with the activation of PI3-kinase through interaction with phosphopeptides containing p85, SH2 domain binding sites (Rodriguez-Viciana *et al.*, 1996). Thus it would appear that PI3-kinase is

another downstream effector of Ras and that full activation of PI3-kinase may require both binding of its p85 subunit to tyrosine phosphorylated proteins, and binding of its p110 subunit to GTP-bound Ras. However other studies using constitutively active PI3-kinase suggest that Ras is downstream of PI3-kinase (Hu *et al.*, 1995). This model has been supported by data which shows that mutants of, or antibodies against the N-terminal SH2 domains of PI3-kinase can block the induction of Fos by insulin, an effect which rescued by activated Ras (Jhun *et al.*, 1994). Although there is no explanation for this conflicting data, it may be that PI3-kinase is both upstream and downstream of Ras or that it is dependent on cell type (Carpenter & Cantley, 1996).

1.3.4 Downstream Effects Of Insulin Signalling

1.3.4.1 Glucose Transport

One of the major effects of insulin is to stimulate the uptake of glucose into tissues, particularly muscle and fat. This is achieved by facilitated diffusion of glucose from the plasma membrane to the interior of the cell via specific glucose transporter proteins (Simpson & Cushman, 1989). A number of glucose transporter genes have been identified which encode a family of homologous proteins (GLUT-1 through GLUT-7) that exhibit different functional properties and tissue-specific expression (Bell *et al.*, 1990; Mueckler, 1990; Bell *et al.*, 1993; Mueckler, 1994). Of these isoforms only GLUT-1 and GLUT-4 are stimulated by insulin. GLUT-1 was the first transporter to be cloned and has the widest tissue distribution. In the basal state the majority of glucose transport is facilitated by GLUT-1. In response to insulin however, GLUT-1 is only increased approximately 2-fold at the plasma membrane which does not account for the increase in glucose transport. GLUT-4 on the other hand is restricted to insulin-sensitive tissues, namely muscle and adipose, and is

responsible for the bulk of the insulin mediated increase in glucose transport (for reviews see James *et al.*, 1994; Mueckler, 1994; Stephens & Pilch, 1995). Insulin stimulation of cells results in a 20-to 30-fold increase in glucose uptake This is due to the translocation of vesicles containing GLUT-4 from an intracellular pool, associated with microsomal membranes, to the cell surface. Here they fuse with the plasma membrane and pick up glucose (Cushman & Wardzala, 1980; Holman *et al.*, 1990; Slot *et al.*, 1991a,b; Marette *et al.*, 1992; Kanai *et al.*, 1993). The mechanism of vesicle retraction from the plasma membrane is not entirely clear, but is thought to involve internalisation via clathrin-coated pits (Slot *et al.*, 1991a,b; Robinson *et al.*, 1992; Piper *et al.*, 1993; Chakrabarti *et al.*, 1994).

1.3.4.1.i Signalling pathways The signal transduction pathway(s) which lead to glucose transport and GLUT-4 translocation in response to insulin are largely unknown. Activated Ras was shown to play a role in the regulation of glucose transport by Kozma *et al.* (1993) and Manchester *et al.* (1994), but the results of these studies were not definitive. MAP kinase activation is not thought to play a role in glucose transport for a number of reasons. First of all, $p42^{MAPK}$ and $p44^{MAPK}$ can both be activated by insulin and EGF, yet only insulin can promote glucose transport (Robinson *et al.*, 1993; Fingar & Birnbaum, 1994a; Lin & Lawrence, 1994) and GLUT-4 translocation (Gould *et al.*, 1994) which suggests that MAP kinase activation is not sufficient to activate glucose transport. Also, the introduction of oncogenically active Raf-1 into 3T3-L1 adipocytes, which should activate MAP kinase, had no effect on glucose transport (Fingar & Birbaum, 1994b). Finally, the time courses of MAP kinase activation and glucose transport stimulation by insulin are not complimentary, at least in 3T3-L1 adipocytes (Robinson *et al.*, 1993).

Present attention is focused on the effect of PI3-kinase on glucose transport and GLUT-4 translocation. The yeast homologue of PI3-kinase, Vps34p, has been shown to have an essential role in vesicle trafficking in yeast (Herman & Erm, 1990; Stack *et al.*, 1993), which suggests that PI3-kinase may have a similar role in mammalian cells. And indeed studies using the PI3-kinase inhibitors wortmannin and LY294002 have shown that these substances can block the insulin-stimulated activation of glucose transport and GLUT-4 translocation in adipocytes (Cheatham *et al.*, 1994; Clark *et al.*, 1994; Okada *et al.*, 1994; Yang *et al.*, 1996), cultured myocytes (Kaliman *et al.*, 1995; Tsakiridis *et al.*, 1995) and rat skeletal muscle (Yeh *et al.*, 1995; Wojtaszewski *et al.*, 1996). Further evidence of a role for PI3-kinase has been given by Haruta *et al.* (1995) who showed that microinjection of the SH2 domains of p85 into 3T3-L1 adipocytes could block insulin stimulated glucose transport. Also, expression of a dominant negative mutant of the p85, lacking the binding site for the catalytic p110 subunit, has been shown to inhibit insulin stimulation of both PI3-kinase and glucose transporter translocation in CHO cells and 3T3-L1 adipocytes (Hara *et al.*, 1994; Kotani *et al.*, 1995). These findings suggest that activation of PI3-kinase by insulin is critical to the stimulation of glucose transport. Other studies however suggest that the activation of PI3-kinase alone is not sufficient to induce these events.

As well as being activated by insulin, PI3-kinase is also activated by PDGF (through association with the tyrosine phosphorylated PDGF receptor) and IL-4 (through association with IRS-1 which is tyrosine phosphorylated in response to IL-4), yet only insulin is able to stimulate glucose transport (Isakoff *et al.*, 1995; Wiese *et al.*, 1995; Navé *et al.*, 1996). This may be explained by differential subcellular targeting of PI3-

kinase. Insulin stimulates PI3-kinase in the microsomal fraction of cells where the intracellular pool of glucose transporters is located (Kelly & Ruderman, 1993; Navé *et al.*, 1996; Yang *et al.*, 1996), whereas PDGF only stimulates PI3-kinase in the plasma membrane-containing fraction (Navé *et al.*, 1996). Thus activation of glucose transporter translocation may require localisation of activated PI3-kinase to the microsomal membranes. The subcellular location of IL-4 activated PI3-kinase has yet to be determined.

Further evidence for other pathways being involved in glucose transport has recently been presented by Frevent & Kahn (1997). They have shown that overexpression of constitutively active PI3-kinase in 3T3-L1 adipocytes could only partial activate glucose transport and GLUT-4 translocation, despite the levels of PI3-kinase activity in the microsomal fractions being higher than those normally induced by insulin. These findings would appear to argue against subcellular localisation of PI3-kinase being responsible for stimulation of glucose transporter translocation. It is however possible that insulin induces PI3-kinase in a specific subfraction of the microsomal compartment to a greater extent than the constitutively active PI3-kinase (Frevent & Kahn, 1997). Studies to determine the precise location of insulin-stimulated PI3-kinase activity have already begun (Heller-Harrison *et al.*, 1996).

The mechanism by which PI3-kinase exerts its effects on glucose transport is still unclear. $p70^{S6K}$ does not appear to be involved as rapamycin, which blocks the activation of $p70^{S6K}$, has no effect on glucose transport (Fingar *et al.*, 1993; Lin & Lawrence, 1994). However, expression of a constitutively active form of PKB in 3T3-L1 adipocytes has been shown to stimulate glucose uptake to the same degree as

insulin and also to induce translocation of GLUT-4 to the cell surface (Kohn *et al.*, 1996a). These findings suggest that glucose transport activation is downstream of PKB activation.

1.3.4.2 Glycogen Synthesis

As well as enhancing uptake of glucose from the bloodstream, insulin also promotes glucose storage. One way insulin does this is to promote glycogen synthesis in the liver, skeletal muscle and adipose tissue (for reviews see Lawrence, 1992; Cohen, 1993).

1.3.4.2.i Glycogen Synthase Conversion of glucose into glycogen is controlled by the enzyme glycogen synthase. This enzyme catalyses the transfer of α-glucosyl residues from UDP-glucose to the growing glycogen chain. Glycogen synthase is regulated both by allosteric interactions and reversible phosphorylation, the enzyme being active in its dephosphorylated form (glycogen synthase $_a$). Insulin increases the rate of glycogen synthesis primarily by dephosphorylating serines C30, C34 and C38 in the carboxy-terminal region of glycogen synthase (Parker *et al.*, 1983), although dephosphorylation of other sites may also play a role (Lawrence & Zhang, 1994). The phosphorylation of these residues is controlled by the relative activities of GSK-3 (Rylatt *et al.*, 1980) and the glycogen-associated form of protein phosphatase-1 (PP-1G) (Ingebristen *et al.*, 1983b,c; Alemany *et al.*, 1986; Stralfors *et al.*, 1985; Hubbard & Cohen, 1989a,b). PP-1G is also responsible for the phosphorylation and inactivation of both glycogen phosphorylase, which catalyses glycogen breakdown, and phosphorylase kinase, the enzyme which is responsible for activating glycogen

phosphorylase (Cohen, 1978; Ingebristen *et al.*, 1983a,b). Glycogen phosphorylase, like glycogen synthase, is regulated by both allosteric interactions and covalent modification through phosphorylation/dephosphorylation. Unlike glycogen synthase, glycogen phosphorylase is active in its phosphorylated form.

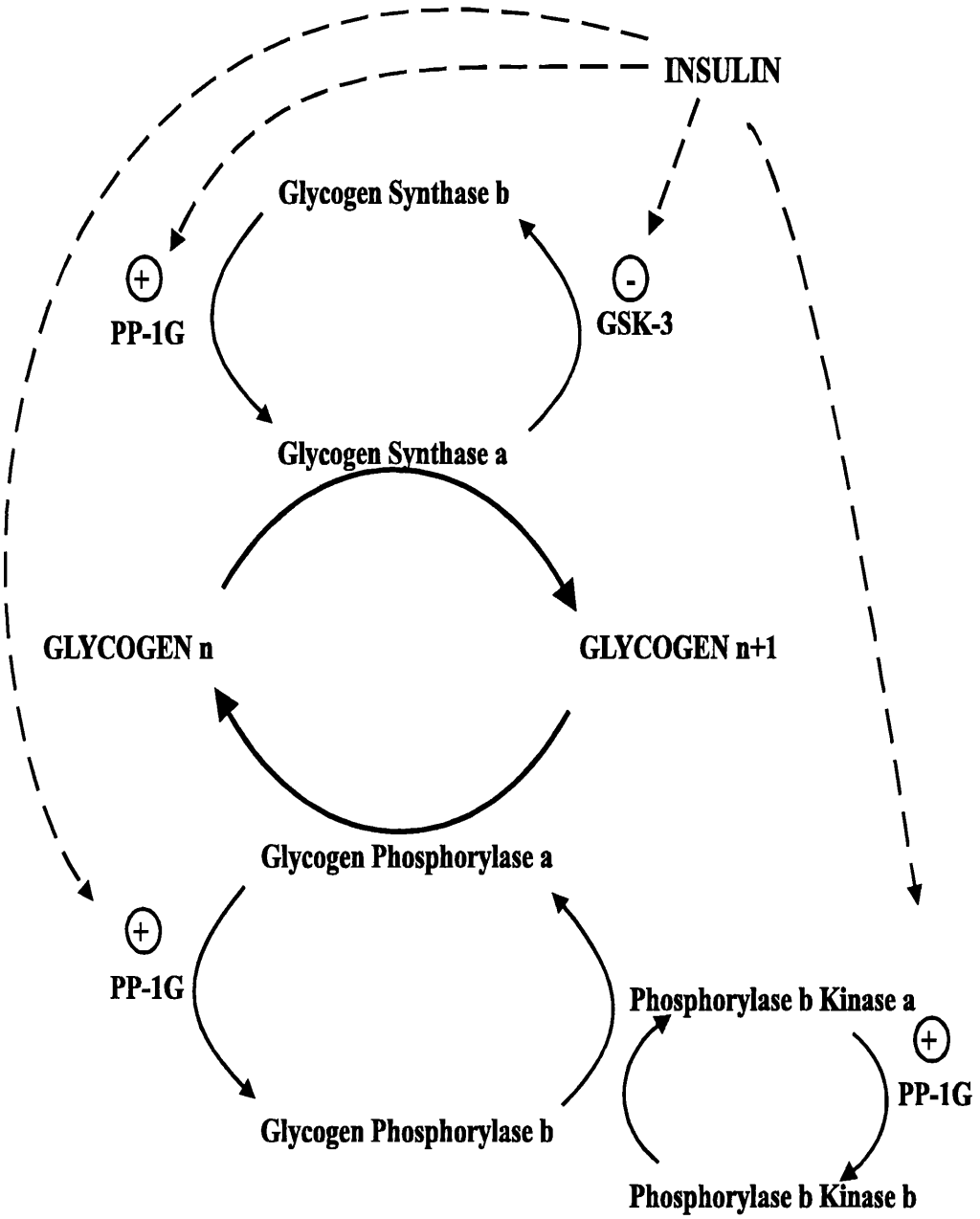
1.3.4.2.ii PP-1G PP-1G is composed of the PP-1 catalytic subunit complexed to a glycogen-binding (G) subunit (Stralfors *et al.*, 1985). Phosphorylation of one serine residue, termed site 1, in the G subunit increases the rate at which PP-1G dephosphorylates and activates glycogen synthase and inactivates phosphorylase kinase (Dent *et al.*, 1990). Insulin stimulates the phosphorylation of this site *in vivo* by activating a kinase which has been identified as MAPKAP-kinase-1 (Lavionne *et al.*, 1991; Sutherland *et al.*, 1993a). More recently insulin has been shown to increase the activity of PP-1 against glycogen phosphorylase in rat adipocytes (Begum, 1995), 3T3-L1 adipocytes and rat L6 myotubules (Lazar *et al.*, 1995), the molecular basis of this however has yet to be determined. This/these mechanism(s) could therefore explain both the activation of glycogen synthesis and the inhibition of glycogen breakdown by insulin (Challis *et al.*, 1987).

1.3.4.2.iii GSK-3 As was mentioned previously GSK-3 can be phosphorylated and inactivated by insulin in a number of cell types (Welsh & Proud, 1993; Cross *et al.*, 1994; Welsh *et al.*, 1994; Borthwick *et al.*, 1995; Hurel *et al.*, 1996) and in the skeletal muscle of insulin treated rabbits (Cross *et al.*, 1994). GSK-3 is responsible for phosphorylating the sites on glycogen synthase which are dephosphorylated in response to insulin (Rylatt *et al.*, 1980). This therefore represents another mechanism by which insulin can stimulate glycogen synthesis i.e. by preventing the inhibitory

effect which GSK-3 has on glycogen synthase. Fig 1.6 gives an overall picture of the control of glycogen synthesis and glycogen breakdown.

1.3.4.2.iv Signalling Pathways The signalling pathway(s) which insulin uses to regulate the activation of glycogen synthesis have not yet been fully elucidated, but appear to be rather complex. Initially it was thought that the MAP kinase pathway alone could be responsible for the regulation of glycogen metabolism by insulin (Cohen, 1993), as MAPKAP kinase-1 can both activate PP-1G (Lavionne *et al.*, 1991; Sutherland *et al.*, 1993a) and inactivate GSK-3 *in vitro* (Sutherland *et al.*, 1993b; Sutherland & Cohen, 1994). More recent evidence however has questioned the involvement of this pathway (for review see Denton & Taveré, 1995). First of all, the half-time for activation of MAPKAP kinase-1 by insulin (5-7 min) is much slower than the half-time for inhibition of GSK-3 (2 min) (Cross *et al.*, 1995; Hurel *et al.*, 1996), suggesting that this is not the rate limiting enzyme for GSK-3 inhibition by insulin. Also EGF, which stimulates the MAP kinase pathway to a similar or greater extent than insulin, has little or no effect on glycogen synthase (Peak *et al.*, 1993; Robinson *et al.*, 1993; Lin & Lawrence, 1994). Again this suggests that the MAP kinase pathway is not sufficient to stimulate glycogen synthesis. It is possible however that EGF and insulin have differing effects on a component(s) outside this pathway (Denton & Taveré, 1995). Expression of mutants which prevented insulin-induced Ras activation and the subsequent stimulation of MAP kinase, had no effect on insulin-stimulated glycogen synthase activation in either CHO (Sakaue *et al.*, 1995a,b) or PC12 cells (Yamamoto-Honda *et al.*, 1995). Finally, PD 98059, which selectively inhibits MAPKK (Alessi *et al.*, 1995), had no effect on the stimulation of glycogen

Fig. 1.6 Regulation of Glycogen Synthesis And Breakdown



synthesis in 3T3-L1 adipocytes, L6 myotubules (Lazar *et al.*, 1995), rat diaphragm(Azpiazu *et al.*, 1996) or human myocytes (Hurel *et al.*, 1996) or on GSK-3 inactivation in L6 myotubules (Cross *et al.*, 1995). Together, these data present convincing evidence that activation of the MAP kinase pathway is not sufficient to stimulate insulin-induced glycogen synthesis.

However, these studies concentrated on the $p42^{MAPK}$ and $p44^{MAPK}$ mediated pathway, and did not take into account other members of the MAP kinase family such as the *jun* N-terminal kinases (JNKs) (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994; Sluss *et al.*, 1994; Gupta *et al.*, 1996) and p38 (Han *et al.*, 1994; Lee *et al.*, 1994). Although it was originally thought that $p42^{MAPK}$ and $p44^{MAPK}$ were the main insulin-stimulated MAPKs, it now appears that both JNK and p38 can also be activated by insulin, in skeletal muscle at least (Moxam *et al.*, 1996). Further, JNK *in vitro* can activate a third isoform of p90rsk/MAPKAP kinase, known as Rsk3 (Moxam *et al.*, 1996). Like Rsk1 and Rsk2 this isoform can be activated by insulin, both *in vitro* (Zhao *et al.*, 1995) and in skeletal muscle *in vivo* (Moxam et al., 1996) and has also been shown to phosphorylate the G subunit of PP-1 (Bjorbaek *et al.*, 1995; Zhao *et al.*, 1995). The study by Moxham *et al.* (1996) presents evidence which suggests that in skeletal muscle, insulin-stimulated glycogen synthase activation is mediated by JNK via the activation of Rsk3.

The PI3-kinase pathway appears to be the most likely candidate for mediating the insulin stimulated activation of glycogen synthase through the regulation of GSK-3. Wortmannin has been shown to block insulin-stimulated glycogen synthesis, activation of glycogen synthase and inhibition of GSK-3 in a variety of cell types (Cross *et al.*,

1994; Moule *et al.*, 1995; Standaert *et al.*, 1995; Shepherd *et al.*, 1995; Yamamoto-Honda, 1995; Hurel *et al.*, 1996). This evidence of a role for PI3-kinase in the activation of glycogen synthesis is consistent with the data which shows that GSK-3 is inactivated by PKB (Cross *et al.*, 1995), and that PKB in turn is activated indirectly via PI3-kinase in response to insulin (Alessi *et al.*,1997) (see section 1.3.3.2).

There is however also evidence against PI3-kinase being involved in this effect. For example, although PDGF stimulates the activation of PI3-kinase to the same extent as insulin in 3T3-L1 adipocytes, it only enhances glycogen synthesis 3-fold, compared to 15-fold by insulin (Weise *et al.*, 1995). Also, overexpression of the insulin receptor and a dominant negative mutant of the p85 subunit of PI3-kinase, which does not bind the p110 catalytic subunit, had no effect on the insulin stimulation of glycogen synthase yet the activation of PI3-kinase was markedly decreased in both the cell lines tested (CHO-IR/p85o and Rat1-IR/p85o). The activation of glycogen synthase was however blocked by wortmannin in these cells. This suggests either that wortmannin inhibits an activator of glycogen synthase that is not PI3-kinase or that a very small increase in PI3-kinase activity is sufficient to activate glycogen synthase (Sakaue *et al.*, 1995b). Finally, in rat adipocytes isoproterenol was able to activate PKB and inhibit GSK-3 in a wortmannin-independent manner, yet it did not stimulate the activation of glycogen synthase (Moule *et al.*, 1997). Thus GSK-3 does not appear to be the primary regulator of glycogen synthase in these cells, suggesting that PP-1G may play a more important role *in vivo*.

$p70^{S6K}$ may also be involved in the activation of glycogen synthase in some cell types. The $p70^{S6K}$ inhibitor rapamycin has been shown to block insulin's activation of

glycogen synthase in rat diaphragm muscle (Azipiazu *et al.*, 1996) and the activation of both glycogen synthase and glycogen synthesis in 3T3-L1 adipocytes (Shepherd *et al.*, 1995). It also partially inhibits glycogen synthase and glycogen synthesis activation in human myoblasts (Hurel *et al.*, 1996), yet it has no effect on these events in rat adipocytes (Lin & Lawrence, 1994; Moule et al., 1994; Cross *et al.*, 1997) or skeletal muscle (Cross *et al.*, 1997). $p70^{S6K}$ has been shown to phosphorylate and inactivate GSK-3 *in vitro*, which would provide a mechanism for the activation of glycogen synthase by this enzyme (Sutherland *et al.*, 1993b; Sutherland & Cohen, 1994). This does not however appear to be the case *in vivo* as rapamycin has no effect on insulin stimulated GSK-3 inhibition in CHO cells (Welsh *et al.*, 1994), L6 myotubules (Cross *et al.*, 1994), rat adipocytes (Cross et al., 1997; Moule *et al.*, 1997); rat skeletal muscle (Cross *et al.*, 1997) or human myoblasts (Hurel *et al.*, 1996). Thus it would appear GSK-3 is not involved in the rapamycin-sensitive regulation of glycogen synthase. A possible explanation may be that another, rapamycin-sensitive, kinase(s) is also able to regulate glycogen synthase activity. The results above seem to suggest that in response to insulin different cells may utilise different pathways to activate glycogen synthase. One study by Chang *et al.* (1995) using skeletal muscle from both normal mice and transgenic mice with a severe insulin receptor defect showed that although glycogen synthase activation by insulin was rapamycin-insensitive in the normal mice, in the transgenic mice this activation was partially impaired by rapamycin. These findings suggest that although $p70^{S6K}$ is not necessary for glycogen synthase activation in this tissue, it may be involved in redundant signalling pathways which also lead to this effect of insulin.

1.3.4.3 Fatty Acid Synthesis

Another method by which insulin promotes the removal of glucose from the bloodstream is to increase the rate of fatty acid synthesis, particularly in adipose tissue, which in turn leads to enhanced lipogenesis. Insulin does this by increasing the activities of pyruvate dehydrogenase (PDH) and acetyl CoA-carboxylase (ACC), the two major regulatory enzymes in the pathway leading to fatty acid synthesis (fig. 1.7).

1.3.4.3.i Pyruvate Dehydrogenase The pyruvate dehydrogenase complex, which is found in the matrix of mitochondria, catalyses the oxidative decarboxylation of pyruvate to acetyl-CoA, the overall reaction being:

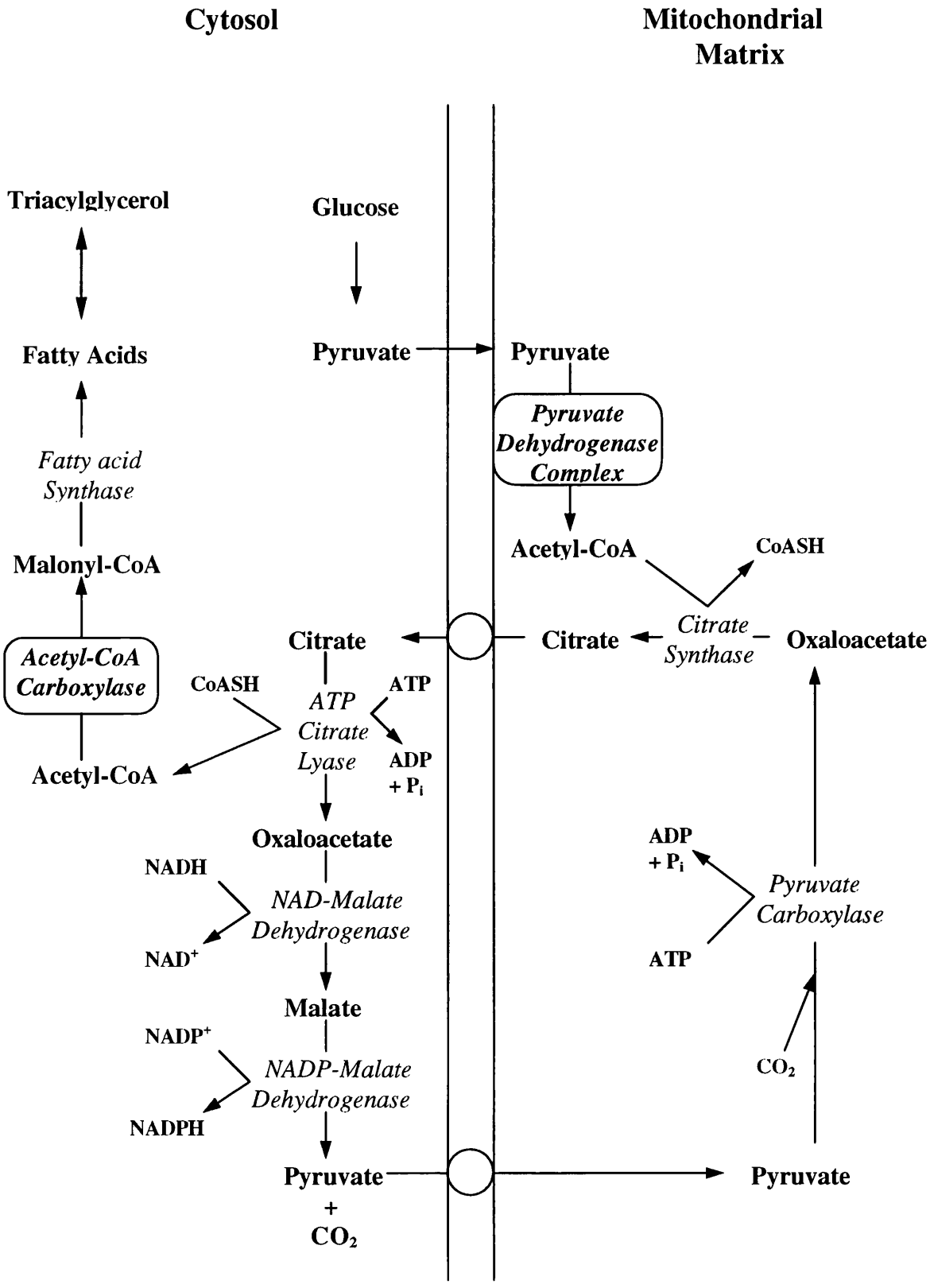
$$\text{pyruvate} + NAD^+ + \text{CoASH} \longrightarrow \text{acetyl-CoA} + NADH + H^+ + CO_2$$

Three components of the complex, namely pyruvate decarboxylase (E_1), lipoate acetyl-transferase (E_2) and dihyrolipoyl dehydrogenase (E_3), are involved in catalysing the five sequential steps which are required to convert pyruvate to acetyl-CoA, the steps being:

1. $CH_3COCO_2H + E_1\,[TPP] \longrightarrow E_1\,[CH_3CHOH\text{-}TPP] + CO_2$
2. $E_1\,[CH_3CHOH\text{-}TPP] + E_2\,[lipS_2] \longrightarrow E_1\,[TPP] + E_2\,[CH_3CO\text{-}S\ lipSH]$
3. $E_2\,[CH_3CO\text{-}S\ lipSH] + CoA\text{-}SH \longrightarrow E_2\,[lip(SH)_2] + CH_3CO\text{-}SCoA$
4. $E_2\,[lip(SH)_2] + E_3\,[FAD] \longrightarrow E_2\,[lipS_2] + E_3\,[FADH_2]$
5. $E_3\,[FADH_2\} + NAD^+ \longrightarrow E_3\,[FAD] + NADH + H^+$

As acetyl-CoA cannot be converted back to glucose, the conversion of pyruvate to acetyl-CoA is a committal step in metabolism. The acetyl-CoA formed can be used either in the tricarboxylic acid (TCA) cycle or for fatty acid synthesis. For reviews of the PDH complex see Wieland, 1983; Patel & Roach, 1990.

Fig. 1.7 Role of Pyruvate Dehydrogenase and Acetyl-CoA Carboxylase in Fatty Acid Synthesis



The first reaction in the sequence, the decarboxylation of pyruvate by pyruvate decarboxylase (E_1), is the only one which is irreversible (Walsh *et al.*, 1976), thus it is the flux through this enzyme which controls the activity of the whole complex. E_1 consists of two polypeptide chains, α and β, which form $\alpha_2\beta_2$ tetramers of molecular weight 154 kDa (Barrera *et al.*, 1972). Regulation of the PDH complex is controlled by phosphorylation-dephosphorylation, the complex being active in its dephosphorylated form PDHa, and inactive in its phosphorylated form PDHb. Conversion between these two forms is catalysed by a specific kinase and a specific phosphatase, both of which are integrated into the PDH-multienzyme complex. The properties of the phosphatase and the kinase appear to be broadly similar in all mammalian tissues (for reviews see Randle, 1981; Reed, 1981; Wieland, 1983)

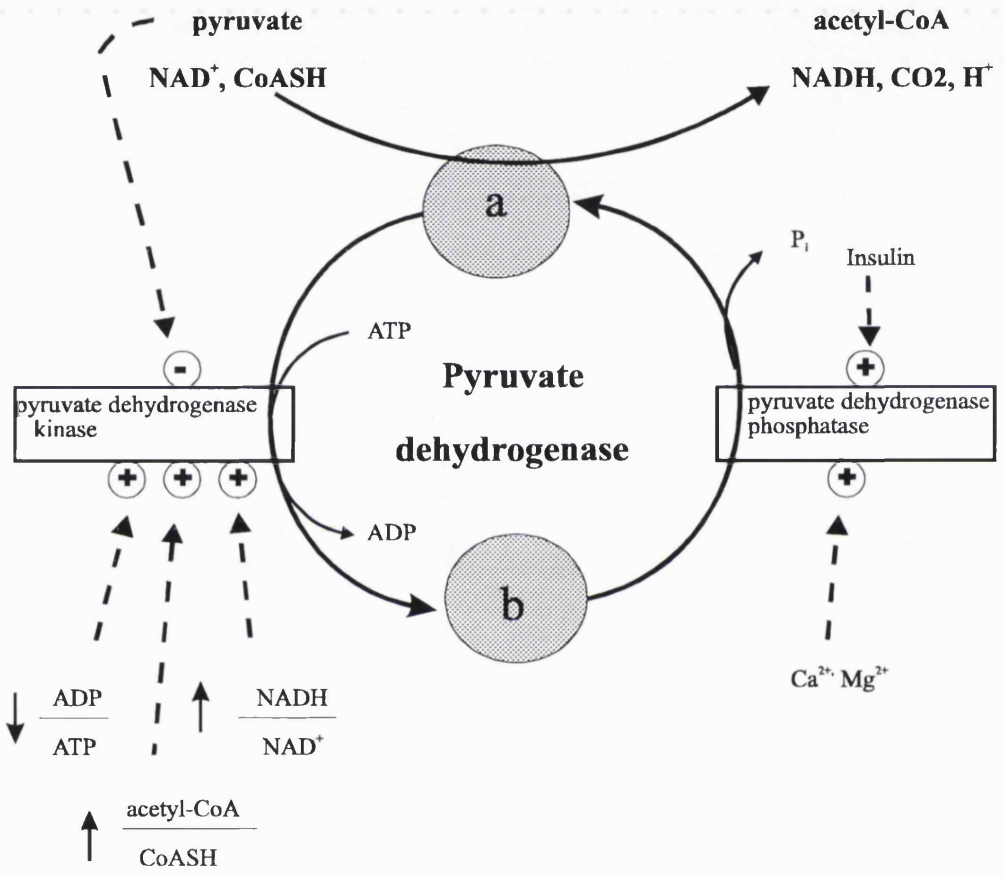
PDH kinase is tightly bound to the lipoate acetyltransferase moiety of the PDH complex (Linn *et al.*, 1969, 1972). This enzyme sequentially phosphorylates three serines on the α-subunit of pyruvate decarboxylase, phosphorylation of site-1 being sufficient to completely inactivate PDH (Yeaman *et al.*, 1978; Sugden *et al.*, 1979; Sale & Randle, 1981). The activity of PDH kinase is regulated by the concentration of a number of metabolites. It is inhibited by pyruvate and increases in the [ADP]/[ATP] ratio, and activated by increases in the [NADH]/[NAD+] and [acetyl-CoA]/[[CoA] ratios (Roche & Reed, 1974; Cooper *et al.*, 1974; 1975; Pettit *et al.*, 1975).

PDH phosphatase which is only loosely bound to the PDH complex (Linn *et al.*, 1969, 1972), is responsible for activating PDH by dephosphorylating the α subunits of E_1 (Hughes *et al.*, 1980). Unlike the kinase, the phosphatase is relatively insensitive to

metabolite concentrations but is activated by Mg^{2+} ($K_{0.5}$=1 mM) and Ca^{2+} ($K_{0.5}$=1 μM) (Denton *et al.*, 1972; Hucho *et al.*, 1972; Siess & Wieland, 1972; Severson *et al.*, 1974; McCormack & Denton, 1980). Studies using intact and toluene-permeabilized mitochondria suggest that the major effect of Ca^{2+} is to lower the K_a of the phosphatase for Mg^{2+} (Thomas *et al.*, 1986; Midgley *et al.*, 1987). Fig. 1.8 gives an overall view of the regulation of PDH.

1.3.4.3.ii Effect of Insulin on PDH Insulin increases the amount of active PDH (PDHa) 2-3 fold in rat epididymal adipose tissue (Jungas, 1971; Coore *et al.*, 1971a,b). This appears to be due to the parallel dephosphorylation of the three serine residues in E_1 which are phosphorylated in response to PDH kinase (Hughes *et al.*, 1980). Evidence suggests that this dephosphorylation is due to the activation of PDH phosphatase, rather than the inhibition of PDH kinase (Hughes & Denton, 1976; Denton *et al.*, 1984; Thomas *et al.*, 1986). Activation of PDH phosphatase by insulin seems to be due to a decrease in the K_a of the enzyme for Mg^{2+} via a Ca^{2+}-independent mechanism (Marshall *et al.*, 1984; Thomas *et al.*, 1986; Thomas & Denton., 1986; Rutter & Denton., 1992; Rutter *et al.*, 1992). The signalling pathway which insulin uses to achieve this effect is a mystery. PDH is not stimulated by EGF, suggesting that the MAP kinase pathway is not involved in its activation, and neither wortmannin nor rapamycin inhibit the activation of PDH by insulin indicating that neither PI3-kinase nor $p70^{S6K}$ are necessary for this effect (Moule *et al.*, 1995). These findings suggest that the signalling pathway involved in the activation of PDH by insulin is distinct from those involved in insulin's other metabolic actions.

Fig. 1.8 Regulation of Pyruvate Dehydrogenase



Shows the regulation of pyruvate dehydrogenase under the influence of PDH kinase and PDH phosphatase which convert PDH from its inactive form *b*, to its active form *a*.

1.3.4.3.iii Acetyl-CoA Carboxylase Acetyl-CoA carboxylase (ACC) is a biotin-containing enzyme which catalyses the carboxylation of acetyl-CoA to form malonyl-CoA. The enzyme catalyses two partial reactions:

1. $ATP + HCO_3^- + Enz.biotin \longrightarrow Enz.biotin.CO_2^- + ADP + Pi$

2. $Enz.biotin.CO_2^- + acetyl\text{-}CoA \longrightarrow Enz.biotin + malonyl\text{-}CoA$

The malonyl-CoA produced indirectly provides C_2 for the *de novo* synthesis of long chain fatty acids (Wakil, 1958; Nugteren, 1965), and acts as a regulator of carnitine palmitoyl transferase (CPT)-1 which is involved in the mitochondrial oxidation of long chain fatty acids (McGarry & Foster, 1980). There are two isoforms of ACC, a 265 kDa isoform which is mainly expressed in lipogenic tissue, and a 280 kDa isoform which can be found in tissues which have a low rate of fatty acid synthesis, but a high capacity for fatty acid oxidation (Bianchi *et al.*, 1990). The two isoforms appear to be encoded by separate genes (Abu-Elheiga et al., 1997), are immunologically distinct (Thampy, 1989; Bianchi *et al.*, 1990; Witters *et al.*, 1994) and exhibit different kinetics towards citrate and acetyl-CoA (Bianchi *et al.*, 1990; Witters *et al.*, 1994). ACC-280 is the predominant isoform found in both heart and skeletal muscle, which are not lipogenic tissue. Thus the primary role of ACC in these tissues is thought to be regulation of fatty acid oxidation (Thampy, 1989; Bianchi *et al.*, 1990 Lopuschuck & Gamble, 1994). ACC-265 appears to be the only isoform expressed in white adipose tissue and brain, and is co-expressed with ACC-280 in brown adipose tissue, liver and mammary glands (Bianchi *et al.*, 1990; Spencer *et al.*, 1993). As the work in this thesis is concerned with the activity of ACC in white adipose tissue, this discussion will concentrate on the regulation of ACC-265.

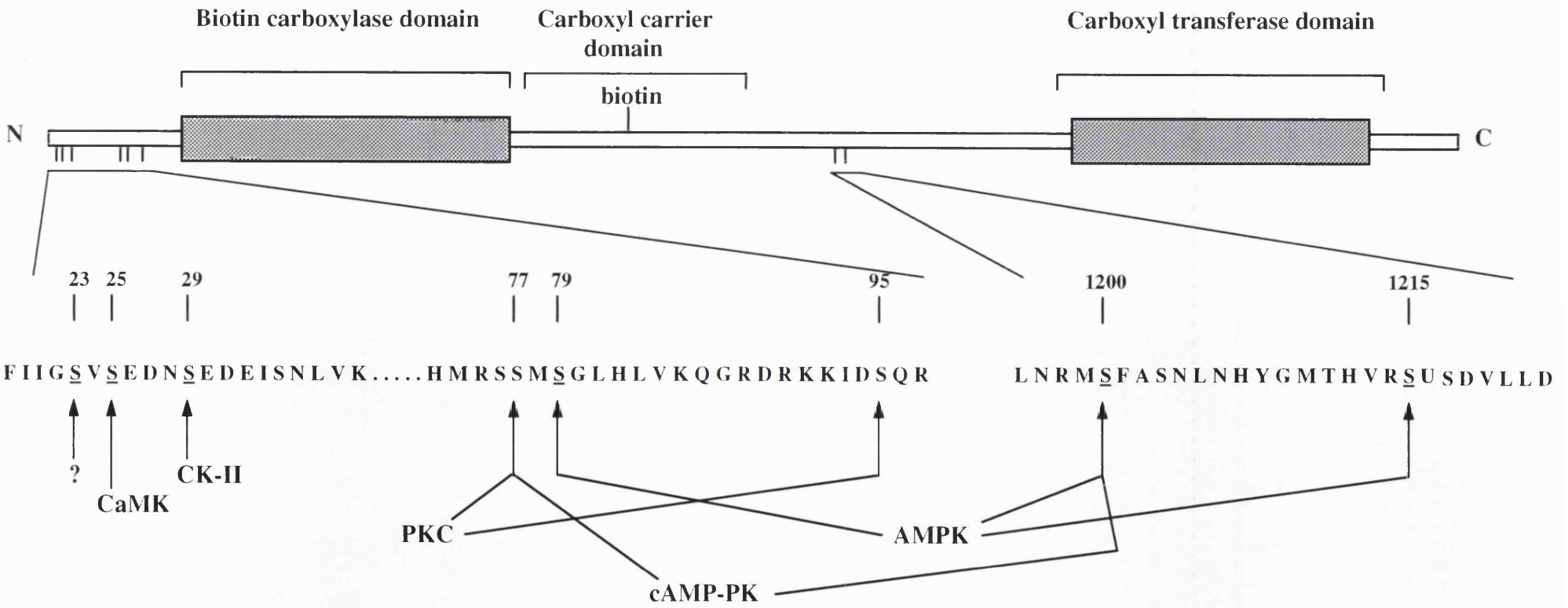
ACC exists as an inactive dimer and as an active polymer, interconversion between these two forms is controlled in the short term by allosteric interactions and reversible phosphorylation (for review see Hardie, 1989) . Long term changes in ACC activity are a result of alterations in the enzyme concentration caused by dietary and hormonal influences at the level of transcription and translation.

Citrate acts as an allosteric activator of ACC promoting its polymerisation and increasing its activity, whilst long chain acyl-CoAs oppose this effect and are inhibitory. Phosphorylation of ACC at certain sites decreases the activity of the enzyme and increases its K_a for citrate. A number of kinases have been shown to act upon ACC *in vitro* including AMP-activated protein kinase (AMPK) (Carling & Hardie, 1986), cAMP-dependent protein kinase (cAMP-PK) (Hardie & Guy, 1980; Tipper & Witters, 1982), protein kinase C (PKC) (Hardie *et al.*, 1986), casein kinases I and II (Tipper *et al.*, 1983; Munday & Hardy, 1984) and calmodulin-dependent multiprotein kinase (CaMK) (Hardie *et al.*, 1986). Despite this, only AMPK and cAMP-PK appear to be involved in the regulation of ACC under physiological conditions. Amino acid sequencing has identified the sites phopshorylated by cAMP-PK and AMPK (Munday *et al.*, 1988); cAMP-PK phosphorylates Ser-77 and Ser-1200 and AMPK phophorylates Ser-79, Ser-1200 and Ser-1215. The relevance of each of these sites in the regulation of ACC has still to be fully elucidated. One study by Ha and co-workers suggests that phosphorylation of Ser-1200 by cAMP-PK is important for the inactivation of ACC *in vitro* (Ha *et al.*, 1994). However, contradictory to this, Davies *et al.* (1990) have reported that removal of the amino terminal region of ACC, containing serines 77 and 79, reversed the effects of phosphorylation by cAMP-PK and AMPK. This rules out a role for Ser-1200

phosphorylation in ACC inactivation. Both studies do agree that phosphorylation of Ser-79 by AMPK is important for inactivation of ACC. The hormones adrenaline and glucagon both inactivate ACC by inducing its phosphorylation and depolymerisation. Studies, both in adipocytes and hepatocytes show that these hormones stimulate the phosphorylation of ACC on Ser-79, not on Ser-77. Thus it would appear that the phosphorylation of ACC in response to hormones which increase cAMP is due to the action of AMPK and not cAMP-PK (Sim & Hardie, 1988; Haystead *et al.*, 1990a). cAMP-PK does however appear to be an essential component in the signalling pathway of these hormones (Haystead *et al.*, 1990a). Fig. 1.9 shows a schematic diagram of the relative positions of the phosphorylation sites of ACC.

Activation of ACC requires dephosphorylation of these inhibitory sites by protein phosphatases. A variety of phosphatases have been identified which are able to remove phosphate from both active and silent phosphorylation sites on ACC. The protein phosphatases 1, 2A and 2C, which are all well characterised, have been shown to reverse the phosphorylation of ACC by various kinases (Krakower & Kim, 1980; Ingebristen *et al.*, 1983a,c; Witters & Bacon, 1985). In addition ACC phosphatases have been purified from rat liver (Thampy & Wakil, 1985) and rat epididymal adipose tissue (Krakower & Kim, 1981), and endogenous ACC phosphatase activities have been characterised in crude preparations of rat liver, mammary gland and adipose tissue (Allred & Roehrig, 1978; Brownsey *et al.*, 1979; McNeillie *et al.*, 1981). The relationship of these phosphatases to protein phosphatase 1, 2A and 2C is uncertain.

Fig. 1.9 Acetyl-CoA Carboxylase Phosphorylation Sites



Shows the linear sequence of rat liver ACC with the proposed domain structure, phosphorylation sites and the kinases that act on these sites.

Those serines which are underlined are phosphorylated *in vivo*.

1.3.4.3iv Effect of Insulin on ACC Activation of ACC by insulin is associated with an increase in the proportion of the enzyme in its active polymeric form which exhibit activity in the absence of citrate (Halestrap & Denton, 1974) This effect is no longer evident when tissues are incubated with sufficient citrate to fully activate ACC and convert it to its polymeric forms (Halestrap & Denton, 1974; Borthwick *et al.*, 1987). Although ACC can be activated by dephosphorylation, this does not appear to be the mechanism insulin uses to activate the enzyme, in adipose tissue at least. Insulin in fact stimulates phosphorylation of ACC at two separate sites. One of these sites is Ser-29 which is phosphorylated by casein kinase-II (Haystead *et al.*, 1988; Lopez-Casillas *et al.*, 1988), but phosphorylation of this site does not effect the activity of the enzyme (Tipper *et al.*, 1983; Munday & Hardie, 1984). Insulin-stimulated phosphorylation of the second site, which lies within a tryptic peptide termed the 'I-peptide', does result in an increase in the activity of the enzyme (Brownsey & Denton, 1982). It was originally thought that phosphorylation of this peptide was responsible for the activation of ACC by insulin. Evidence now however suggests that although the I-peptide is phosphorylated in response to insulin, this phosphorylation does not actually activate the enzyme. Protein phosphatase treatment of crude extracts from insulin-stimulated adipocytes did not affect the activation of ACC despite the I-peptide being completely dephosphorylated (Haystead & Hardie, 1986). Also, the phorbol ester TPA mimicked the effect of insulin on phosphorylation of ACC, but did not affect its activation indicating that these two events can be dissociated (Haystead & Hardie, 1988).

An insulin-stimulated protein-serine kinase has been identified in rat adipocytes which phosphorylates the I-peptide (Borthwick *et al.*, 1990). As well as phosphorylating

ACC, it also appears to block the inhibition of ACC by coenzyme A (Borthwick *et al.*, 1990; Moule *et al.*, 1992). This kinase binds to the inactive dimeric form of ACC, but not the active polymeric form, a property which has allowed purification of the enzyme to near homogeneity (Heesom *et al.*, 1995). In one report this kinase co-eluted from Mono-Q chromatography at 175mM NaCl in the presence of citrate with an 'activator' protein which appeared to activate ACC without changing its phosphorylation state (Heesom *et al.*, 1995). A similar non-phosphorylated 'activator' protein has been identified in high speed supernatant fractions from rat liver (Quayle *et al.*, 1993). The exact roles which the insulin-stimulated kinase and the protein 'activator' play in the activation of ACC by insulin have yet to be fully determined.

The signalling pathway(s) which leads to the activation of ACC by insulin is also unclear. In one study, as well as being stimulated by insulin, ACC and lipogenesis were also stimulated by EGF (Haystead & Hardie, 1986), which suggested that a MAP kinase pathway may be involved in its activation. However another study by Moule *et al.* (1995) did not see any effect of EGF on ACC activity either in isolated rat adipocytes or epididymal fat pads. This study did however show that insulin-stimulated ACC activation could be inhibited by wortmannin suggesting that PI3-kinase may be involved. Insulin-stimulated MAP kinase activation was also inhibited by wortmannin in this study, so the lack of EGF effect on ACC in this case does not necessarily rule out a role for the MAP kinase pathway. GSK-3 has been shown to phosphorylate and inactivate ACC in vitro (Ramakrishna & Benjamin, 1983,1985; Sheorain *et al.*, 1985). Thus, as is the case for glycogen synthase, insulin may prevent this inactivation by inhibiting GSK-3 via the PI3-kinase dependent pathway described

in section 1.3.3.2. However, no evidence of an effect on ACC by GSK-3 has been found *in vivo*.

1.3.4.4 Lipolysis

As well as increasing the level of stored lipids by promoting fatty acid synthesis, insulin also prevents their breakdown by inhibiting lipolysis.

1.3.4.4.1 Hormone Sensitive Lipase The rate limiting enzyme in lipolysis is hormone-sensitive lipase (HSL) which catalyses the hydrolysis of triacylglycerol (TAG). The activity of HSL is regulated acutely by reversible phosphorylation (For review see Yeaman, 1990). There are two phosphorylation sites on the HSL polypeptide which can be phosphorylated *in vitro* and *in vivo* (Yeaman, 1990). Site 1, Ser-563 in the rat HSL sequence (Garton *et al.*, 1988; Holm *et al.*, 1988), is a regulatory site and is responsible for the activation of HSL in response to lipolytic hormones (Stralfors *et al.*, 1984; Stralfors & Honner, 1989). The enzyme responsible for the phosphorylation of this site is cAMP-PK (Stralfors & Belfrage, 1983; Garton *et al.*, 1988). Site 2, or the basal site, is located at position 565 in the rat HSL sequence (Olsson *et al.*, 1986; Holm *et al.*, 1988). As is suggested by the name, this site is phosphorylated under basal conditions of lipolysis (Stralfors *et al.*, 1984). Site 2 can be phosphorylated by a number of kinases *in vitro*, but AMP-activated kinase (AMPK) appears to be the most likely candidate *in vivo* (Garton *et al.*, 1989; Sullivan *et al.*, 1994). *In vitro* phosphorylation of one of these sites prevents phosphorylation of the other i.e. the two sites are mutually exclusive (Garton *et al.*, 1989; Garton & Yeaman, 1990). This suggests that site 2 phosphorylation may have an anti-lypolytic effect *in vivo* (Garton *et al.*, 1989). Indeed one study has shown that the AMPK

activator AICAR (5-amino-4-imidazolecarboxamine ribonucleoside) can inhibit isoprenaline-stimulated lipolysis in isolated rat adipocytes (Sullivan *et al.*, 1994).

Protein phosphatases (PP) 1, 2A and 2C, which can be found in rat adipocytes, can all dephosphorylate HSL *in vitro* (Olsson & Belfrage, 1987; Wood *et al.*, 1993). A study by Olsson & Belfrage which used purified protein phosphatase preparations and non-physiological concentrations of HSL found that PP-2A was the major HSL phosphatase activity and that all three phosphatases preferentially dephosphorylated the basal site. PP-1 was 20% more active towards this site and PP-2A and PP-2C were 80% more active (Olsson & Belfrage, 1987). A more recent study by Wood and co-workers, which was carried out on isolated adipocytes extracts, also found PP-2A to be the major phosphatase for both sites. In contrast to the previous study however, PP-2C was also found to be a major phosphatase for these sites. As PP-2C is also known to dephosphorylate AMPK and HMG-CoA reductase (Ingebristen *et al.*, 1983a,c; Hardie., 1992), this finding suggests that PP-2C may play a specific role in the overall regulation of lipid metabolism (Wood *et al.*, 1993). As with Olsson and Belfrage's study, Wood *et al.*, found that the total phosphatase activity was higher against the basal site than the regulatory site. What effect hormonal treatment has on the activities of these protein phosphatases has yet to be established.

1.3.4.4.ii Effect of Insulin on HSL Insulin appears to mediate its antilipolytic actions by decreasing the phosphorylation of HSL (Nilsson *et al.*, 1980). It does this primarily by lowering the level of cellular cAMP, which in turn decreases the activity of cAMP-PK (Butcher *et al.*, 1966; Wong & Loten, 1981; Londos *et al.*, 1985). Insulin appears to exert this effect by activating cGMP-inhibited cAMP

phosphodiesterase (cGI-PDE), through phosphorylation on serine residue(s) (Eriksson *et al.*, 1995). This insulin-induced phosphorylation/activation is catalysed by an insulin-stimulated serine kinase (cGI-PDE IK) (Shibata & Kono, 1990a,b; Lopez-Apriciao *et al.*, 1993) The identity of the kinase and the site(s) of phosphorylation have still to be determined. There is also some evidence to suggest that insulin may also exert a cAMP-independent antilipolytic effect, mediated by a protein phosphatase (Londos *et al.*, 1985; Stralfors & Honner, 1989). This evidence is not however conclusive.

The signal transduction pathway which insulin uses to inhibit lipolysis, as with the majority of insulin's metabolic effects, appears to involve PI3-kinase. Wortmannin was able to block the antilipolytic effects of insulin on isoproterenol, noradrenaline and forskolin-stimulated adipocytes (Okada *et al.*, 1994; Rahn *et al.*, 1994; Fain *et al.*, 1997). This inhibitor also blocked the phosphorylation and activation of cGI-PDE and the activation of cGI-PDE IK. These two enzymes were not inhibited by wortmannin in *in vitro* assays indicating that PI3-kinase mediates the antilipolytic effect upstream of cGI-PDE IK activation (Rahn *et al.*, 1994).

1.4 AIMS OF PROJECT

The main aims of this project were:

1. To produce malarial extracts which would synergistically enhance insulin signalling

2. To determine where in the insulin signalling these extracts were exerting their effects

In order to try and achieve these aims the following protocol was followed:

(a) Malarial extracts were prepared using the methods adopted by Playfair and co-workers to produce malarial antigens which could stimulate the release of TNF by macrophages, as these same methods had been used to produce the malarial antigens/ extracts which were originally shown to enhance insulin signalling (Taylor *et al.*, 1992b)

(b) To determine the ability of the extracts to enhance insulin signalling, each sample was then screened for its ability to enhance insulin-stimulated lipogenesis by measuring the incorporation of radiolabelled glucose into lipid. This particular screen was chosen as enhancement of lipid synthesis was one of the effects which the malarial extracts were shown to exert (Taylor *et al.*, 1992b). Also, the actual assay procedure was relatively quick and simple and a number of extracts could be screened at the one time.

(c) The malarial extracts which were shown to enhance insulin's activation were then used in subsequent experiments to determine where in the insulin signalling network the extracts were exerting their effects.

CHAPTER TWO :

MATERIALS AND METHODS

2.1 COMMERCIAL PREPARATIONS

All reagents routinely used were of the highest grade available, purchased from Sigma Chemical Company, Poole, Dorset, U.K. and British Drug House Ltd., Poole, Dorset, U.K. In addition the following reagents were supplied by:-

Sigma Chemical Co. Ltd., Poole, Dorset: Glycogen type II, glutathione (GSH), fatty acid poor bovine serum albumin fraction V (BSA), 5-5'dithiobis(2-)nitrobenzoic acid (DTNB), PMSF, α-D glucose-1-phosphate (G1P), D-glucose-6-phosphate (G6P), uridine 5' diphosphoglucose (UDPG), Epidermal growth factor (EGF), cocarboxylase, L-α-phosphatidylinositol, adenosine 5'-triphosphate (ATP), L-isoproterenol, polymixin B-agarose, diaphorase, indomethacin.

Boeringer Mannheim U.K., Lewes, East Sussex, U.K.: Collagenase D (lot no: 83762821-11), insulin (bovine), nicotinamide adenine dinucleotide (NAD), reduced nicotinamide adenine dinucleotide (NADH).

British Drug Houses Ltd., Poole, Dorset, U.K.: Dithiothreitol (DTT), thin layer chromatography (t.l.c.) plates (Merck silica gel 60, aluminium backed).

Amersham International, Bucks., U.K.: [U-^{14}C]glucose, α-D[U-^{14}C]glucose-1-phosphate, uridine diphosphoglucose ([U-^{14}C]glucose), sodium [^{14}C]bicarbonate, D-[1-^{3}H]glucose, D-[6-^{3}H]glucose.

New England Nuclear (NEN), Stevenage, Herts., U.K.: γ^{33}P adenosine 5'triphosphate salt.

Calbiochem-Novobiochem (U.K.) Ltd., Beeston, Nottingham, U.K.: Nonidet - P40.

ICN Pharmaceuticals Ltd., Thame, Oxon., U.K.: Antiphosphotyrosine antibody PY20.

Autogen Bioclear, Mile Elm Calne, Wiltshire, U.K.: Protein A-agarose.

National Diagnostics, Manville, New Jersey, U.S.A.: Ecoscint A.

Gibco Ltd., Paisley, Renfrewshire, U.K.: Foetal Calf Serum (FCS).

BOC Ltd., London, U.K.: Liquid nitrogen, pressurised gasses.

Flow Laboratories, Irvine, Ayrshire, U.K.: 0.2 mm Millipore filters.

Maxisorp, Nunc, Roskilde, Denmark: Microtitre plates.

2.2 ANIMALS

2.2.1 Mice

Outbred female CD1 mice, at least six weeks old, were obtained from Charles River Ltd., Margate, Kent, UK. Male C57Bl/6J mice, at least 7 weeks old, and female *ob/ob* C57BL/6J mice were obtained from Harlan-Olac, Oxon, U.K..

2.2.2 Rats

Unless otherwise stated all rats used were male Sprague-Dawleys weighing 140-170 g bred in house at UCL.

All animals had constant access to drinking water and Rat and Mouse No. 3 Breeding Diet (Special Diet Services, Witham, Essex, U.K.) which contains (w/w) 21%

digestible crude protein, 4% digestible crude oil and 39% starches and sugars. The light/dark cycle was 13 hours/11 hours with light from 06:00h to 19:00h.

2.3 PREPARATION OF MALARIAL EXTRACTS

2.3.1 Parasites

Both the lethal YM line of *Plasmodium yoelii* strain 17X (from Dr A Holder, National Institute of Medical Research, London, U.K.) and *Plasmodium chabaudi* 2722 AS (from Dr K.N. Brown, NIMR, London, U.K.) were used. Infection of the mice was initiated with either intravenous (i.v.) or intraperitoneal (i.p.) injection of 10^4 parasitized erythrocytes and the parasitaemias were determined from blood films stained with geimsa.

2.3.2 Measurement of Blood Glucose

Blood glucose concentrations were determined from a drop of tail blood, using Glucostix and an Ames Glucometer (Miles Ltd., Stoke Poges, UK) according to the manufacturer's instructions.

2.3.3 Preparation of Malarial Extracts

The preparation of the malarial extracts was derived from the methods of Bate *et al.* (1992c) and Sheikh *et al.* (1996).

Parasitized red blood cells (prbcs) were obtained from mice with parasitaemias (the percentage of rbcs containing parasites) greater than 50% via heart puncture after anaesthesia with halothane. The erythrocytes were then washed and resuspended in PBS (phosphate buffered saline) at a concentration of 10^8 prbcs/ml before being incubated overnight on rollers at 37°C. The following day, in order to stimulate release of the active component, the erythrocytes were subjected to one of the following treatments :-

1. Sonication for 2 x 30 secs in a cuphorn attached to an XL2020 sonicator

2. Freeze-thawing x 3

3. Centrifugation at 500g for 10 mins followed by boiling of the resulting supernatant for 5 mins

Following each of the above treatments the samples were spun at 500g for 10 mins. The supernatants were then removed and the pellets resuspended to the same volume as the supernatant with PBS. Both supernatants and resuspended pellets were then incubated overnight with 10 mg/ml pronase E. The following day the supernatants and resuspended pellets were boiled for 5 mins (to kill the pronase), and centrifuged at 500g for 10 mins. The resulting supernatants were then mixed with polymixin B-agarose and centrifuged to remove any bacterial endotoxin. Finally the preparations were filtered through 0.2 μm Millipore filters and stored at 4°C.

2.4 ASSAY OF TUMOUR NECROSIS FACTOR (TNF)

This assay was derived from the method of Sheikh *et al.* (1996)

2.4.1 Collection of Macrophages

Mice (normally 3-4) were injected intraperitonealy with 1 ml of thioglycolate (4%) per mouse. Macrophages were then collected 3-5 days later by lavaging the peritoneum of the mice with 5-7 ml Earles Balanced Salt Solution (BSS) containing 1U of heparin. The collected macrophages were washed twice with BSS, discarding the supernatant after each wash. The resulting pellet was resuspended in RPMI with 5% Foetal Calf Serum (FCS) to a final volume of 2 ml. The number of macrophages in the sample were then determined by staining with Acridine orange and ETBr at a 1/5 dilution. Once collected and counted the macrophages were then seeded at 5×10^5 to 1×10^6 macrophages per well of a microtitre plate (100 µl per well). The plate was then incubated at 37°C in a CO_2 incubator for 1-2 hours to allow the macrophages to adhere.

2.4.2 Macrophage Stimulation

Less than 30 mins. before stimulation of the macrophages, 100 µl/well of 2 ng/ml Indomethacin/RPMI was added to the microtitre plate (this was to block any prostaglandin effect, which inhibits TNF production). After the incubation was complete the microtitre plate was washed three times with warm BSS to remove any non-adherent cells. To stimulate the cells, 200 µl/well of sample with 5 µg/ml Polymixin B in RPMI was added. The plate was then incubated overnight at 37°C in a CO_2 incubator before being checked for macrophage growth and stimulation. Once activated the macrophages were diluted 1 in 5 with 2% FCS (or BSA) in RPMI (100 µl of activated macrophages in 400 µl FCS/RPMI).

2.4.3 ELISA for Murine TNF

Maxisorp microtitre plates were coated with 50 µl/well of a 1/1000 dilution of Hamster anti-murine TNF monoclonal antibody (αMuTNF Mab) in coating buffer. These were then stored overnight at 4°C, or for a minimum of 3 hours at 37°C. Once coated the plates were then blocked with 200 µl/well of blocking buffer containing 1% Marvel, 0.3% Gelatine in PBS for 3 hours at 37°C or overnight at 4°C. Following blocking, 100 µl/well of the activated macrophage samples were added to the plates in duplicate along with a rTNF standard curve and incubated for 1 hour at 37°C. The plates were then washed x 3 with 200 µl/well of 0.2% BSA in PBS (wash buffer). After washing the plates were coated with a 1/5000 dilution of Rabbit αMuTNF at 50 µl/well and incubated at 37°C for 30-45 mins. Following this incubation the plates were washed again x 3 with 200 µl/well of wash buffer before being coated with 50 µl/well of peroxidase conjugated anti-rabbit IgG (1/3000 dilution) and incubated at 37°C for 45 mins. Once incubated, the plates were again washed x 3 with wash buffer (200 µl/well). Fresh substrate consisting of 1 mg/ml *o*-phenylenediamine/H_2O_2 was then added to each plate at 100 µl/well. To allow the colour to develop the plate was placed in the dark for 5-10 mins. At this point the OD of the wells was read at 450 nm using a Dynatech MR5000 ELISA reader.

2.5 ISOLATION OF ADIPOCYTES

The adipocytes were isolated using a method described by Rodbell (1964) and modified by Cushman (1970).

The epididymal fat pads were excised from male SD rats (140-170g), cut into small pieces and incubated in a shaking water bath at 37°C, 200 rev/min for 15 mins in Krebs Ringer Bicarbonate/HEPES buffer (KRB/HEPES) consisting of 140 mM NaCl, 2.5 mM KH_2PO_4, 1.25 mM $Mg_2SO_4.7H_2O$, 4.7 mM KCl, 2.5 mM $CaCl_2H_2O$, 10 mM $NaHCO_3$(fresh) and 30 mM HEPES, pH 7.4. Also present in the incubation medium was 1% Bovine Serum Albumin (BSA) and 0.15% Collagenase D. Following incubation the digested tissues were filtered through bolting cloth, and the resulting cell suspension was washed three times by suspension and gentle centrifugation in fresh KRB/HEPES buffer supplemented with 1% BSA. Typically the cells from one fat pad occupied 0.3 ml packed volume.

2.6 MEASUREMENT OF CARBOHYDRATE METABOLISM IN ADIPOCYTES

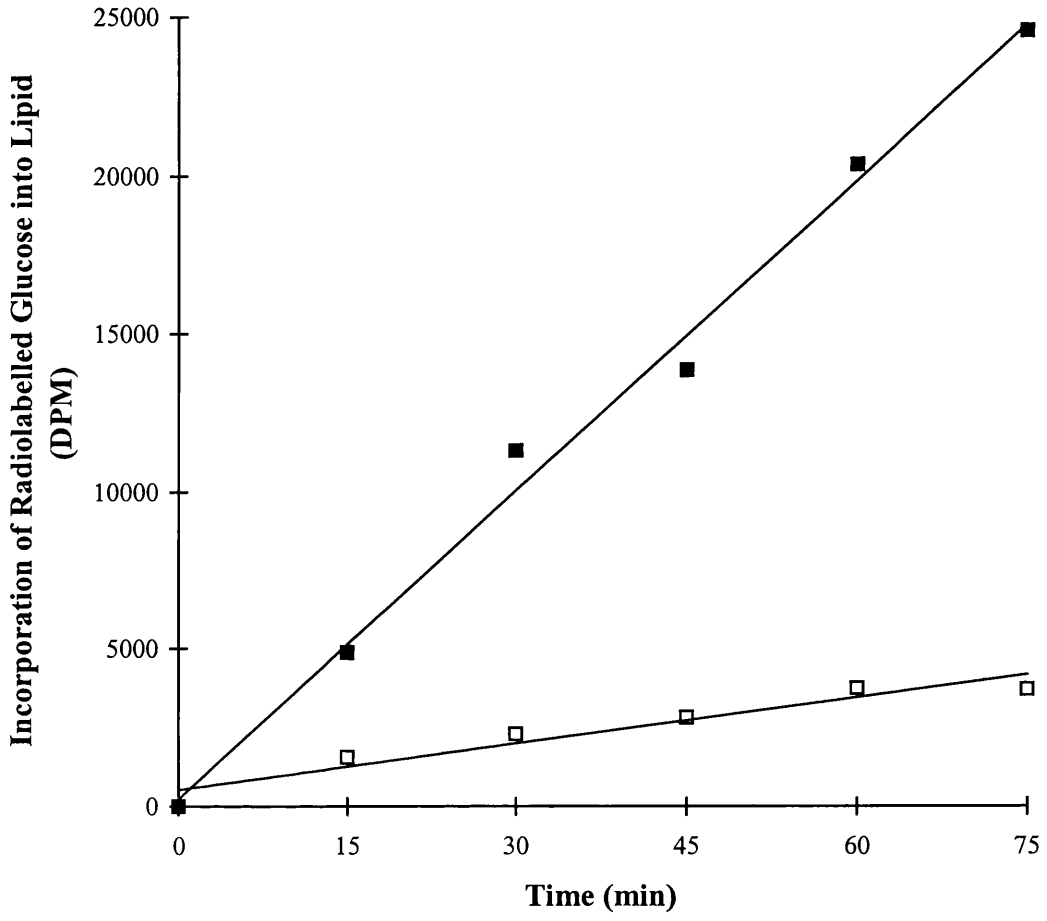
2.6.1 Radiolabelled Glucose Incorporation into Lipids

2.6.1.1 ***[^{3}H] Adipocyte Assay*** Lipid accumulation in isolated adipocytes was measured by the incorporation of [1-^{3}H] and [6-^{3}H]glucose into lipids using a method derived from that of Moody *et al.* (1974). Isolated adipocytes were incubated in 1 ml volumes (1/5 fat pad /ml) of KRB/HEPES medium supplemented with 1% BSA and 5 mM [1-3H] and [6-3H]glucose (0.5 μCi/assay) on a shaking water bath (75 rev/min) for one hour at 37 °C with the additions stated in the text. The incubations were terminated by addition of 4 ml BBOT/toluene (4g/litre). They were then left to stand for at least one hour to allow extraction of the lipids into the toluene before quantitation by scintillation counting.

2.6.1.2 ***[U-^{14}C] Adipocyte Assay*** Lipid accumulation in isolated adipocytes was measured by the incorporation of [U-^{14}C]glucose into lipids using a method derived from that of Taylor *et al.* (1992b). Isolated adipocytes were incubated in 1ml volumes (1/5 fat pad /ml) of KRB/HEPES medium supplemented with 1% BSA and 5 mM [U-^{14}C] glucose (0.5 μCi/ assay) on a shaking water bath (75 rev/min) for one hour at 37°C with additions stated in the text. The incubations were terminated by addition of propan-2-ol/hexane/0.5 M H_2SO_4 (40:10:1), 2.5 ml per assay, followed by extraction of lipids into hexane (Dole, 1956). A 1 ml aliquot of the extracted lipids was then added to 4 ml of Ecoscint A for quantitation by scintillation counting.

2.6.1.3 ***[U-^{14}C] Fat Piece Assay*** Lipid accumulation in epididymal fat pieces was measured by the incorporation of [U-^{14}C]glucose into lipid using a method derived from that of Taylor *et al.* (1992b). The epididymal fat pads were removed from male SD rats (140-170 g) and each fat pad was cut into five pieces. The pieces were then washed in KRB/HEPES, blotted on tissue paper and weighed. Each fat piece was incubated in a 1 ml volume of KRB/HEPES medium supplemented with 1% BSA and 5 mM [U-^{14}C]glucose (0.5 μCI/assay) on a shaking water bath (75 rev/min) for one hour at 37 °C with additions stated in the text. The incubations were terminated by addition of propan-2-ol/hexane/0.5 M H_2SO_4 (40:10:1), 2.5 ml per assay, and left to stand overnight to allow the fat pieces to dissolve. The following day the lipids were extracted into hexane (Dole, 1956) and 1 ml aliquots of the extracted lipids were added to 4 ml Ecoscint A for quantitation by scintillation counting.

Fig. 2.1. Lipogenesis Time Course



Shows the time course of U-[^{14}C]glucose incorporation into lipid in isolated rat adipocytes, carried out under conditions described in section 2.6.1.2 in the absence (❑) and presence (■) of 10 nM insulin. Results are the mean of triplicate measurements carried out on one adipocyte preparation. In the absence and presence of insulin the rates of incorporation of glucose into lipid was 2.20 and 9.77 μmol/hr/g dry weight of cells respectively.

2.6.2 Radiolabelled Glucose incorporation into Glycogen

The accumulation of glycogen in isolated adipocytes was measured by the incorporation of [U-^{14}C]glucose into glycogen as described by Muchmore *et al.* (1981). Cells (2/5 fat pad/ml) were incubated in a final volume of 0.21 ml KRB/HEPES medium containing 1% BSA, 3 mM [U-^{14}C]glucose (0.562 μCi/ assay) for one hour at 37°C on a shaking water bath (75 rev/min) with additions stated in the text. The reactions were stopped by addition of 0.2 ml 10 N NaOH and the glycogen extracted at 90°C for one hour. Following centrifugation at $1000g_{av}$ for 2 mins, 0.1 ml aliquots were spotted on 2 cm squares of Whatman no. 31ET paper (Thomas *et al.*, 1968). The wet papers were then dropped into 600 ml of 66% ethanol at -20°C for 15 mins to allow glycogen precipitation. This was followed by two, 600 ml washes with 66% ethanol for 30 mins at room temperature and one overnight wash at 4°C. The papers were finally rinsed in acetone, dried and counted in scintillation vials containing 10 ml Ecoscint A.

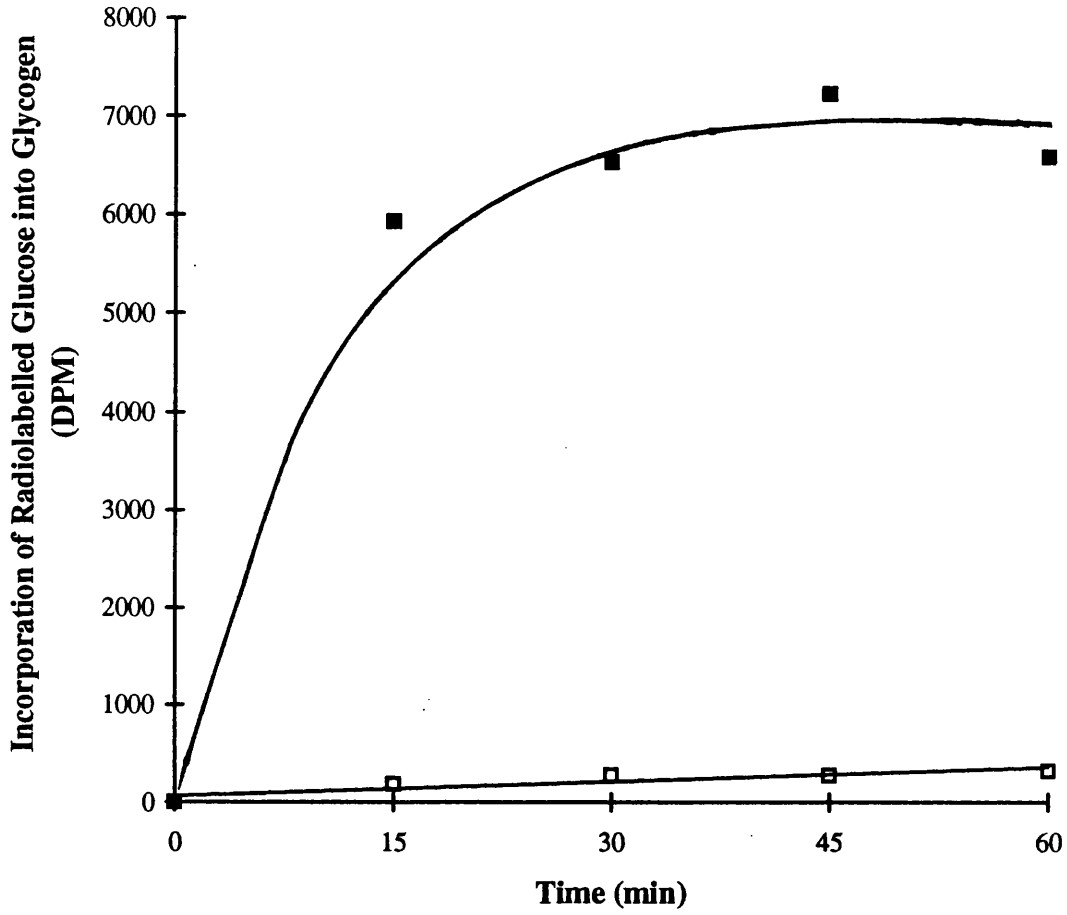
The incubations for the above assays were all carried out in 5 ml polypropylene tubes

2.7 RADIOCHEMICAL ENZYME ASSAYS

2.7.1 Assay of Acetyl-Coenzyme A Carboxylase (ACC) Activity

This assay was derived from the method of Halestrap and Denton, 1973.

Fig 2.2 Glycogen Synthesis Time Course



Shows the time course of incorporation of U-[^{14}C]glucose into glycogen in the absence (❑) and presence (■) of 10 nM glucose, carried out under conditions described in section 2.6.2. The results are the means of triplicate measurements from two separate adipocyte preparations. The rates of incorporation of glucose into glycogen in the absence and presence of insulin were 0.0365 and 0.581 μmol/hr/g dry weight of cells respectively.

2.7.1.1 ***Activation of ACC*** The distal ends of epididymal fat pads were removed from male rats and cut into two pieces of approximately equal size, giving a total of four pieces per rat. The fat pieces were collected in KRB/HEPES medium at 37°C, blotted on tissue paper and weighed (~100 mg/fat piece). Each fat piece was then pre-incubated for 30 minutes at 37°C in 5 ml of KRB/HEPES buffer supplemented with 11 mM glucose before being transferred to 5 ml of fresh medium containing 11 mM glucose plus the additions stated in the text and incubated on a shaking water bath (100 rev/min) for a further 30 minutes at 37°C. Following incubation the fat pieces were lightly blotted and rapidly frozen in liquid Nitrogen.

2.7.1.2 ***Extraction of Fat Pads for Enzyme Analysis*** The fat pieces were extracted at 0-4°C using 3 x 15 sec bursts of an Ultra-Turrax homogenizer in 1 ml of 100 mM potassium phosphate, pH 7.3 containing 2 mM EDTA, 4 mM GSH and 10 mg/ml BSA (dialysed against distilled H_2O). After extraction the homogenates were centrifuged at $1500g_{av}$ for 1 min, 4°C, to remove any fat and cell debris. The infranatants were removed using a syringe and assayed within 5 mins of extraction.

2.7.1.3 ***ACC Assay*** Acetyl-CoA carboxylase (EC 6.4.1.2) was assayed using the method of Halestrap and Denton (1973) which measures the incorporation of [^{14}C]bicarbonate into malonyl-CoA. Assays were carried out at 30°C and were initiated by the addition of 50 µl of infranatant to 0.45 ml of 100 mM Tris-HCl, pH 7.4 containing 10 mM $MgSO_4$, 0.5 mM EDTA, 1 mM GSH, 150 µM Acetyl-CoA, 1 mM DTT, 10 mg/ml BSA (dialysed against distilled H_2O), 2.5 mM ATP and 15 mM

$KH^{14}CO_3$ (0.5 μCi/mmol). After 2 mins the assays were terminated by addition of 6 M HCl (0.1 ml). They were then left on ice for 5 mins before being centrifuged at $1500g_{av}$ for a further 5 mins. Samples of the supernatants (0.4 ml) were then evaporated to dryness in scintillation vials by blowing with a hairdryer, thus removing radioactive CO_2. Following drying the residues were dissolved in 0.5 ml water before adding 10 ml of Optiphase safe scintillation fluid and counting for radioactivity.

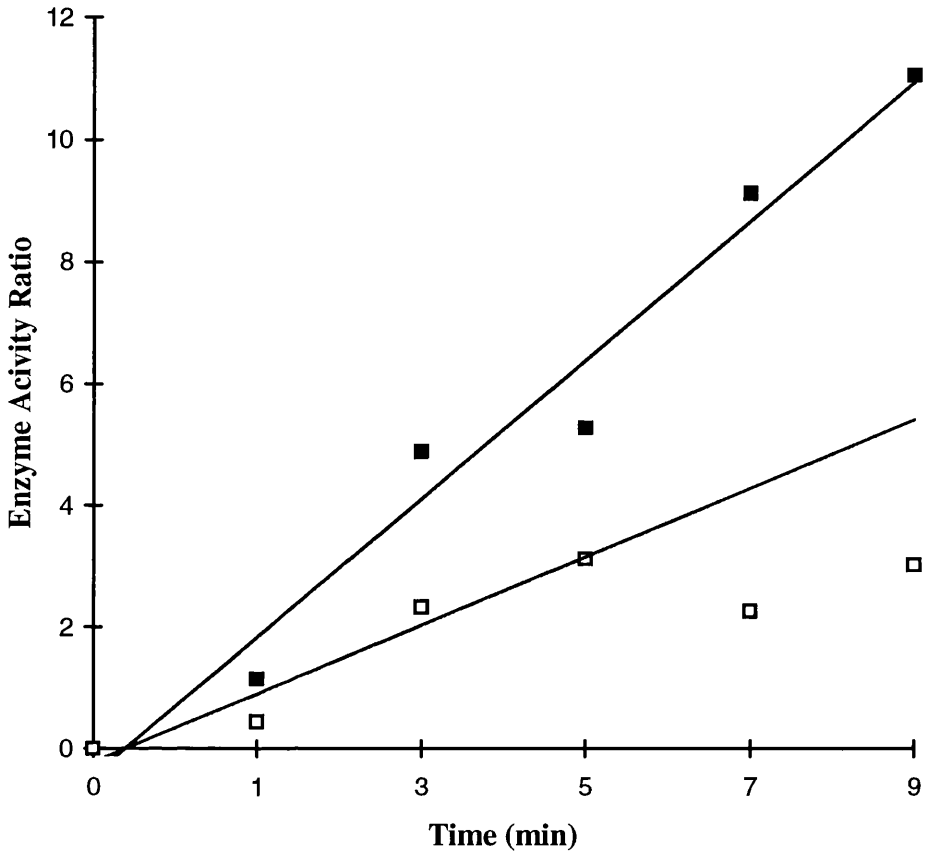
The total ACC activity of the extracts was determined by pre-incubating the infranatants at 30°C for 20 mins with 20 mM trisodium citrate (final concentration) before assaying as above. To prevent chelation of Mg^{2+} by citrate an equamolar concentration of $MgCl_2$ (i.e. 20 mM) was also included in the pre-incubation.

2.7.2 Assay of Glycogen Synthase Activity

Glycogen synthase (EC 2.4.1.11) activity was determined by measuring the incorporation of UDP glucose into glycogen. The assay is derived from the method of Lawrence *et al.* (1977).

2.7.2.1 ***Activation of the Enzyme*** Adipocytes, isolated from the epididymal fat pads as described previously, were incubated in 5 ml volumes (2/5 fat pad/ml) of KRB/HEPES medium containing 1% BSA and 5 mM glucose, plus additions stated in text. After incubating for 15 mins at 37°C on a shaking water bath (75 rev/min) the cells were centrifuged for 15 secs in a bench centrifuge and the medium aspirated. To

Fig. 2.3 Acetyl-CoA Carboxylase Time Course



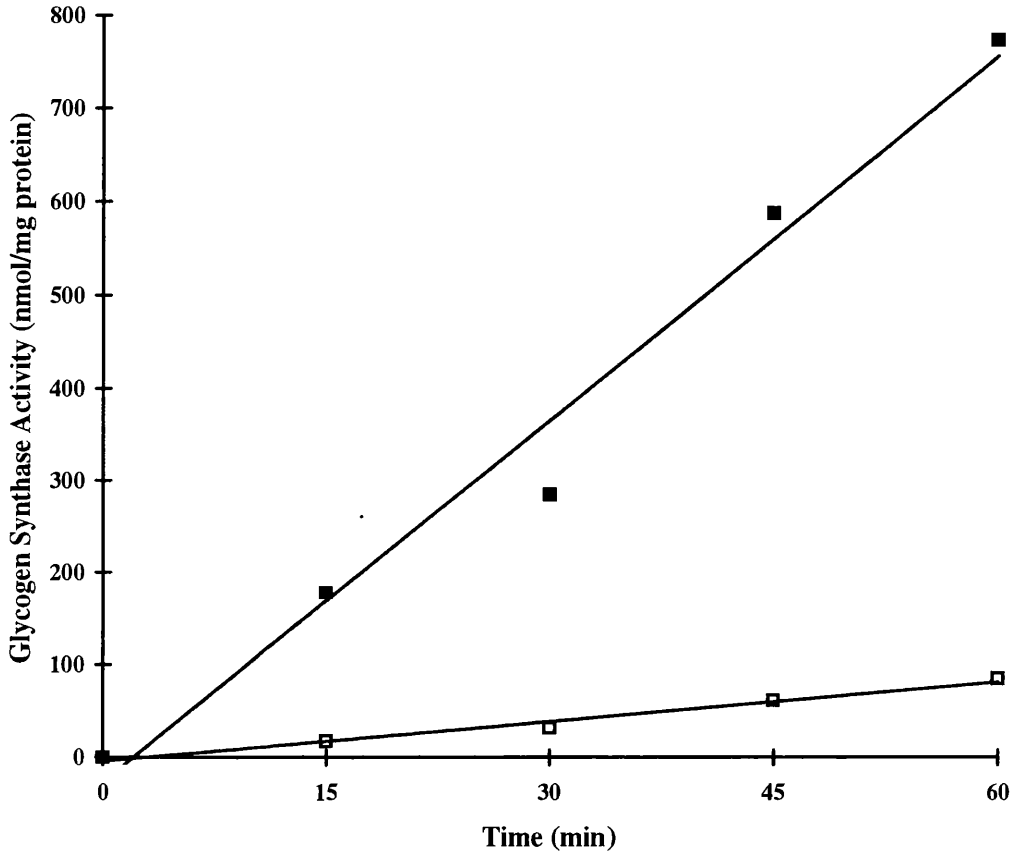
Shows the time course of the ACC assay with (■) and without (❑) a 20 min pre-incubation with 20 mM citrate, carried out under conditions described in section 2.7.1. (n = 2). Values are given as the Enzyme Activity Ratio i.e. the ratio of ACC to LDH activity in the extracts x 1000.

terminate the incubation the cells were snap frozen by plunging the glass vials containing the cells into liquid N_2.

2.7.2.2 ***Extraction of Cells for Enzyme Analysis*** For each incubation 1 ml of ice cold buffer, containing 100 mM KF, 10 mM EDTA, pH 7.0 and 1 mM benzamidine, was added and the cells homogenised at 0-4°C using 3 x 5 sec bursts of an Ultra-Turrax homogenizer. The homogenates were then centrifuged at $10,000g_{av}$ for 5 mins and the infranatants removed for assay.

2.7.2.3 ***Glycogen Synthase Assay*** Samples of the infranatant (30 µl) were added to 60 µl of assay buffer containing 50 mM Tris-HCl, pH 7.8, 20 mM EDTA, 25 mM KF, 10 mg/ml glycogen and 6.7 mM UDP-[U-^{14}C]glucose (0.1µCi/assay). They were then incubated for 20 mins at 30°C at which point 75 µl aliquots were taken and spotted on 2 cm squares of Whatman no. 3MM filter paper. The filter papers were immediately dropped into a beaker containing 600 ml of 66% ethanol, washed twice for 30 mins at room temperature and then overnight at 4°C. After the final wash the filter papers were rinsed in acetone, air dried and placed in scintillation vials containing 10 ml Ecoscint A for determination of radioactivity. To measure the total synthase activity of the infranatants, 10 mM glucose 6-phosphate (G6P) was included in the assay buffer.

Fig. 2.4 Glycogen Synthase Time Course



Shows the time course of the glycogen synthase assay in the absence (❑) and presence (■) of 10 mM glucose 6-phosphate, carried out under conditions described in section 2.7.2. (n = 1).

2.7.3 Assay of Glycogen Phosphorylase Activity

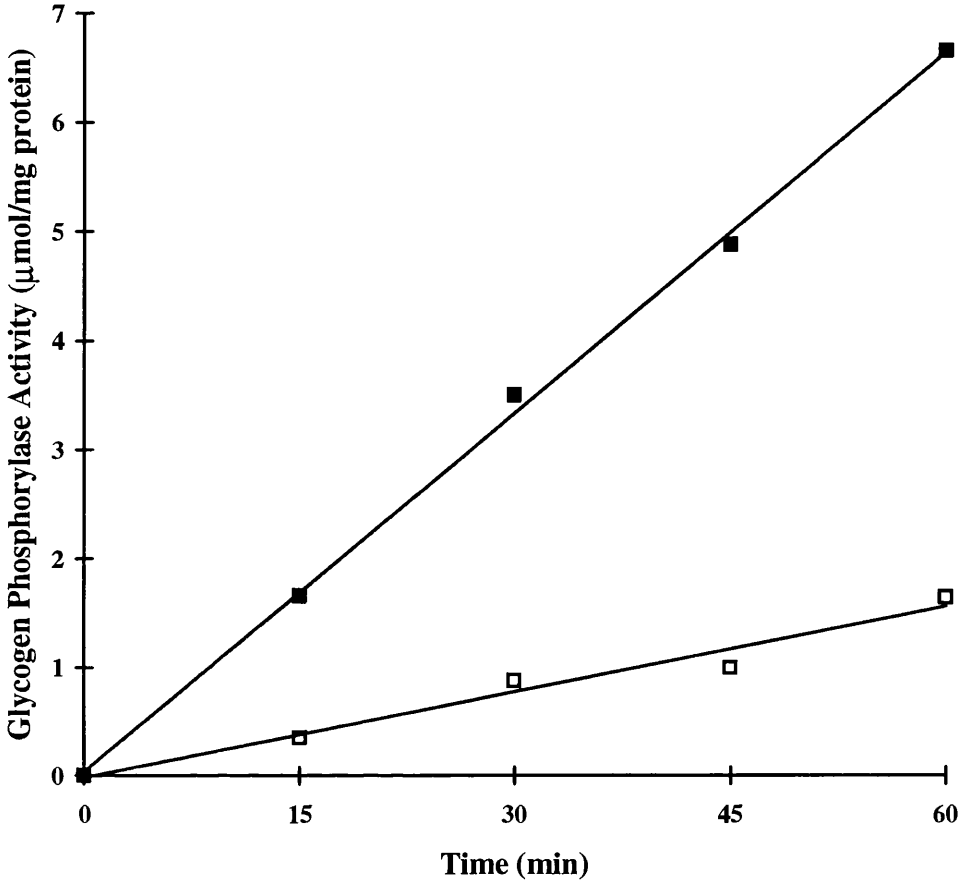
2.7.3.1 ***Activation and Extraction of the Enzyme*** This was carried out using exactly the same method as for the glycogen synthase assay.

2.7.3.2 ***Glycogen Phophorylase Assay*** This assay was derived from the method of Gilboe *et al.* (1972) and measures the incorporation of radiolabelled glucose 1-phosphate (G1P) into glycogen by glycogen phosphorylase (EC 2.4.1.1.). Samples of the infranatants (30 µl) were added to 60 µl of assay buffer containing 200 mM KF, 10 mg/ml glycogen, 100 mM D-[U-^{14}C]glucose 1-phosphate (0.0727 µCi/assay) and the assay mixture was adjusted to pH 6.1. After incubating for 20 mins. at 30°C, 75 µl aliquots of the assay mixture were removed and spotted on to 2 cm squares of Whatman no. 3MM filter paper. As for the glycogen synthase assay, the filter papers were immediately dropped into a beaker containing 600 ml of 66% ethanol, washed twice for 30 mins at room temperature and then overnight at 4°C. After a final rinse in acetone the filter papers were air-dried, placed in scintillation vials containing 10 ml Ecoscint A and counted for radioactivity. To determine the total phosphorylase activity in the samples 2.0 mM AMP was added to the assay buffer.

2.7.4 Assay of Phosphatidylinositol 3-Kinase Activity

2.7.4.1 ***Activation and Extraction of the Enzyme*** Adipocytes were isolated from the epididymal fat pads of male SD rats as described previously. The isolated adipocytes were then incubated at 37°C on a shaking water bath (75/ rev/min) for 15 mins in 5 ml volumes of KRB/HEPES medium (2/5 fat pad/ml) containing 5 mM

Fig. 2.5 Glycogen Phosphorylase Time Course



Shows the time course of the glycogen phosphorylase assay in the absence (❑) and presence (■) of 2 mM AMP, carried out under conditions described in section 2.7.3. (n = 1).

glucose, 1% BSA and additions stated in the text. Incubations were terminated by quickly aspirating the medium, washing the cells once in ice-cold PBS and adding 1 ml of ice-cold lysis buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1% w/v NP-40, 10% w/v glycerol, 20 mM Tris-HCl, pH 8.0, 0.2 mM PMSF, 10 mg/ml leupeptin and 10 mg/ml aprotonin. The adipocytes were then lysed by vigorous vortexing and the fat cake and cell debris were removed by centrifuging in a microfuge (Eppendorf 5142) for 5 mins at 4°C. Following centrifugation the infranatants were removed for assay.

2.7.4.2 ***Immunoprecipitation of Cell Lysates*** To immunoprecipitate any tyrosine phosphorylated protein present, 0.8 ml of the cell lysate was incubated with 8 µl of antiphosphotyrosine antibody PY 20 and protein A-agarose (80 µl of a 1:4 slurry of protein A-sepharose washed in lysis buffer) for between 3 and 12 hours at 4°C. The protein-antibody-protein A-agarose complex was then pelleted in a microfuge for 15 secs and the supernatant removed.

2.7.4.3 ***Phosphotidylinositol 3-kinase assay*** This assay was developed from the method of Jackson, *et al.* (1992). Before assay the immunoprecipitated beads were washed x 3 in lysis buffer, 2 x in Wash 1 (0.5 M LiCl, 0.1 M Tris-HCl, pH 8.0, 4°C), 1 x in Wash 2 (0.15 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.6, 4°C) and 1 x in Wash 3 (1 mM DTT, 5mM $MgCl_2$, 20 mM HEPES, pH 7.6, 4°C). Once washed the excess buffer was removed using a syringe and the immunoprecipitates were resuspended in 40 µl of kinase assay buffer which contained 20 mM β-

glycerophosphate, 5 mM sodium pyrophosphate, 30 mM NaCl, 1 mM DTT, pH 7.2 at 4°C. The beads were then mixed with 20 µl of the substrate, phosphatidylinositol (3 mg/ml stock solution in kinase assay buffer containing 1% cholate), and incubated in a 37°C waterbath for 5 mins. To this, 40 µl of kinase assay buffer containing 20 µCi γ-[^{33}P]-ATP, 3 mM Na_2ATP and 7.5 mM $MgCl_2$ was added and the mixture incubated for a further 10 mins at 37°C. The reaction was stopped by the addition of 0.45 ml $CHCl_3$/MeOH (1:2 v/v).

2.7.4.4 ***Lipid Extraction*** After completion of the kinase assay the lipid products were then extracted. To do this 150 µl chloroform and 150 µl 0.1 M HCl were added to each tube, the tubes were vortexed well and centrifuged for 10 mins at room temp. in a microfuge. The bottom layer was then carefully removed into a fresh tube and the top layer (aqueous phase) discarded. To the bottom layer, 300 µl chloroform and 300 µl 0.1 M HCl were added, again the tubes were vigorously vortexed and centrifuged for 10 mins at room temp in a microfuge. The bottom layer was subsequently removed and dried under nitrogen. Samples could then be stored at -70°C if wrapped in Nescofilm.

2.7.4.5 ***Separation of Phospholipids by Thin Layer Chromatography*** The dried samples were resuspended in 25 µl $CHCL_3$/MeOH/0.1 M HCL (200:100:1 [v/v/v]) by vortexing and spotted onto silica gel 60 coated (layer thickness 0.2 mm), aluminium backed, 20 x 20 cm t.l.c. plates. The t.l.c. plates had been pre-treated by dipping them briefly in freshly mixed MeOH/H_2O (containing 1% potassium oxalate, 2 mM EDTA)

(1:1 v/v), air drying them and baking in an oven at 150°C for 2 hours. The t.l.c. plates were developed for 2.5 to 3 hours in a t.l.c. tank that had been equilibrated for 24 hours with MeOH/$CHCl_3$/NH_3 (88% spec. grav.)/H_20 (75:52.5:20:10 [v/v/v/v]). Following development the plates were dried and the radioactive spots visualised and quantitated using a Fuji BAS 1000 phosphoimager.

2.8 SPECTROPHOTOMERIC ASSAYS

2.8.1 Assay of Pyruvate Dehydrogenase (PDH) Activity

The pyruvate dehydrogenase (EC 1.2.2.2.) assay was derived from the method of Elnageh and Gaitonde (1988).

2.8.1.1 ***Activation of PDH*** The distal ends of epididymal fat pads were removed from male rats and cut into two pieces of approx. equal size, giving a total of four pieces per rat. The fat pads were collected and washed in KRB/HEPES buffer containing 1% BSA and 5 mM glucose. The fat pieces were then incubated in a shaking water bath (100 rev/min) for 30 mins at 37°C in 4 ml volumes of KRB/HEPES medium again containing 1% BSA and 5 mM glucose plus additions stated in the text. The activation of PDH was terminated by dropping the fat pieces into liquid N_2.

2.8.1.2 ***Extraction of the Tissue for Enzyme Analysis*** The fat pieces were homogenised in 1ml of ice-cold buffer containing 10 mM potassium phosphate, pH 7.2, 2 mM EDTA and 1 mM DTT, with 3 x 15 sec bursts of an Ultra-Turrax homogeniser, keeping on ice at all times. Following homogenisation the suspensions

were centrifuged at 2,400g for 2 mins at 4°C to remove fat and cell debris. The resulting infranatants were removed for assaying.

2.8.1.3 ***PDH Assay*** To each of two 1 ml cuvettes was added 0.7 ml assay buffer made up of 50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.2% (w/v) Triton x-100, 1 mM $MgCl_2$, 0.5 mM NAD^+, 0.1 mM Coenzyme A, 0.1 mM sodium oxalate, 1 mg/ml BSA, 0.2 mM cocarboxylase, 7 units diaphorase, 0.6 mM p-Iodonitrotetrazolium violet dye (INT) and 20 µl tissue extract. The contents of each cuvette were mixed and a stable baseline at 500 nm, 37 °C was obtained for the reaction mixture in a dual beam UNICAM SP 8-100 spectrophotometer. The reaction was started by addition of 10 mM (20 µl) pyruvate to the sample cuvette and assay buffer (20 µl) to the reference cuvette. The absorbance of the reaction mixture at 500 nm, 37°C was recorded for 5 mins. The activity of PDH was calculated using the extinction coefficient 15.4 x 10^3 $M^{-1}.cm^{-1}$. To measure the total PDH activity of the extracts 50µl samples were incubated at 30°C with 0.2 µM $MgCl_2$ (10µl), 20 µl rabbit serum and 20 µl PDH phosphatase. After 5mins a 20 µl aliquot was removed and assayed for PDH activity.

2.8.1.4 ***Preparation and Assay of PDH Phosphatase*** The assay and preparation of PDH phosphatase were derived from the method of Siess & Wieland (1972). The PDH phosphatase enzyme was prepared by centrifuging a sample of purified PDH/PDH phosphatase complex (kindly supplied by Prof. R. Denton, Bristol) at 100,000 rpm for 2 hours. A 20 µl aliquot of the resulting supernatant, which contained no PDH activity, was incubated for 15 mins at 30°C with 50 µl

phosphorylated PDH, 0.2 mM $MgCl_2$ (10 µl) and 20 µl rabbit serum. Following incubation a 20 µl aliquot was removed and assayed for PDH activity. PDH phosphatase was considered to be present if the supernatant could dephosphorylate and reactivate the phosphorylated PDH substrate which had previously been assayed for PDH activity.

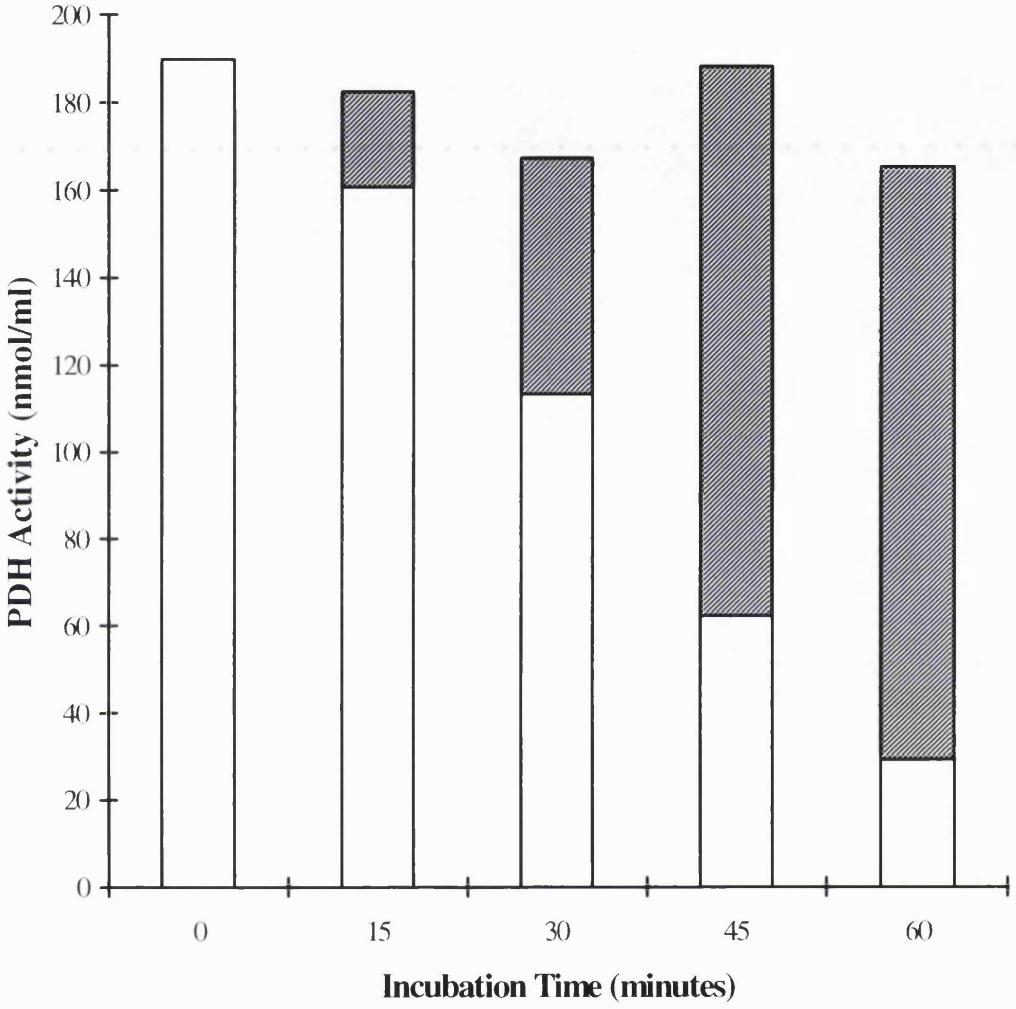
The phosphorylated PDH substrate used in the assay was prepared from the pellet produced by the centrifugation of the purified PDH/PDH phosphatase complex, following removal of the supernatant containing the PDH phosphatase. The pellet was resuspended in 1 ml of buffer containing 20 mM potassium phosphate, 0.2 mM ATP and 0.1 mM $MgCl_2$ and incubated at 30°C. This buffer allowed PDH kinase, which is tightly bound to the PDH complex and would not have been removed during centrifugation, to phosphorylate and inactivate PDH. At regular time intervals 20 µl aliquots of the incubation mixture were removed and assayed for PDH activity. In this way the time course of inactivation of the PDH could be determined.

2.8.2 Citrate Synthase Assay

In order to standardise the PDH assay, a citrate synthase (EC 4.1.3.7.) assay was carried out on each tissue extract. This assay was derived from the method of Shepherd and Garland (1969).

To each of two 1 ml cuvettes was added 0.7 ml assay buffer containing 100 mM Tris-HCl, 0.1% Triton x-100, pH 8.0, 50 nM acetyl-CoA, 200 mM DTNB and 20 µl tissue extract (from PDH assay). The contents of each cuvette were mixed and a stable

Fig. 2.6 Assay of PDH Phosphatase Activity



Purified PDH, containing PDH kinase but not PDH phosphatase, was incubated with 0.1 mM $MgCl_2$ and 0.2 mM ATP for the indicated times followed by measurement of PDH activity (open bars). Also at these times, aliquots were taken for a subsequent 15 min. incubation with purified PDH phosphatase followed by measurement of PDH activity (combined open + hatched bars). This was carried out under the conditions described in section 2.8.1.4, n = 1.

baseline at 412 nm, 37°C for the reaction mixture was obtained in a dual beam Shimadzu UV-2101PC spectrophotometer. The reaction was started by addition of 0.05 nM oxaloacetic acid (20 µl) to the sample cuvette and water (20 µl) to the reference cuvette. The absorbance of the reaction mixture was recorded at 412 nm, 37°C for approx. 3 mins. Citrate synthase activity was calculated using the extinction coefficient 13.6 x 10^3 $M^{-1}.cm^{-1}$.

2.8.3 Lactate Dehydrogenase Assay

Lactate dehydrogenase (LDH) (EC 1.1.1.27) activity was measured at 20°C by following the oxidation of NADH using the method of Saggerson (1974). In a final volume of 1 ml, the assay mixture contained 65 mM Tris-HCl, pH 7.4, 0.3 mM NADH and 10 µl tissue extract. The assays were initiated by the addition of sodium pyruvate (final concentration 1.3 mM) and the resulting decrease in absorbance at 340 nm was measured against a blank minus sodium pyruvate. LDH activity was calculated using the extinction coefficient for NADH of 6.22 x 10^3 M^{-1}. cm^{-1}.

2.9 PROTEIN DETERMINATION

2.9.1 Bicichinoic Acid Protein Assay

This assay was carried out using a kit purchased from Sigma following the manufacturers instructions.

2.10 LIQUID SCINTILLATION COUNTING

Scintillation counting was carried out on a Packard Tri-Carb 1900CA analyser (Canberra Packard, Pangbourne, Berks., U.K.) which had been pre-programmed for

counting ^{14}C and ^{3}H isotopes. The counter was equipped with a computer which stored the quench curves for ^{14}C and ^{3}H allowing direct measurement of radioactivity in disintegrations per minute (DPM).

2.11 STATISTICAL ANALYSIS

Statistical significance was determined by Student's *t*-test for paired samples. Values are the mean $\pm$ SEM for the stated number of separate measurements (n). Where SEM bars in figures are not visible they lie within the symbol.

CHAPTER THREE:

RESULTS and DISCUSSION

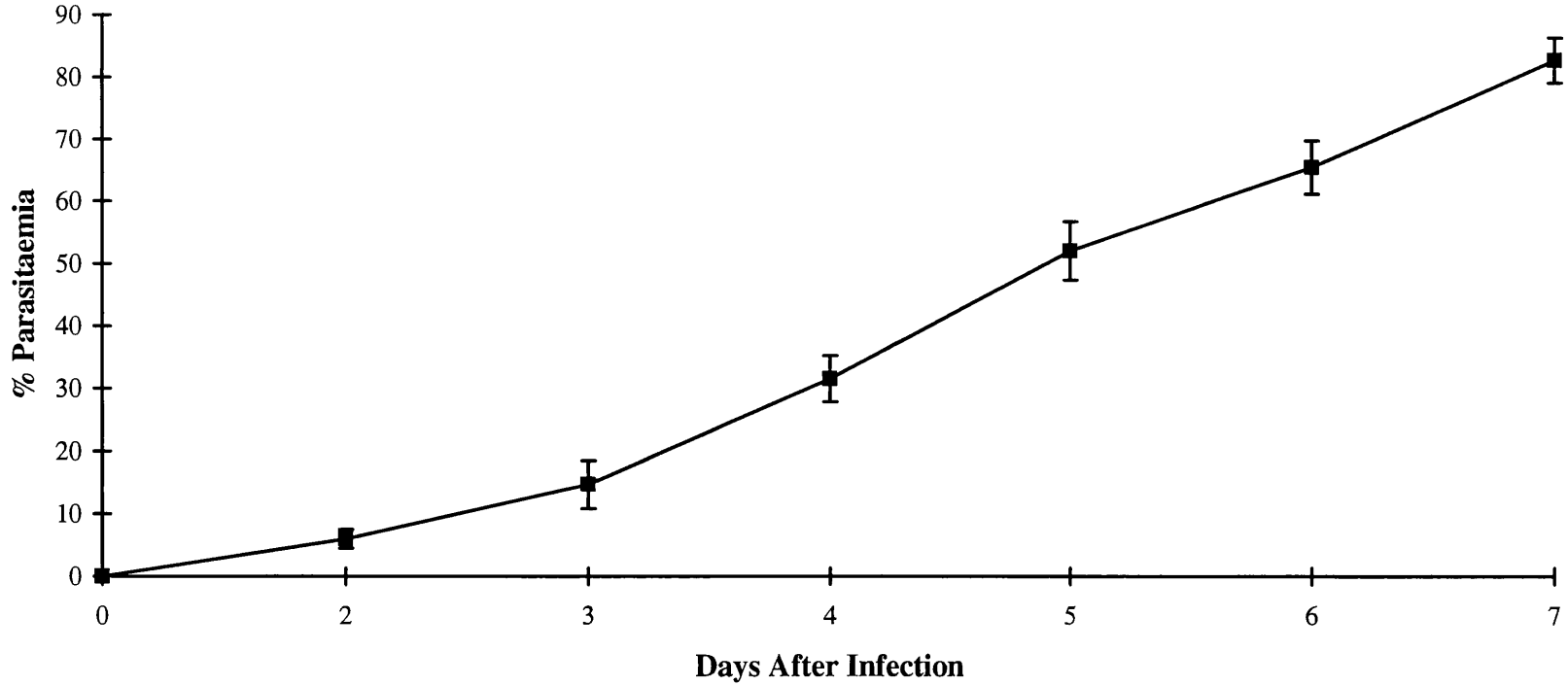
3.1 PREPARATION OF MALARIAL EXTRACTS

3.1.1 Malarial Parasites

In the paper which this project stems from, Taylor and co-workers demonstrated that malarial extracts (MEs) derived from the blood stages of a lethal *P. yoelii* infection could not only induce hypoglycaemia when injected into normal mice, but could also synergise with insulin to enhance this effect. In addition these MEs potentiated insulin's effects on lipogenesis and lipolysis in isolated adipocytes (Taylor *et al.*, 1992b). Mice infected with lethal *P. yoelii* also develop hypoglycaemia during the terminal stages of the infection (Elased & Playfair, 1994). *P. yoelii* however is not the only *Plasmodium* strain which can cause hypoglycaemia in rodents. Rats infected with *P. berghei* (Holloway *et al.*, 1991) and mice infected with non-lethal *P. chabaudi* (Elased & Playfair, 1994) have also been shown to develop hypoglycaemia. It would therefore seem reasonable to hypothesise that these strains could also enhance insulin's effects. To test this theory, extracts were prepared from both lethal *P. yoelii* infected and non-lethal *P. chabaudi* infected mice.

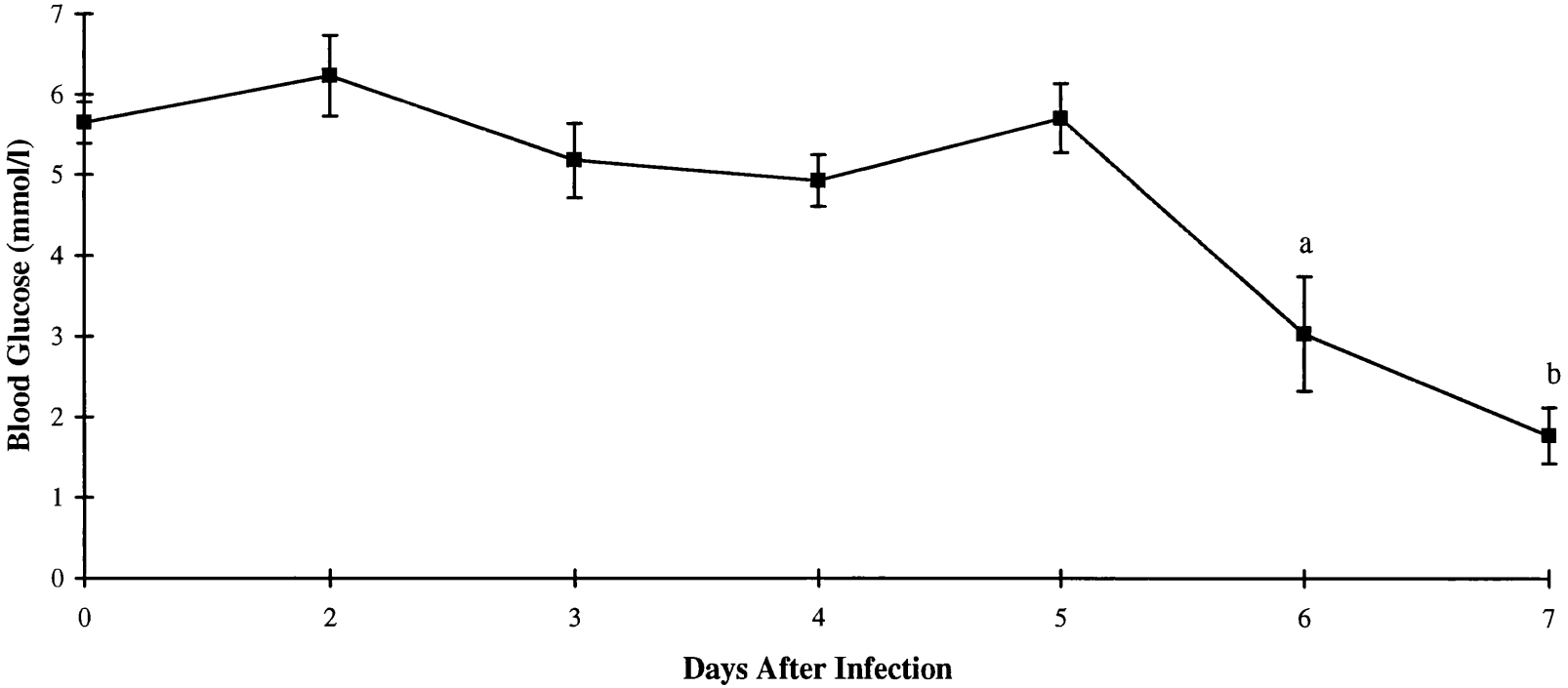
1.3.1.1 P. Yoelii The *P. yoelii* strain used came from the lethal YM line. This was taken from liquid nitrogen and passaged through at least one mouse before being used to infect the CD1 mice used for the preparation of the MEs. The mice were infected either i.p or i.v. with approximately 10^4 parasitized erythrocytes. On each day following infection the % of parasitized red blood cells (parasitaemia) was measured using a blood film stained with geimsa. At the same time the blood glucose concentration was determined from a drop of tail blood using glucostix and an Ames glucometer. As can be seen from fig. 3.1, the parasitaemias of the mice rose steadily

Fig. 3.1 Parasite Levels During a *P. yoelii* Infection



Shows the parasite levels during infection of CD1 mice with lethal *P. yoelii* YM. % Parasitaemia is the percentage of parasitized red blood cells per ml blood. Values are means ± SEM (five to sixteen mice per time point).

Fig 3.2 Blood Glucose Concentrations During a *P. yoelii* Infection

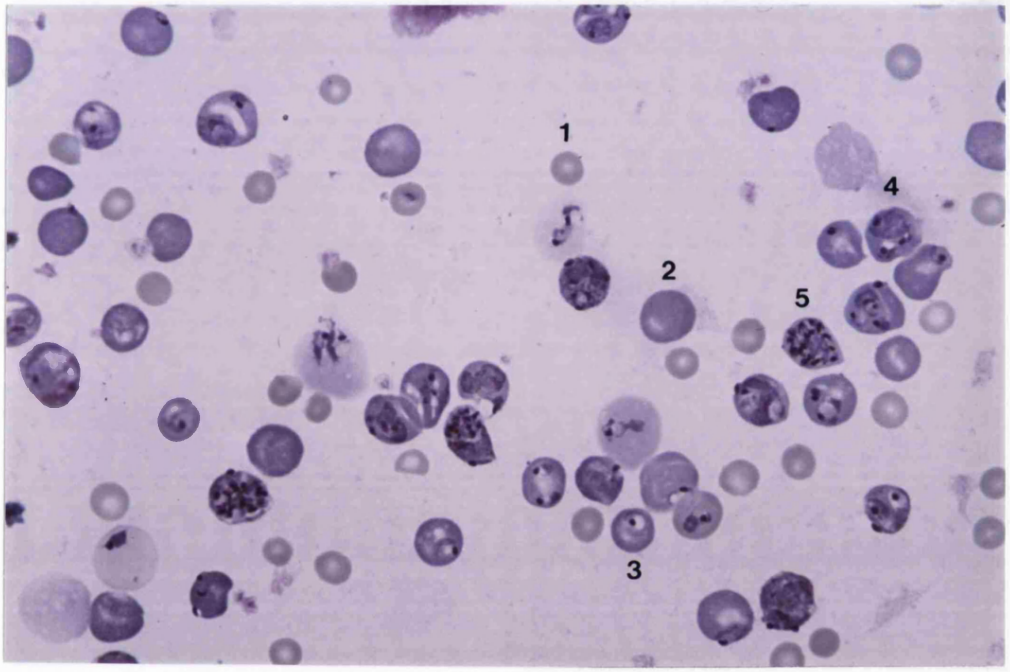


Shows the blood glucose concentrations during infection of CD1 mice with lethal *P. yoelii* YM. Values are mean ± SEM (five to thirteen mice per time point). a and b indicate $p<0.01$ and $p<0.001$ respectively for comparison with blood glucose concentration on Day 0.

to reach a maximum of approx. 80% on day 7. Conversely the blood glucose levels remained normal until day 5 of the infection, then fell rapidly to hypoglycaemic levels by day 7 (fig 3.2). At this stage the majority of the parasites were in their schizont form. Fig. 3.3 shows an example of a schizont-filled erythrocyte along with other stages of the parasite. The mice were normally bled on day 6 or 7 of the infection to ensure a high percentage of parasites in the blood and a large number of schizonts. Had the mice been left, the schizonts would have ruptured and the mice would have died. Fig. 3.4 shows an example of a blood slide from a *P. yoelii* infected mouse with a very high parasitaemia (approx. 95%).

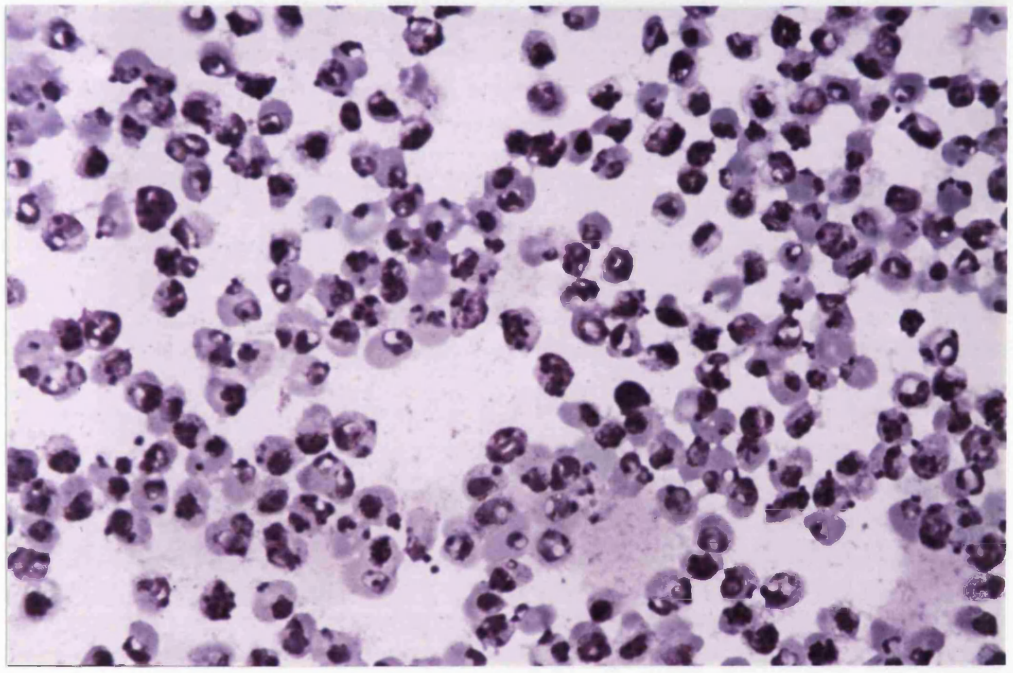
1.3.1.2 P. chabaudi In this case, the non-lethal AS strain of *P. chabaudi* was used. Like *P. yoelii*, it was taken from liquid nitrogen and passaged through at least one mouse before being used to infect the mice for ME preparation. The same methods of infection and screening were also used. With this parasite, after a lag phase of approx. 4 days the parasite levels rose to reach a maximum of approx. 55% by day 7 (fig.3.5). As was the case with *P. yoelii*, the majority of the parasites at this stage were in the schizont form. Unlike *P. yoelii* however, if the mice were left their parasitaemias began to fall, returning to normal levels by day 14-16. Why the mice recover from this infection but not the *P. yoelii* infection is unclear. The fact that the parasitaemias do not reach as high levels with this strain probably plays a role. When *P. chabaudi* infected mice reach 'crisis', i.e. the point at which the schizonts rupture the red blood cells releasing merozoites, they will still have enough uninfected red cells to survive. The *P. yoelii* infected mice on the other hand have such high parasitaemias that when their schizonts rupture the resulting anaemia would probably kill them .

Fig. 3.3 Intra-Erythrocytic Stages of *Plasmodium*



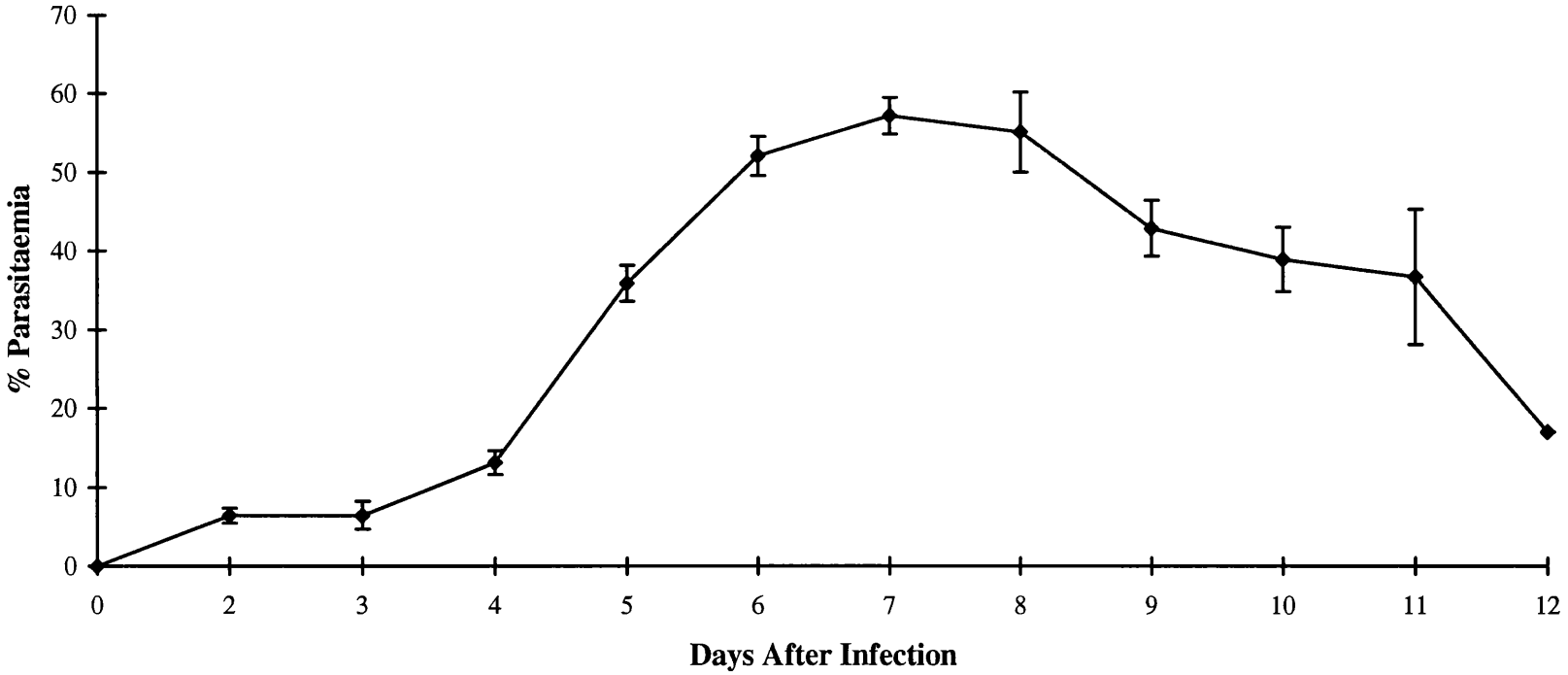
Shows a blood film taken from a mouse on day 5 of a *P. yoelii* infection. Labelled on the picture are no.1 a normal mature red blood cell, no.2 a reticulocyte, no.3 an early trophozoite (ring stage), no.4 a late trophozoite and no.5 a schizont containing a number of merozoites.

Fig. 3.4 Parasitized Red Blood Cells



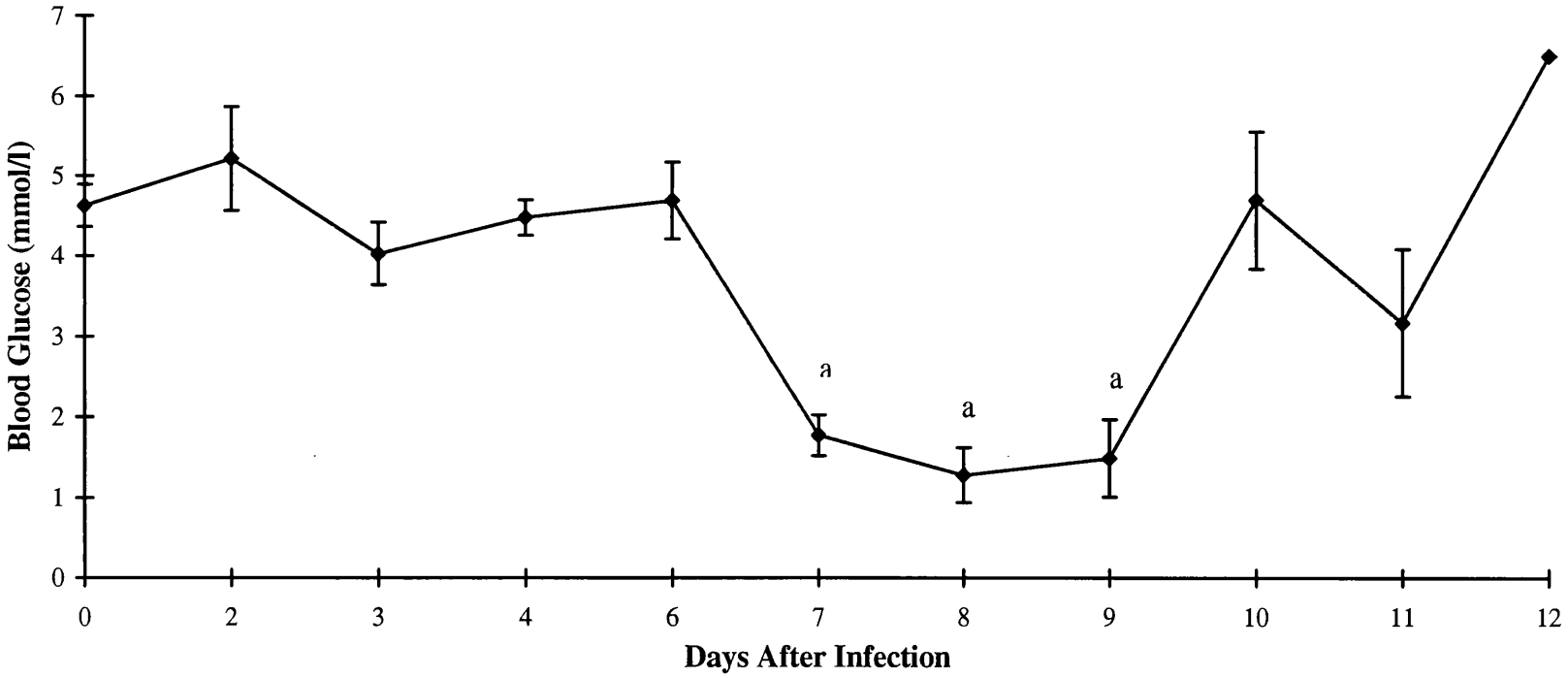
Shows a blood film taken from a mouse on day seven of a *P. yoelii* infection. As can be seen from the picture the mouse has a parasitaemia of >90% and the erythrocytes contain mainly schizonts.

Fig. 3.5 Parasite Levels During a *P. chabaudi* Infection



Shows the parasite levels during infection of CD1 mice with non-lethal *P. chabaudi* AS. % Parasitaemia represents the percentage of parasitized red blood cells per ml of blood. Values are means ± SEM (two to twelve mice per time point).

Fig. 3.6 Blood Glucose Concentrations During a *P. chabaudi* Infection



Shows the blood glucose concentrations during infection of CD1 mice with non-lethal *P. chabaudi* AS. Values are Means ± SEM (two to twenty three mice per time point). a indicates $p<0.001$ when compaired with blood glucose concentration at Day 0.

The changes in blood glucose levels also differ between the *P. chabaudi* infection and the *P. yoelii* infection. With *P. chabaudi* the blood glucose levels remained normal until day 6 of the infection then fell rapidly to hypoglycaemic levels by day 7. If the mice were left the levels remained hypoglycaemic until day 9 at which point they returned to normal (fig 3.6). Thus with this strain there appears to be an inverse correlation between the parasite levels and the blood glucose levels (fig. 3.5 and 3.6) i.e. when the parasite levels were high, the glucose levels were low.

The *P. chabaudi* infected mice were normally bled around day 7 or 8, before crisis took place. As with the *P. yoelii* this ensured the highest parasitaemias and largest number of schizonts. This however was sometimes difficult to achieve as this strain of the parasite has a synchronous cycle, and normally reaches crisis around midnight therefore it was often difficult to catch them at their highest parasitaemias.

3.1.2 Methods of Malarial Extract Preparation

All preparation of the malarial extracts was carried out under sterile conditions. The blood derived from mice infected with *P. yoelii* was treated separately to that derived from *P. chabaudi* infected mice.

Once the blood had been taken from each mouse via heart puncture (normally 3-4 mice per preparation) it was pooled and washed twice in PBS. The number of red blood cells per ml (rbc/ml) was then determined using a haemocytometer. From this and the parasitaemia, which was measured prior to the mice being bled, the number of parasitized rbc/ml (prbc/ml) was determined. This was then adjusted to 10^8 prbc/ml by resuspending the rbcs in PBS.

The resuspended blood cells were then put on rollers overnight at 37°C. This was done to encourage the schizont-filled erythrocytes to rupture, hopefully releasing the determinant(s) which enhance insulin's effects. The following day, to ensure all the prbcs were burst the resuspended blood cells were subjected to one of three different treatments:

1. Sonication with two 30 second bursts from a XL2020 sonicator
2. Freeze/thawing x3
3. Centrifuging for 10 mins. at 500g before removing the supernatant and boiling both the supernatant and the pellet which had been resuspended in PBS.

After each of these treatments the preparations were centrifuged at 500g for 10 mins. In the case of the sonicated and freeze/thawed samples the supernatants were then removed and the pellets were resuspended to the same volume as the supernatants with PBS. With the boiled preparations, the supernatants were removed and the pellets were discarded. All the samples were then treated with pronase E and left overnight at 37°C.

The following day the preparations were boiled to kill the pronase and centrifuged to remove any debris. The supernatants were then treated with polymixin B-agarose and filtered before being stored at 4°C. The purpose of treating the preparations with pronase, centrifuging with polymixin B-agarose, filtering the samples and preparing them under sterile conditions was to prevent contamination of the samples, particularly with bacterial endotoxin. Endotoxins, such as lipoplysaccharide (LPS), have been implicated in the pathogenesis of malaria (Clark, 1978; Tubbs, H., 1980) and high levels have been shown to cause hypoglycaemia *in vivo* by impairing glucose

production and increasing glucose disposal in certain tissues (Lang *et al.*, 1993). Therefore these measures were adopted to rule out any possibility that the effects of the MEs might be caused by such contamination.

Pronase treatment did not inhibit the activity of the preparations. Fig 3.7 shows the lipogenesis screen of a *P. yoelii* ME and a *P. chabaudi* ME which were prepared with and without pronase treatment. Form the results of this screen, using the equations

$$(1) \qquad \text{Mimetic Factor} = \frac{P}{M}$$

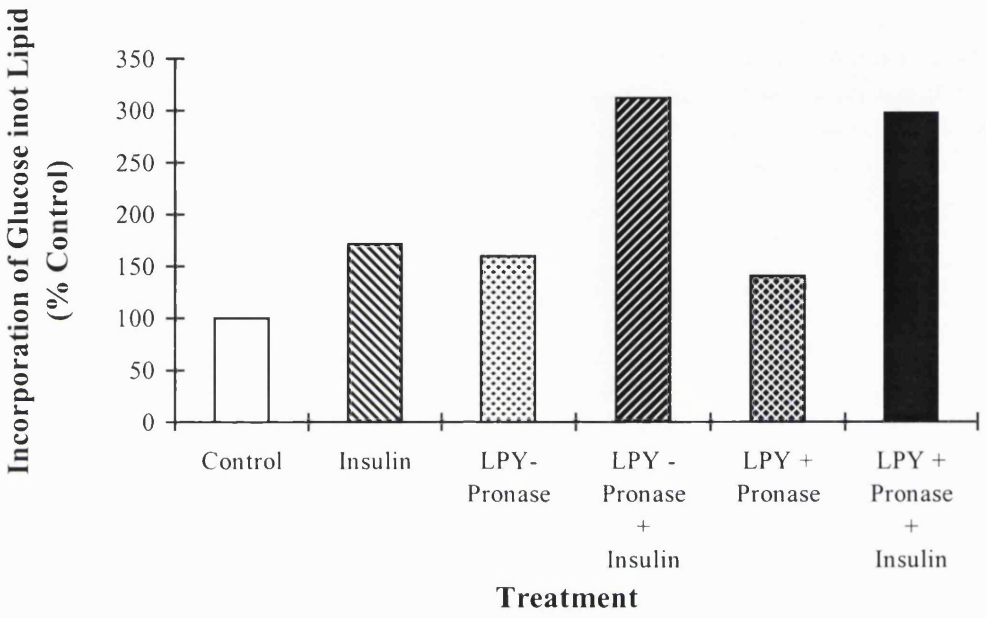
and

$$(2) \qquad \text{Synergy Factor} = \frac{(T - M)}{(I - M) + (P - M)}$$

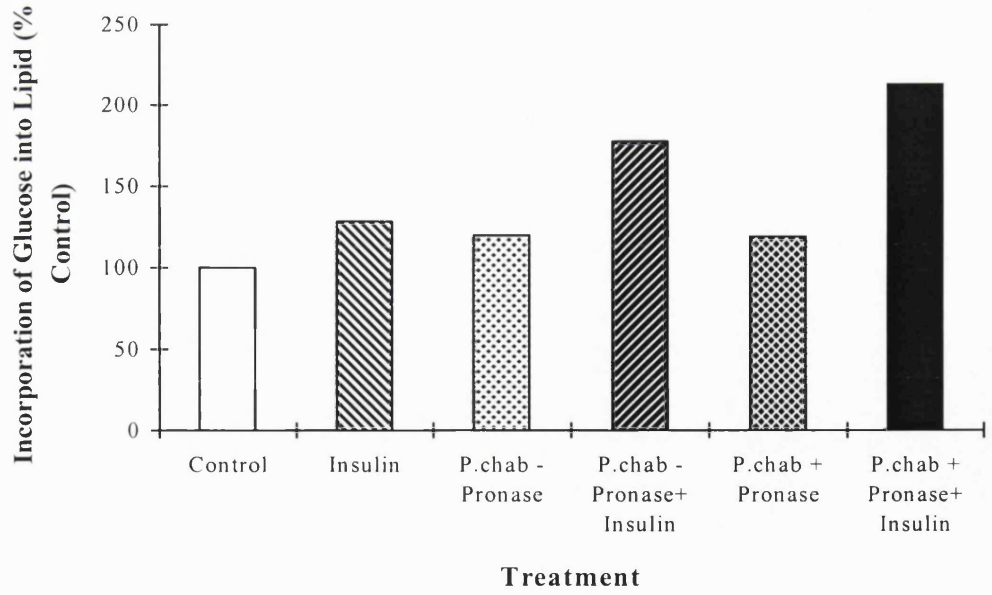
where M is the amount of radiolabelled glucose incorporated into lipid in the presence of medium alone, I is the amount incorporated in the presence of insulin, P is the amount incorporated in the presence of ME and T is the amount of radiolabelled glucose incorporated in the presence of both insulin and ME, it was possible to calculate the mimetic effects of the MEs, equation (1), and their synergistic effects, equation (2), in the absence and presence of pronase. Values greater than one for equation (1) and equation (2) indicate insulin mimicry and synergy with insulin respectively. From Table 3.1 it can be seen that, if anything, the pronase treatment actually enhanced the synergistic activity of the preparations.

Fig. 3.7 Effect of Pronase Treatment on ME Activity

(A) *P. yoelii* Derived Extract



(B) *P. chabaudi* Derived Extracts



Shows the screens of (A) a *P. yoelii* derived extract and (B) a *P. chabaudi* derived extract prepared with and without pronase treatment, incubated in the absence and presence of 10 nM insulin. For each experiment the results are the mean of triplicate measurements from one adipocyte preparation. The control values for the *P. yoelii* and *P. chabaudi* derived extracts are 1.229 and 2.016 μmol/hr/g dry weight of cells respectively.

Table. 3.1 Effect of Pronase Treatment on ME Activity

Malarial Extracts	Insulin (10 nM)	Pronase Treatment	Mimetic Factor	Synergy Factor
P. yoelii	-	-	1.59	
	+	-		1.62
	-	+	1.40	
	+	+		1.77
P. chabaudi	-	-	1.19	
	+	-		1.63
	-	+	1.19	
	+	+		2.40

This representative experiment shows the effect of pronase treatment on the activity of both a *P. yoelii* derived and a *P. chabaudi* derived malarial extract. For each strain n = 1. The mimetic factor was calculated using equation (1), the synergy factor was calculated using equation (2).

(Equation (1) and equation (2) are used regularly throughout this thesis to determine the mimetic and synergistic properties of the MEs and will be explained more fully in section 3.2.3)

The purpose of using three different treatments to prepare the MEs was to try and find a method which would consistently produce 'active' samples, i.e. samples which would synergise with insulin and enhance its effects. Unfortunately this was not achieved. When screened for their ability to enhance insulin-stimulated lipogenesis, examples of MEs prepared using each of the three methods and both parasite strains were shown to synergise with insulin (table 3.2). However, it proved impossible to consistently produce active preparations, despite using exactly the same methods of preparation. Table 3.3 shows the synergistic and mimetic properties of ten separate *P. yoelii* extracts all prepared using the freeze/thaw method. (For table 3.2 and 3.3 mimicry and synergy were calculated using equations (1) and (2) respectively). As can be seen from this table, five of these preparations were able to synergise with insulin, while the other five could not. The same inconsistency was also seen with the insulin mimetic effects of the extracts, only three of the ten preparations showed signs of insulin mimicry. There also appeared to be no relationship between the mimetic effects of the extracts and their synergistic effects, two of the preparations which mimicked insulin did not enhance insulin's effects and four of the five synergistic MEs had no effect in the absence of insulin. This difference between the insulin-mimetic properties of the MEs and their insulin-enhancing properties will be discussed more fully in section 3.2.3.

Table 3.2 Activities of MEs Prepared Using Both Parasite Strains and Each Method of Preparation

Strain	Method of Preparation	Mimetic Factor	Synergy Factor
P. yoelii	Sonication	1.15	4.21
P. yoelii	Freeze/Thaw	1.03	4.49
P. yoelii	Centrifuge/Boil	1.71	2.13
P. chabaudi	Sonication	1.08	2.88
P. chabaudi	Freeze/Thaw	1.32	3.15
P. chabaudi	Centrifuge/Boil	1.25	2.61

This table shows the mimetic and synergistic properties of six separate malarial extracts, three prepared from lethal *P. yoelii* infected mice and three prepared from *P. chabaudi* infected mice. For each strain, an example of a ME prepared using each of the methods described in section 2.3.3 is given. Each sample was screened using a lipogenesis assay, for each preparation n = 1. Mimicry and synergy were calculated from the results of those screens using equations (1) and (2) respectively.

Table 3.3 Activity of *P. yoelii* Extracts Prepared Using Freeze/Thaw Method

Strain	Method of Preparation	Mimetic Factor	Synergy Factor
P. yoelii	Freeze/Thaw	1.03	4.49
P. yoelii	Freeze/Thaw	1.0	1.47
P. yoelii	Freeze/Thaw	0.95	1.85
P. yoelii	Freeze/Thaw	0.79	2.02
P. yoelii	Freeze/Thaw	1.41	1.40
P. yoelii	Freeze/Thaw	1.41	0.64
P. yoelii	Freeze/Thaw	1.08	0.96
P. yoelii	Freeze/Thaw	1.0	0.68
P. yoelii	Freeze/Thaw	1.27	0.83
P. yoelii	Freeze/Thaw	0.9	1.07

Shows the properties of ten *P. yoelii* derived extracts all prepared using the freeze/ thaw method. Mimicry and synergy were determined from the lipogenesis assay screens of the extracts using equations (1) and (2) respectively. For each extract n =1.

To ensure that the insulin-enhancing factor from the parasitized red cells was not being in lost in the initial washing of the cells following collection of the blood, the washes were routinely kept, treated in the same way as the resuspended blood cells and screened to determine if the had any insulin-enhancing properties. Table 3.4 gives an example of the activities of the first and second washes from two lots of *P. chabaudi* infected blood, one which produced a ME which could enhance the incorporation of radiolabelled glucose into lipid and one which could not. It can be seen from the table that there was little or no synergistic activity in any of the washes whether the resulting ME was active or not. The washes from the blood which produced the inactive ME did display insulin-mimetic activity however this does not appear to have any relation to the insulin-enhancing activity. Therefore the insulin-enhancing factor at least does not appear to be being lost during washing of the red blood cells.

A similar inability to produce active preparations was experienced by Playfair and co-workers who were producing MEs using the same methods. They were unable to consistently produce preparations which would stimulate the production of TNF from macrophages (personal communications).

The reasons why this should be the case both for ourselves and for Playfair and co-workers are unclear, but it was probably due to a number of factors such as:-

1. Variability within individual mice in their response to the infection
2. Variability within the parasites, i.e. the stage of replication they were at when the blood was taken.

Table 3.4 Activity of Washes from *Plasmodium* Infected Blood used to prepare Malarial Extracts

Strain	MEs Synergistic	Wash no.	Mimetic Factor	Synergy Factor
P. chabaudi	Yes	1	0.76	1.08
		2	0.94	0.67
P. chabaudi	No	1	1.55	1.03
		2	1.16	0.76

Shows the insulin-mimetic and insulin-enhancing properties of the first and second washes derived from two lots of *P. chabaudi* infected blood used to prepare MEs. The ME derived from one lot of infected blood was able to enhance insulin-stimulated lipogenesis, while the other was not. As before the mimetic and synergistic properties were determined from the results of a lipogenesis screen using equations (1) and (2) respectively. For each wash, n = 1.

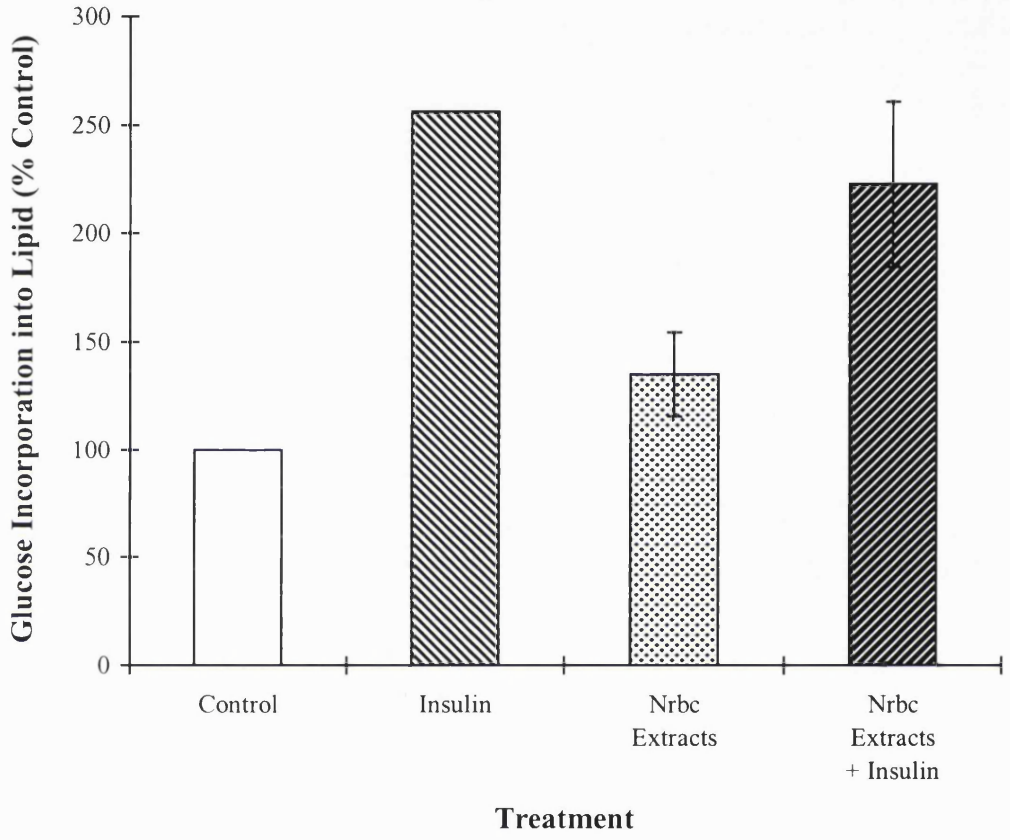
3. Where the actual determinants are derived from. It is still not known whether the determinants which synergise with insulin (or induce TNF) are derived from the parasites themselves or from the erythrocytes in response to the parasite.

To try and determine if the insulin-enhancing effect of the MEs could be derived from the erythrocytes, extracts were prepared from the blood of normal mice which had not been infected with malarial parasites. Eight separate nrbc (normal red blood cell) extracts were screened to determine if they had any ability to mimic or enhance the insulin-stimulated incorporation of radiolabelled glucose into lipids and the results were pooled (fig. 3.8). Although in the absence of insulin the nrbc extracts were able to stimulate lipogenesis slightly, this effect was not significantly different from the control. Likewise the slight inhibitory effect the nrbc extracts appeared to have on the insulin response was not significant. These results suggest that if the active factors are derived from the erythrocytes, it must be in response to the parasite and not merely a result of the preparation methods.

Whatever the reason for not being able to consistently produce active extracts, this proved to be a major stumbling block in the project. A lot of time was wasted preparing MEs which did not enhance insulin's effects when they were screened.

To try and overcome this problem some attempts were made by Dr Michael Orford to isolate and characterise the active determinant(s) in the preparations. The insulin-enhancing factor (IEF) in the MEs was found to have an M_r of approx. 1200 on Sephadex G50. Upon extraction of ME with $CHCl_3$/methanol to give aqueous and organic fractions, approx. 50% of the IEF activity was soluble in the organic fraction.

Fig. 3.8 Lipogenesis Screen of Normal Red Blood Cell Extracts



Shows the amount of U-[^{14}C]glucose incorporated into lipid in isolated adipocytes in the absence and presence of 10 nM insulin, with and without normal red blood cell extracts. Values are means $\pm$ SEM, where appropriate, for the assay of eight Nrbc extracts using two separated adipocyte preparations. Thus for control and insulin n = 2, and for Nrbc extracts and Nrbc extracts + insulin n = 8. The rate of incorporation for the control was 1.239 μmol/hr/g dry weight of cells.

This organic fraction was then further fractionated by t.l.c. using $CHCl_3$/ methanol/H_20/ammonia (48/40/7/5) in the first dimension and $CHCl_3$/methanol/formic acid (55/25/5) in the second dimension. At this stage the IEF activity was still heterogeneous as five of the spots visualised with iodine vapour were able to enhance insulin-stimulated lipogenesis. As well as being fractionated by t.l.c, the organic fraction was also treated with PI-specific phospholipase C or nitrous acid followed by a second $CHCl_3$/methanol extraction to give a second lot of aqueous and organic fractions. Nitrous acid treatment of the first organic fraction totally abolished the IEF. Phospholipase C treatment on the other hand rendered the IEF activity water soluble without inactivating it. These findings suggest that the IEF could be a GPI-type molecule and/or a headgroup thereof which is consistent with the findings of Taylor *et al.*, (1992a) which showed that the hypoglycaemic agents(s) in *P. yoelii* malarial extracts contained an inositol phosphate motif.

Unfortunately the methods used to isolate the IEF activity, such as 2-D t.l.c. were very inefficient. By the time the IEF activity had been extracted from the spots on the t.l.c. plate and screened using the lipogenesis assay there was no sample left for further experimentation. Consequently only the crude malarial extracts which had been shown to have insulin-enhancing activity were used in subsequent experiments.

3.1.3 Screening Procedures

As mentioned previously the activity of the MEs was determined by screening their ability to enhance insulin stimulated lipogenesis. This was carried out using adipocytes which had been isolated from rat epididymal fat pads (see section 2.5). The original method used to measure the incorporation of glucose into lipids came from a paper by

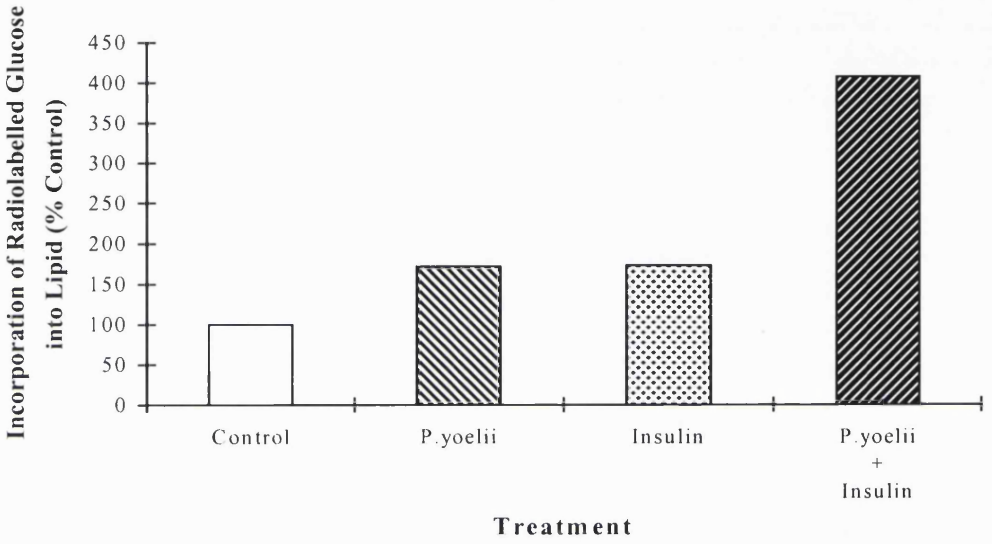
Moody *et al.* (1974) which used 1-[^{3}H]glucose and 6-[^{3}H]glucose. However, although this method was successfully screened active ME preparations, the effect of insulin on glucose incorporation into lipids was very poor. Fig 3.9 (A) and (B) give examples of the results from the screen of a *P. yoelii* and a *P. chabaudi* extract respectively. These show that the MEs can enhance insulin-stimulated lipid synthesis with synergy factors of 2.13 for the *P. yoelii* extract and 2.61 for *the P. chabaudi* extract, yet the actual insulin responses are only just above control level.

In case the screening procedure was at fault it was decided to change to a method which measured the incorporation of [^{14}C]glucose into lipids (see section 2.6.1.2). This however made no difference as the insulin effects were still very poor (results not shown). To test whether the isolation of the adipocytes was affecting the insulin response the assay was carried out using fat pieces rather than isolated adipocytes (section 2.5.1.3). Using this method an approx. 5-7 fold stimulation of glucose incorporation into lipids in response to insulin was consistently produced (fig. 3.10), which is similar to published observations. Fat pieces were therefore used to screen the MEs, and for some of the other experiments described in later sections, until the problem with the insulin response in the adipocytes was resolved.

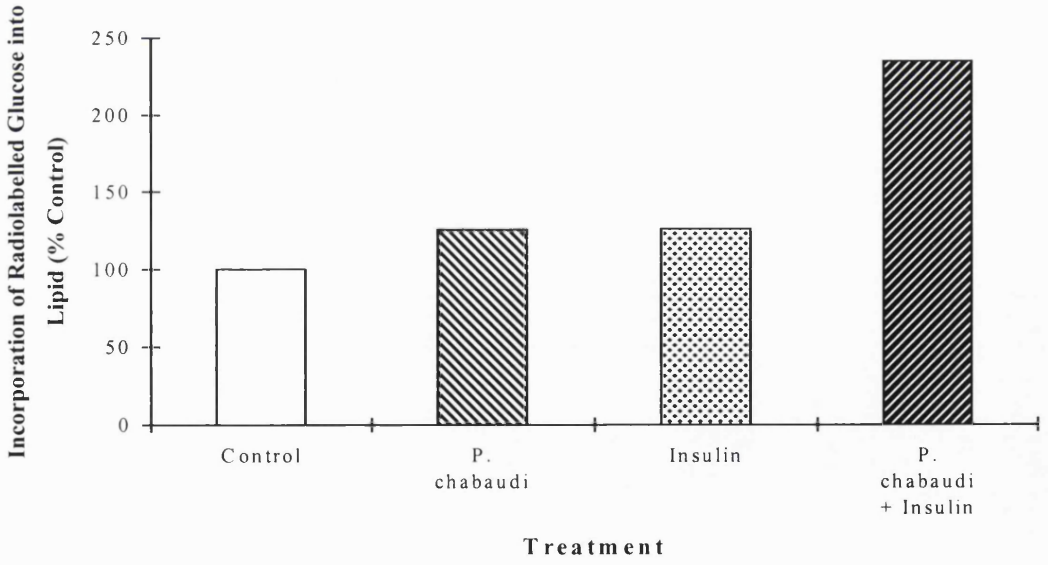
The effect which insulin had in fat pieces clearly showed that the lack of insulin response was related to the isolation of the adipocytes. As the only treatment which the fat pads undergo during adipocyte isolation is digestion with collagenase it was thought that the collagenase treatment may be affecting the insulin response. Consequently Dr Orford screened numerous batches of collagenase from various sources until he found one particular batch which did not affect the insulin response.

Fig. 3.9 [^{3}H] Lipogenesis Screen Of (A) a *P. yoelii* Extract and (B) a *P. chabaudi* Extract

(A)

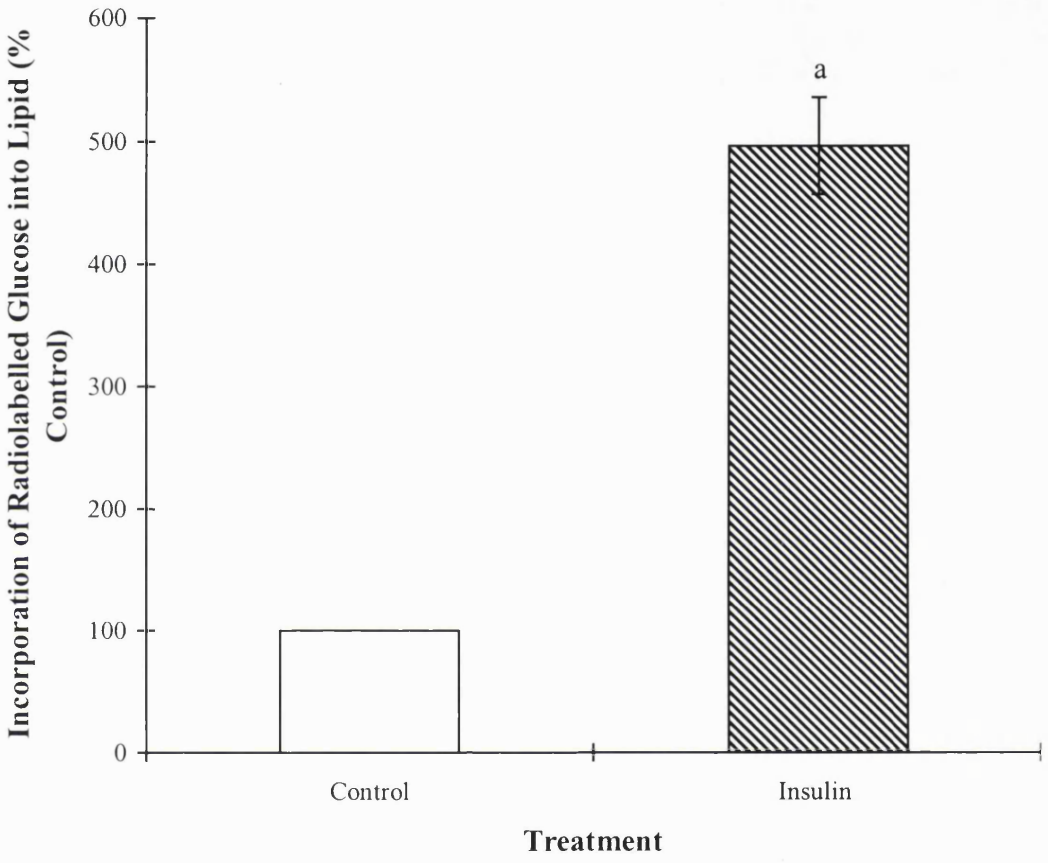


(B)



Shows the incorporation of [^{3}H]glucose into lipid in the absence and presence of 10 nM insulin, with and without (A) a *P. yoelii*-derived malarial extract and (B) a *P. chabaudi* derived extract. For each experiment the results are the mean of triplicate measurements from one adipocyte preparation. The control values for the *P. yoelii* and *P.chabaudi* screens were 2.309 and 1.525 μmol/hr/g dry weight of cells respectively.

Fig. 3.10 [U-^{14}C] Lipogenesis Assay Using Fat Pieces



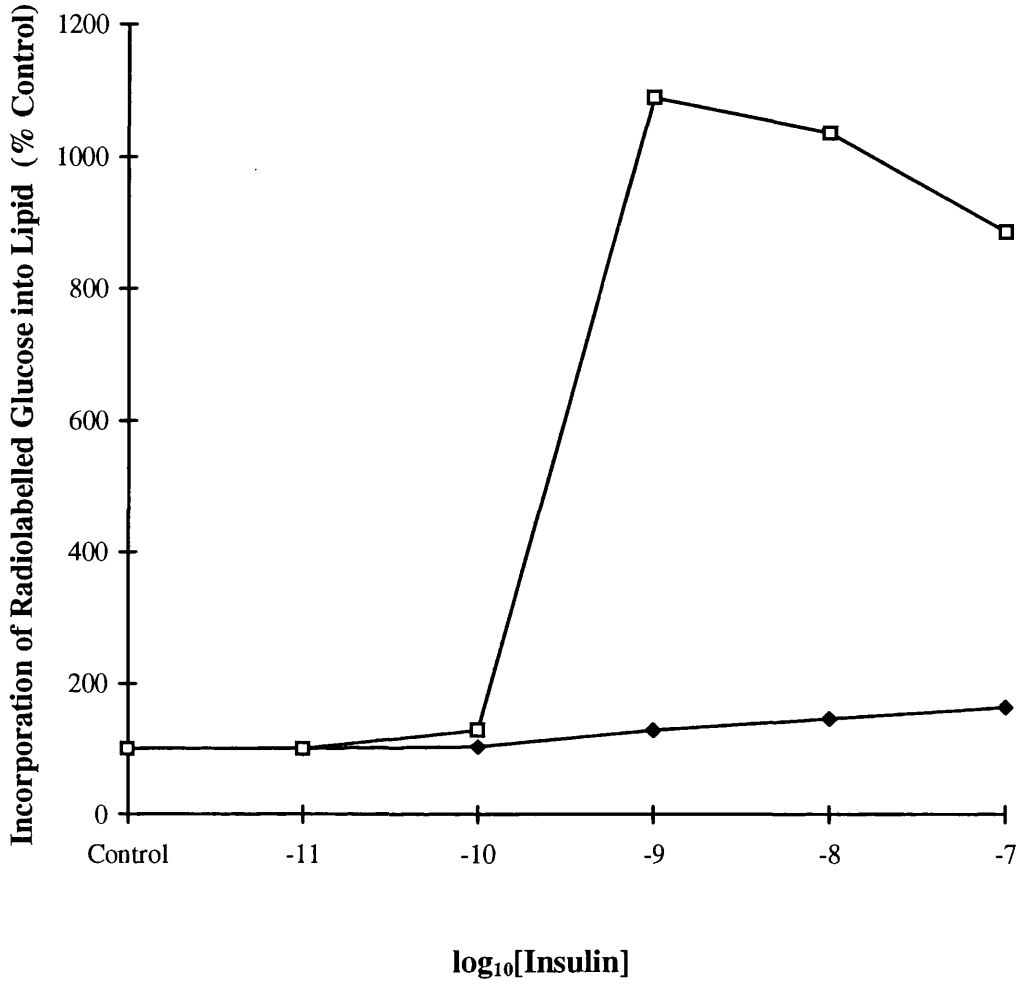
Shows the effect of 10 nM insulin on the incorporation of [U-^{14}C]glucose into lipid in fat pieces. Results are given as a percentage of the radiolabelled glucose incorporated into lipid in the presence of medium alone, following normalisation per g tissue. The rate of incorporation for the control was 2.324 $\pm$ 0.3 μmol/hr/g wet weight of tissue. Values are means $\pm$ SEM, n = 6. a indicates $p<0.0005$ when compared to control.

Fig. 3.11 shows the insulin-dose response curves for the incorporation of radiolabelled glucose into lipids in adipocytes isolated using the original collagenase and adipocytes isolated using the non-inhibitory collagenase batch. At the highest insulin concentrations using the original collagenase batch lipogenesis only reached a maximum of approx. 1.6-fold over basal, whereas with the non-inhibitory batch the maximum effect was approx. 10-fold over basal. All subsequent screens were therefore carried out using adipocytes derived from fat pads which had been digested using this non-inhibitory batch of collagenase.

For the screening of the MEs 25µl of the preparations were incubated with 1ml of isolated adipocytes in the absence or presence of 10 nM Insulin. This concentration of insulin is maximally effective in this assay (fig. 3.11), hence any increase in lipogenesis seen when the MEs were present had to be due to the preparations themselves. As there was no way of determining the concentration of the active determinants in the preparations, 25µl of ME preparation per ml of resuspended adipocytes derived from 1/5 of a fat pad was the standard ratio used throughout the experiments.

Table 3.5 gives a list of the active preparations which were used in subsequent experiments. This includes the strain of parasite which they were derived from and their method of preparation. Mimicry and synergy with insulin were calculated using the equations (1) and (2) respectively. As mentioned previously a value greater than 1 from equation (1) indicates insulin mimicry and a value greater than 1 from equation (2) indicates that the ME enhances insulin's effects.. Although the mimetic effect of the MEs is included in this table, only their synergistic effects were taken into account when deciding which preparations to use for subsequent experiments. Once it had

Fig. 3.11 Lipogenesis Assay Insulin-Dose Response Curves



Shows the incorporation of radiolabelled glucose into lipid at various concentrations of insulin using adipocytes derived from fat pads which had been digested with the inhibitory collagenase batch ◆ and the non-inhibitory collagenase batch ❑ . Results are given as percentage of the radiolabelled glucose incorporated into lipid in the presence of medium alone. For each condition n = 1.

been shown that all the methods of preparation could produce active ME it was decided to concentrate on one method, namely freeze/thawing. As a result the majority of the MEs used for subsequent experiments were prepared using this method (fig.3.5).

Table 3.5 Shows the properties of the malarial extracts which were used to try and determine where in the insulin signalling network the insulin-enhancing properties of these preparations were having their effect. The MEs were selected by screening for their ability to enhance insulin stimulated lipogenesis. The table gives the strain of the parasite the extracts were derived from, their preparation method, their mimicry and synergy factors as determined by equations (1) and (2) (which have been described previously) and their insulin-enhancing effects which were calculated using the equation;

$$\text{Insulin enhancing effect} = \frac{T}{I}$$

where I was the amount of radiolabelled glucose incorporated into lipid in the presence of insulin alone and T was the amount incorporated in the presence of both insulin and ME.

These insulin-enhancing MEs account for approximately 40% of the total extracts prepared. Of the other 60%, 10% showed synergistic activity but also had high mimetic activity and hence were not used as their synergy factor was less than one. The other 50% showed no insulin-enhancing properties.

Table 3.5 Properties of Malarial Extracts Used in Subsequent Experiments

ME	Strain	Preparation Method	Mimetic Factor	Synergy Factor	Insulin-Enhancing Effect
A	*P. chabaudi*	Centrifuge/Boil	1.28	2.27	1.76
B	*P. chabaudi*	Centrifuge/Boil	1.25	2.61	1.86
C	*P. chabaudi*	Freeze/Thaw	1.39	1.93	2.67
D	*P. chabaudi*	Freeze/Thaw	0.84	2.42	2.79
E	*P. chabaudi*	Freeze/Thaw	1.47	1.16	1.25
F	*P. chabaudi*	Freeze/Thaw	1.30	1.76	2.49
G	*P. chabaudi*	Freeze/Thaw	1.00	1.51	1.51
H	*P. chabaudi*	Freeze/Thaw	1.01	1.52	1.43
I	*P. chabaudi*	Freeze/Thaw	0.82	1.39	1.13
J	*P. yoelii*	Centrifuge/Boil	0.88	1.29	1.34
K	*P. yoelii*	Freeze/Thaw	0.86	1.47	1.10
L	*P. yoelii*	Freeze/Thaw	0.91	1.78	1.48
M	*P. yoelii*	Freeze/Thaw	0.80	2.02	1.41
N	*P. yoelii*	Freeze/Thaw	0.84	1.39	1.27
O	*P. yoelii*	Freeze/Thaw	2.01	2.27	2.58
P	*P. yoelii*	Freeze/Thaw	2.14	1.29	1.51
Q	*P. yoelii*	Freeze/Thaw	1.04	1.32	1.29
R	*P. yoelii*	Freeze/Thaw	1.09	1.27	1.25
S	*P. yoelii*	Freeze/Thaw	1.06	1.39	1.22

3.2 CORRELATION STUDIES

As was mentioned previously, Playfair and co-workers had originally been investigating the ability of ME preparations to stimulate the production of TNF by isolated macrophages when they found the same preparations could also induce hypoglycaemia in normal mice (Taylor *et al.*, 1992a). TNF is thought to play a role in many of the clinical symptoms of malaria, including hypoglycaemia (see section 1.2). The induction of hypoglycaemia and the stimulation of TNF production by the ME preparations appeared to be due to the same, or similar phosphatidylinositol (PI)-containing components (Taylor *et al*, 1992a). At least two merozoite surface proteins (MSPs) from *P. falciparum* are known to be anchored to the merozoite surface via glycosylphosphatidlyinositol (GPI) moieties which contain PI (Smythe *et al.*, 1988). Thus it is possible that these parasite GPI's, or cleavage products from them are released during the rupture of parasitized erythrocytes and are responsible for the effects which the MEs exert. Indeed, Schofield and co-workers have managed to purify GPI molecules from falciparum malarial parasites which can induce high levels of TNF and IL-1 from macrophages, mimic insulin's effects on lipogenesis and glucose oxidation in adipocytes, and induce hypoglycaemia *in vivo* (Schofield & Hackett, 1993). These findings therefore suggested that all the effects of the MEs could be caused by a single TNF-inducing entity.

Contradictory to this however Taylor *et al.* (1992b) found that while the MEs could induce hypoglycaemia and synergise with insulin to enhance its effects on lipogenesis and lipolysis, they had little or no ability to mimic insulin's effects. Also, although Schofield and Hackett demonstrated that the GPIs could mimic insulin's effects, they

did not show any ability of the GPIs to synergise with insulin (Schofield & Hackett, 1993). More recent work by Playfair and co-workers has shown that there is no correlation between the ability of the ME preparations to induce hypoglycaemia and stimulate the production of TNF by macrophages (Taverne *et al.*, 1995). These findings therefore suggest that more than one entity could be responsible for the effects of the MEs and hence the symptoms of malaria.

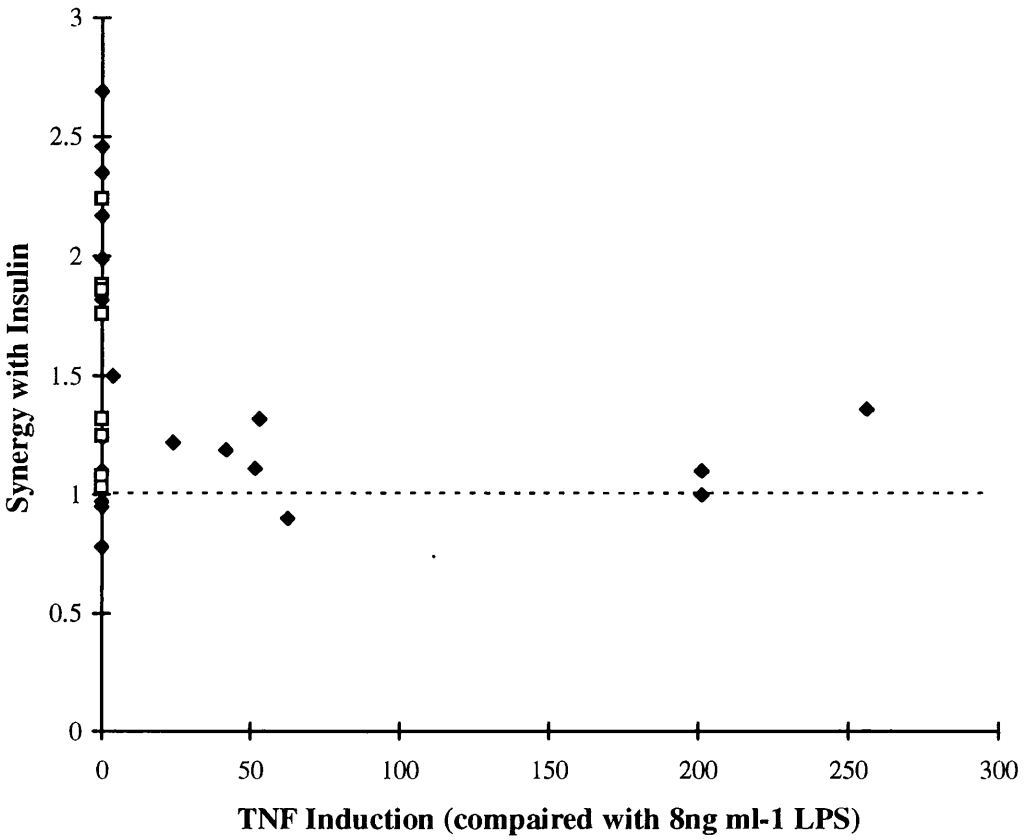
3.2.1 TNF v's Synergy

In an attempt to try and clarify whether a TNF-inducing entity could be responsible for all the effects of the ME preparations a collaboration was set up with Playfair's lab. The ability of 28 different MEs (some prepared by myself and some prepared by Nadeem Sheikh from Playfair's lab) to enhance insulin's effect on lipogenesis and to induce TNF production by macrophages was compared. The ability of the extracts to synergise with insulin was calculated using equation (2), therefore values greater than one indicated synergy. The amount of TNF produced by the macrophages was measured using the ELISA described in section 2.4.3., the results being standardised to the amount of TNF induced by 8 ng ml^{-1} of LPS. As can be seen from fig. 3.12, no correlation was seen between these two properties of the malarial extracts, $r = -0.264$. (Taverne *et al.*, 1996).

3.2.2 TNF v's Mimicry

Although Taylor *et al.* (1992b) found that the MEs had little or no ability to mimic insulin's stimulation of lipogenesis, in our hands some preparations could induce this effect. As Schofield and Hackett had clearly shown that malarial-derived GPIs could induce TNF and mimic insulin, it was decided to look for a correlation between these

Fig. 3.12 TNF Induction versus Synergy with Insulin



Comparison of the ability of ◆ *P. yoelii*-derived and ❑ *P. chabaudi*-derived MEs to induce the production of TNF from macrophages (X-axis) and to synergise with insulin and enhance lipogenesis (Y-axis) as calculated by equation (2). A total of 28 preparations were tested. r = -0.264.

Values >1 on the Y-axis indicate synergy with insulin

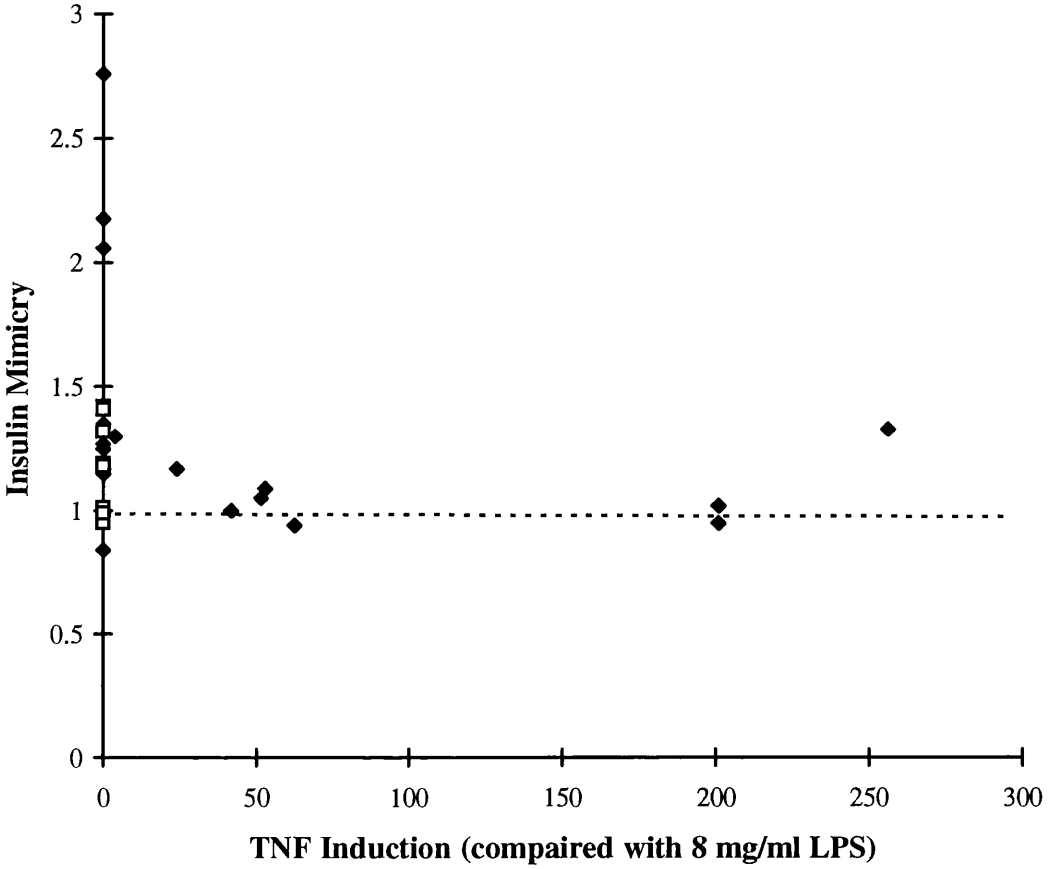
two effects of the preparations. Insulin mimicry was calculated by the formula P/M. Again, as can be see in fig. 3.13, no correlation was seen between these two effects of the malarial extracts, r = -0.142. (Taverne *et al.* 1996).

It is interesting also to note that while the majority of the *P. chabaudi* derived extracts were able mimic insulin (fig. 3.13) and enhance it effects (fig. 3.12), none of these preparations were able to induce the production of TNF from macrophages. Also the induction of TNF by similar preparations was unaffected by treatment with nitrous acid (Bate *et al.*, 1992b) which abolishes insulin enhancing activity, and PLC treatment of Schofield and Hacketts purified *P. falciparum*-GPI abolished its stimulation of TNF release (Schofield & Hackett, 1993), whereas PLC treatment renders the insulin enhancing activity water soluble. It would therefore seem from the results of the correlation studies, and these observations that a single TNF-inducing entity is not responsible for all the effects which MEs exert

3.2.3 Synergy v's Mimicry

The fact that some of our MEs could mimic insulin's effects as well as enhancing them could suggest that the apparent synergistic effects of these preparations were merely a result of insulin mimicry. In an attempt to rule out this possibility a number of steps were taken. First of all, in all experiments the concentration of insulin used was maximally effective, therefore any mimetic effects the preparations had should have been cancelled out when insulin was present. Secondly, the ability of each ME to synergise with insulin was calculated using the formula mentioned above (i.e. equation (2)). This takes into account any mimetic effect the preparations have, hence only MEs which could enhance insulin-stimulated lipogenesis, over and above the insulin

Fig. 3.13 TNF Induction versus Insulin Mimicry



Comparison of the ability of ◆ *P. yoelii*-derived and ❑ *P. chabaudi*-derived MEs to induce the production of TNF from macrophages (X-axis) and to mimic insulin's stimulation of lipogenesis (Y-axis) as calculated by equation (1). A total of 28 preparations were tested. r = -0.142.

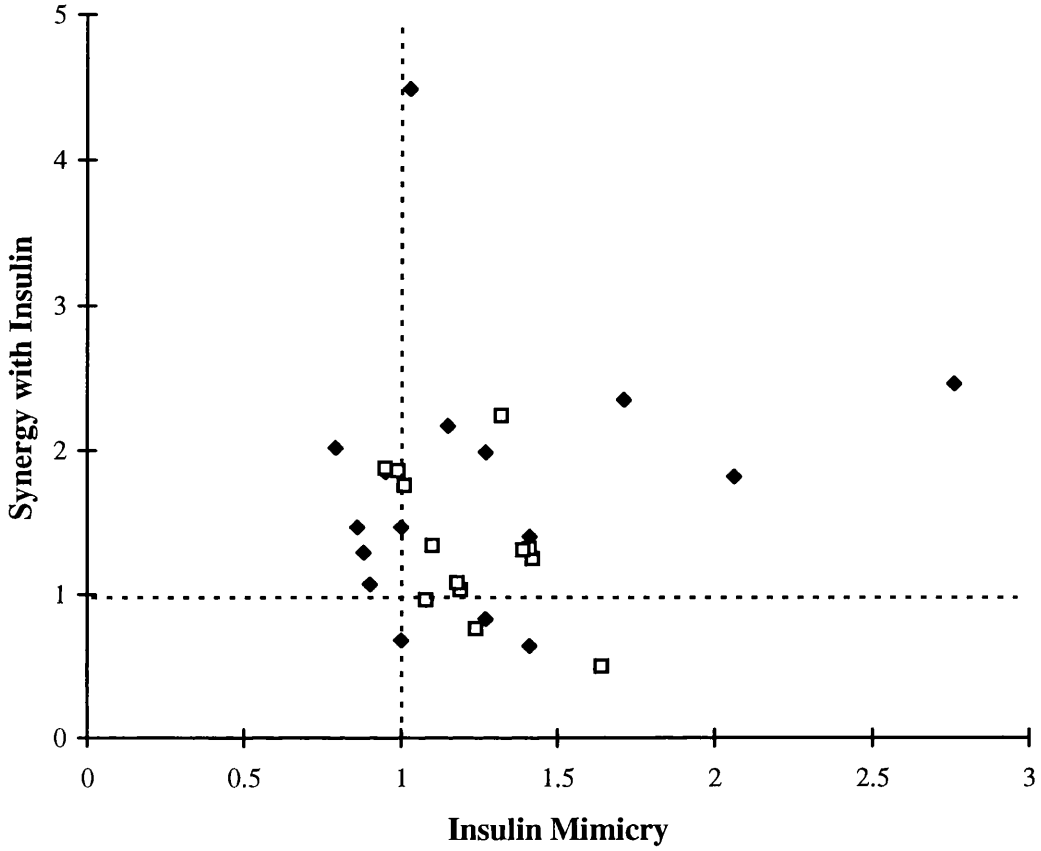
Values >1 on the Y-axis indicate insulin mimicry.

response and any mimetic response, were used in subsequent experiments. Finally, not all ME preparations which synergised with insulin were also able to mimic insulin (see table 3.3), which suggested that these effects, like the other effects of the extracts, were induced by separate entities. To test this I compared the insulin mimetic properties of 30 extracts, with their ability to synergise with insulin. As expected, no correlation was seen between the two, r = 0.0905 (fig. 3.14). This not only demonstrates that the insulin synergistic and mimetic effects of these preparations are separate, but also gives further evidence that separate entities are involved in ME's effects.

3.2.4 Conclusions

The results of these correlation studies and the other observations appear not only to back up the evidence which suggests that the TNF-inducing entity derived from *plasmodium* infected blood is not responsible for the other effects which these preparations can exert, but also that the other effects of the MEs such as insulin mimicry and insulin synergy are also caused by separate entities. These findings therefore allow us to rule out the possibility that the insulin mimetic effects of our preparations are responsible for their insulin enhancing effects. This evidence does not however rule out the possibility that each of these entities could be derived from the same GPI-type precursor molecule.

3.14 Insulin Mimicry versus Synergy with Insulin



Comparison of the ability of ◆ *P. yoelii*-derived and ❑ *P. chabaudi*-derived MEs to mimic (X-axis) and to enhance (Y-axis) insulin's effects on lipogenesis. A total of 30 extracts were tested. r = 0.0905.

Values>1 on the X-axis indicate insulin mimicry.

Values >1 on the Y-axis indicate synergy with insulin.

3.3 EFFECT OF MALARIAL EXTRACTS ON FATTY ACID SYNTHESIS

The main aim of this project was to try and determine where in the insulin signalling network the malarial extracts were exerting their effects. Having produced active preparations it was then necessary decide where in the signalling pathways to begin looking for an effect. Initial studies by Dr Orford had shown that the MEs had no effect on the binding of [^{125}I]insulin to cells or on insulin receptor internalisation, nor did they increase the rate of 3-O-methylglucose uptake (results not shown). Therefore the only insulin effects which the preparations were known to enhance were the stimulation of lipogenesis and the inhibition of lipolysis (Taylor *et al.*, 1992b). As the extracts had already been screened for their ability to enhance lipogenesis, this seemed the logical place to begin the investigation.

As was discussed in section 1.3.4.3, there are two enzymes which insulin stimulates to enhance fatty acid synthesis, and hence lipogenesis, namely pyruvate dehydrogenase (PDH) and acetyl-CoA carboxylase (ACC). Therefore it was decided to begin by looking at the effect of MEs on these two enzymes.

3.3.1 Effect on Pyruvate Dehydrogense

Insulin activates PDH by stimulating dephosphorylation of the enzyme through activation of PDH phosphatase. A number of studies have shown that inositol phosphate glycans, possibly derived from GPIs can mimic the effects of insulin on PDH activation (for review see Field, 1997). One such 'insulin-mediator' which activates PDH phosphatase and PP-2C has been characterised as a pseudo-disaccharide of pinitol (3-O-methyl-D-chiro-inositol) and galactosamine chelated to

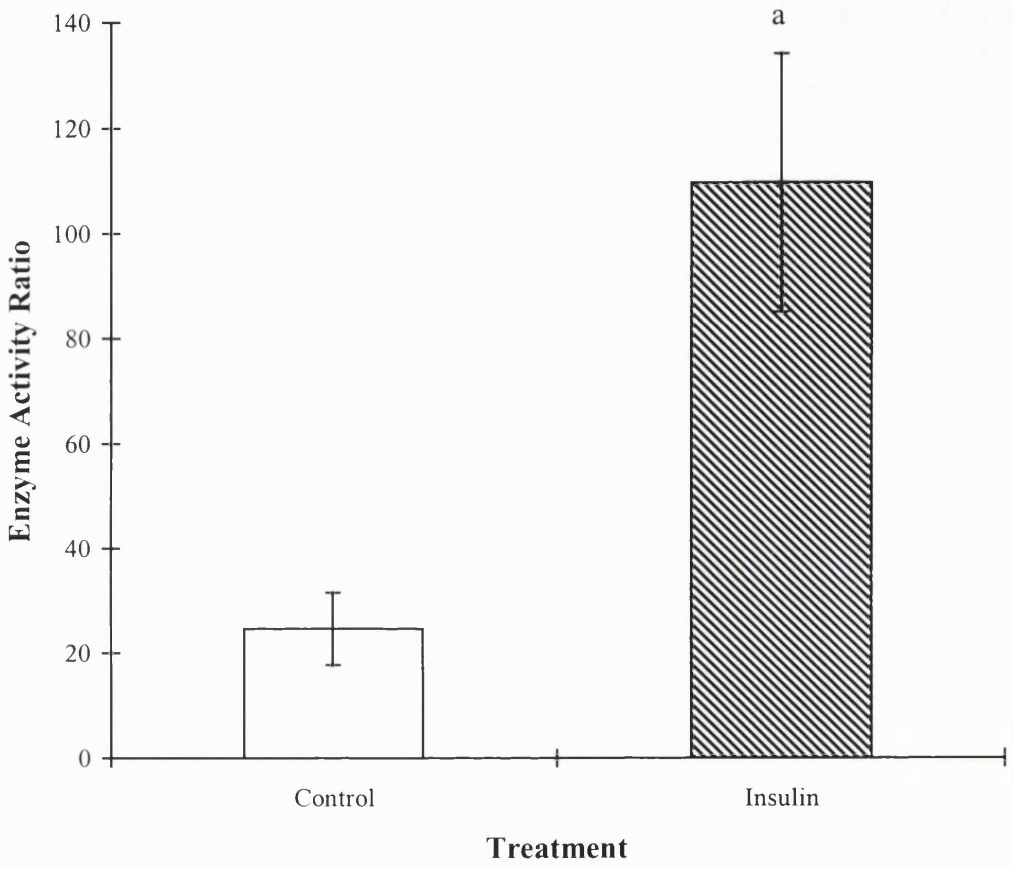
Mn^{2+} (Fonteles *et al.*, 1996). It would therefore seem reasonable to consider PDH as a possible target for the insulin enhancing effects of the apparently PI-containing malarial extracts.

At this stage in the project there were still problems isolating adipocytes which were responsive to insulin (see section 3.1.3). Consequently it was decided to carry out these experiments using fat pieces from rat epididymal fat pads rather than cells. To ensure that the assay was working properly and that insulin could indeed stimulate the enzyme, the activities of extracts prepared from fat pieces which had been incubated in the absence and presence of 100 nM insulin were measured. A high concentration of the hormone was used to ensure a maximal response. As can be seen from fig 3.15, PDH activity was stimulated approximately 4-fold in response to insulin. The activity of the enzyme is given as the ratio of the PDH to citrate synthase activity in the extracts x 1000 ie. the Enzyme Activity Ratio. Citrate synthase is a mitochondrial matrix marker enzyme which is unaffected by insulin.

Having established an insulin response, the effect of the malarial extracts on PDH activity was then examined, both in the absence and presence of 100 nM insulin. To determine if there was any difference between the effect of the *P. chabaudi* extracts and the *P. yoelii* extracts, preparations from each strain were assayed separately. In each case 100 µl of ME was added to the incubation medium of the test samples, while 100 µl of PBS was added to the controls.

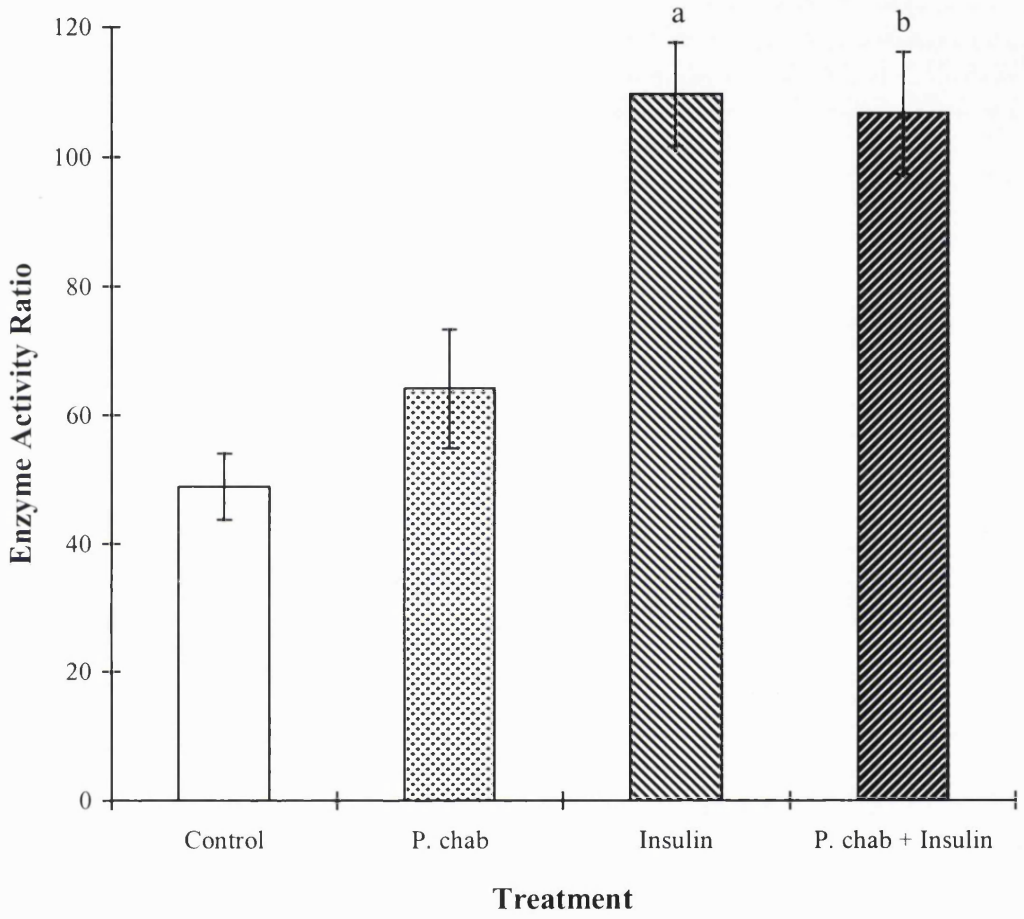
Two separate *P. chabaudi* extracts, extracts A and B from Table 3.5., were used to examine the effect of this strain on PDH and the results were pooled (fig. 3.16). The

Fig. 3.15 Effect of Insulin on PDH Activity



Shows the activity of PDH in extracts prepared from fat pieces which had been incubated in the absence and presence of 100 nM insulin. Results are given as the ratio of the PDH to citrate synthase activity x 1000 (Enzyme Activity Ratio) in the extracts. For the controls the mean PDH and citrate synthase activities were 0.11 and 4.47 nmol/hr/g wet weight of tissue respectively. Values are means $\pm$ SEM for three experiment. a indicates $p<0.05$ compared to control.

Fig. 3.16 Effect of *P. chabaudi* Malarial Extracts on PDH Activity



Shows the activity of pyruvate dehydrogenase in extracts from fat pieces incubated in 5 ml volumes in the absence and presence of 100 nM insulin, with and without 100 µl of MEs derived from *P. chabaudi*-infected blood. Results are given as the ratio of PDH to citrate synthase activity in the extracts. Values are means ± SEM for eleven experiments.

a indicates $P<0.0005$ versus control

b indicates $P<0.0005$ versus incubation with *P. chabaudi* MEs

P.chabaudi extracts alone stimulated PDH activity slightly, but this effect was not significantly different from the control. In the presence of insulin the extracts had no effect on PDH activity.

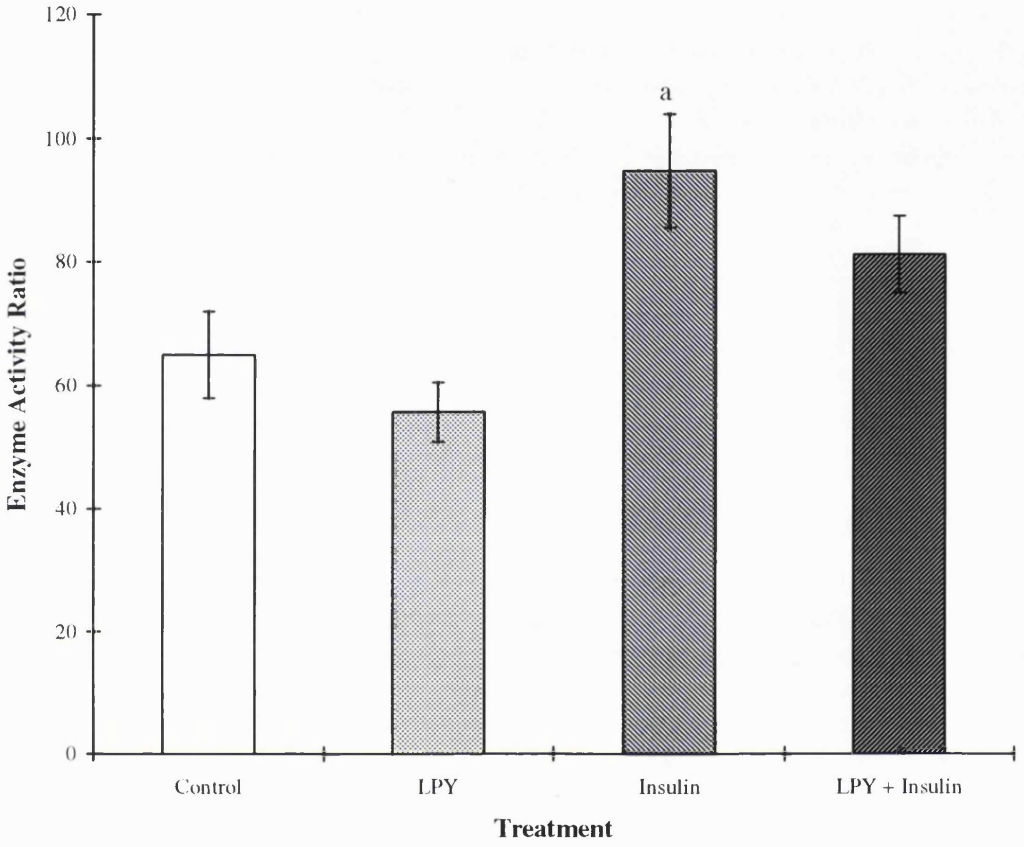
PDH activity was also assayed in the presence of two separate *P. yoelii* extracts (extracts J and K form table 3.5). As with the *P. chabaudi* extracts, the results were pooled (fig. 3.17). Both in the presence and absence of insulin, the *P. yoelii* extracts inhibited PDH activity slightly, however neither effect was significant.

Therefore it appeared that neither the *P. chabaudi* nor the *P. yoelii* derived extracts had any significant effect on PDH activity. To ensure that this was not due to the enzyme already being fully activated in the presence of insulin, control and insulin-treated fat piece extracts were incubated in the absence and presence of PDH phosphatase. As described in section 2.8.1.4 the PDH phosphatase was separated from a PDH/PDH phosphatase complex kindly donated by Prof R. Denton, Bristol and assayed for its ability to reactivate PDH which had previously been inactivated by PDH kinase. Figs. 3.18 and 3.19 show that in the absence of PDH phosphatase, insulin can only activate PDH to 75 $\pm$5% of its total activity measured in the presence of PDH phosphatase. Therefore, as insulin does not totally activate PDH these results do indicate that the MEs have no effect on the activity of this enzyme.

3.3.2 Effect on Acetyl-CoA Carboxylase

As the MEs had no effect on the ability of insulin to stimulate PDH, it seemed likely that they would have an effect on the activity of ACC as this is the only other enzyme involved in the regulation of fatty acid synthesis which has been shown to be activated

Fig. 3.17 Effect of *P. yoelii* Malarial Extracts on PDH Activity

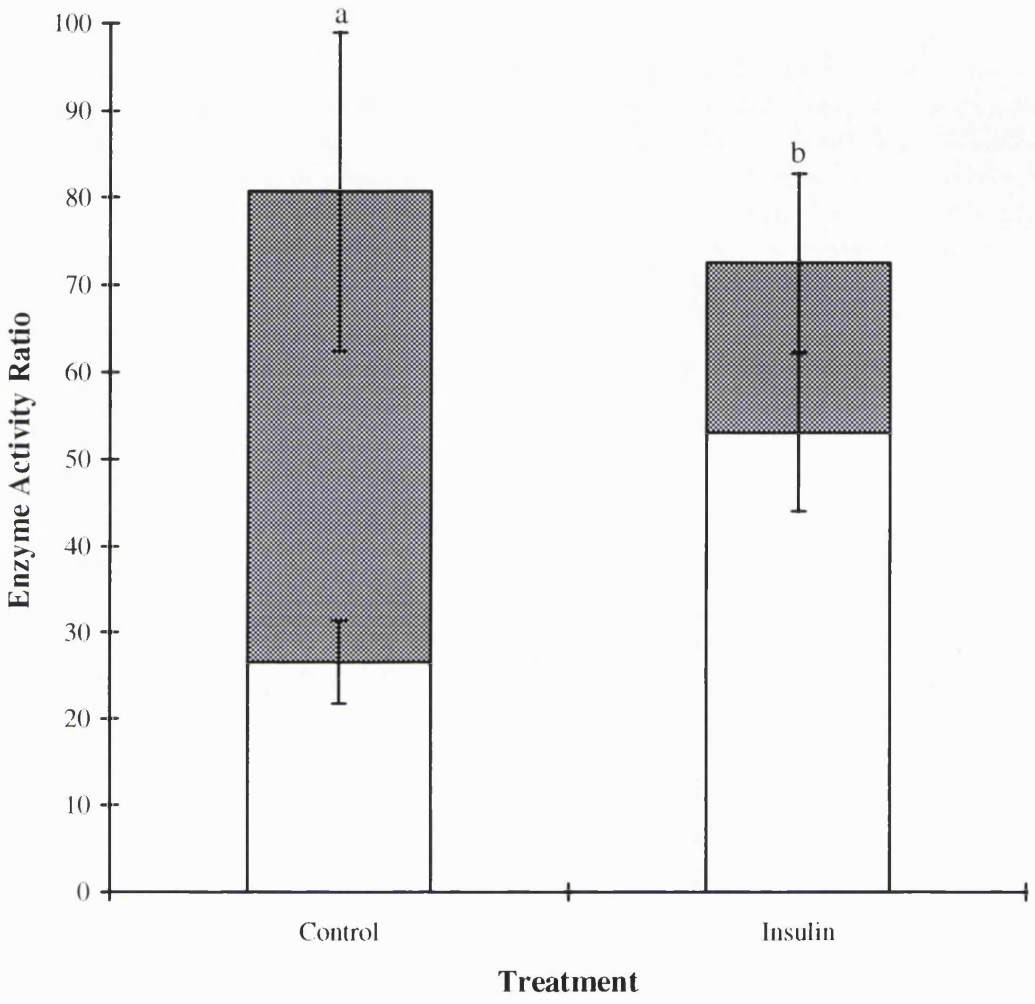


Shows the activity of PDH in extracts from fat pieces incubated in the absence and presence of 100 nM insulin, with and without MEs derived from *P. yoelii*-infected blood. Results are given as the ratio of PDH to citrate synthase activity. Values are means ± SEM for seven experiments.

a indicates $p<0.005$ versus the control

b indicates $p<0.005$ versus incubation with *P. yoelii* MEs

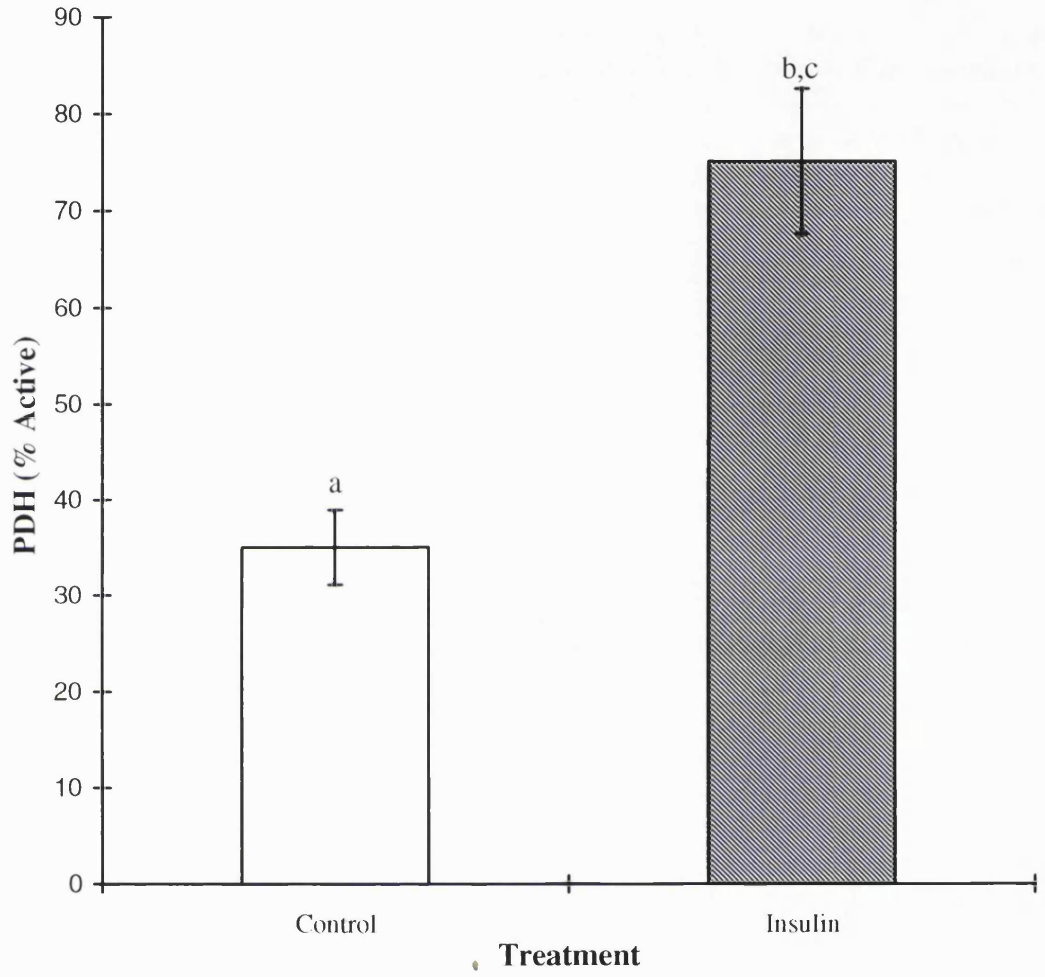
Fig. 3.18 Total PDH Activity



Shows the activity of PDH in the absence (open bars) and presence (open + hatched bars) of PDH phosphatase, with and without 100 nM insulin. Results are given as the ratio of PDH to citrate synthase activity in the extracts. Values are means ± SEM, where n = 8 for controls and n = 5 for insulin treated extracts.

a indicates $P<0.005$ versus PDH activity in the absence of PDH phosphatase
b indicates $P<0.05$ versus PDH activity in the absence of PDH phosphatase

Fig. 3.19 PDH as a Percentage of Total Activity



Shows PDH activity in extracts prepared from fat pieces which have been incubated in the absence and presence of 100 nM insulin. Results are given as a percentage of the total PDH activity in the extracts measured in presence of PDH phosphatase. Values are means ± SEM, for control n = 8 and in presence of insulin n = 5 .

a indicates P<0.0005 versus total PDH activity

b indicates P<0.025 versus total PDH activity

c indicates P<0.001 versus control

by insulin. This assay was again carried out on extracts derived from fat pieces which had been incubated in the absence and presence of 100 nM insulin. As can be seen from fig. 3.20 insulin stimulates ACC activity almost 2-fold. The activity of ACC is given as a percentage of the total activity of the enzyme which is found by pre-incubating the extracts with 20 mM citrate.

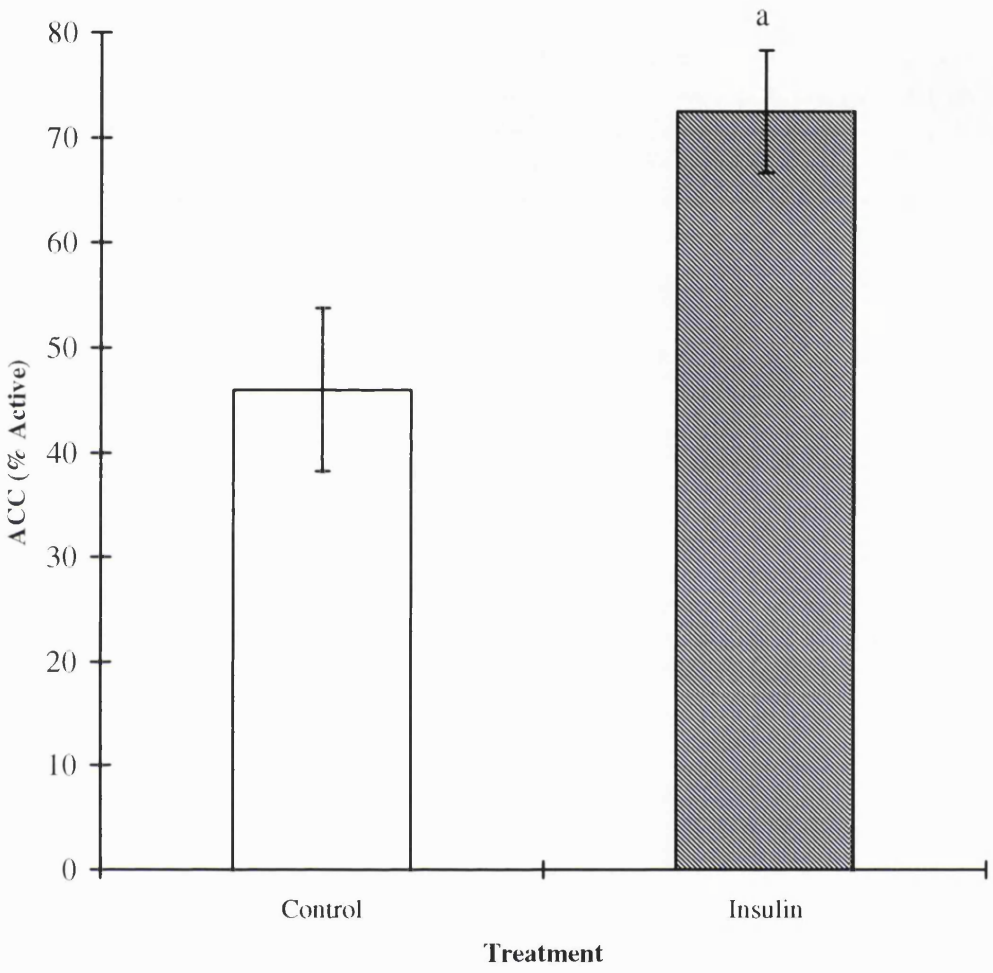
To test the effect of the MEs on this enzyme, 125 µl of ME was included in the incubation medium of the test samples, while 125 µl of PBS was added to the control incubations. As there had been no difference in the effect of the *P. chabaudi*-derived and *P. yoelii*-derived extracts on either lipogenesis or PDH, it appeared that they were working in a similar manner. Therefore for this and all the subsequent experiments the results for extracts derived from each strains were pooled. In this assay extracts C, L, M and N from table 3.5 were used to determine the effect on ACC activity

Fig. 3.21 shows that in the absence of insulin, the MEs significantly stimulated ACC activity. In the presence of insulin the MEs were also able to enhance ACC activity significantly. This effect was more than additive, giving a synergy factor of 1.43. Neither insulin, nor the MEs had any effect on the total activity of the enzyme (fig. 3.22)

3.3.3 Conclusions

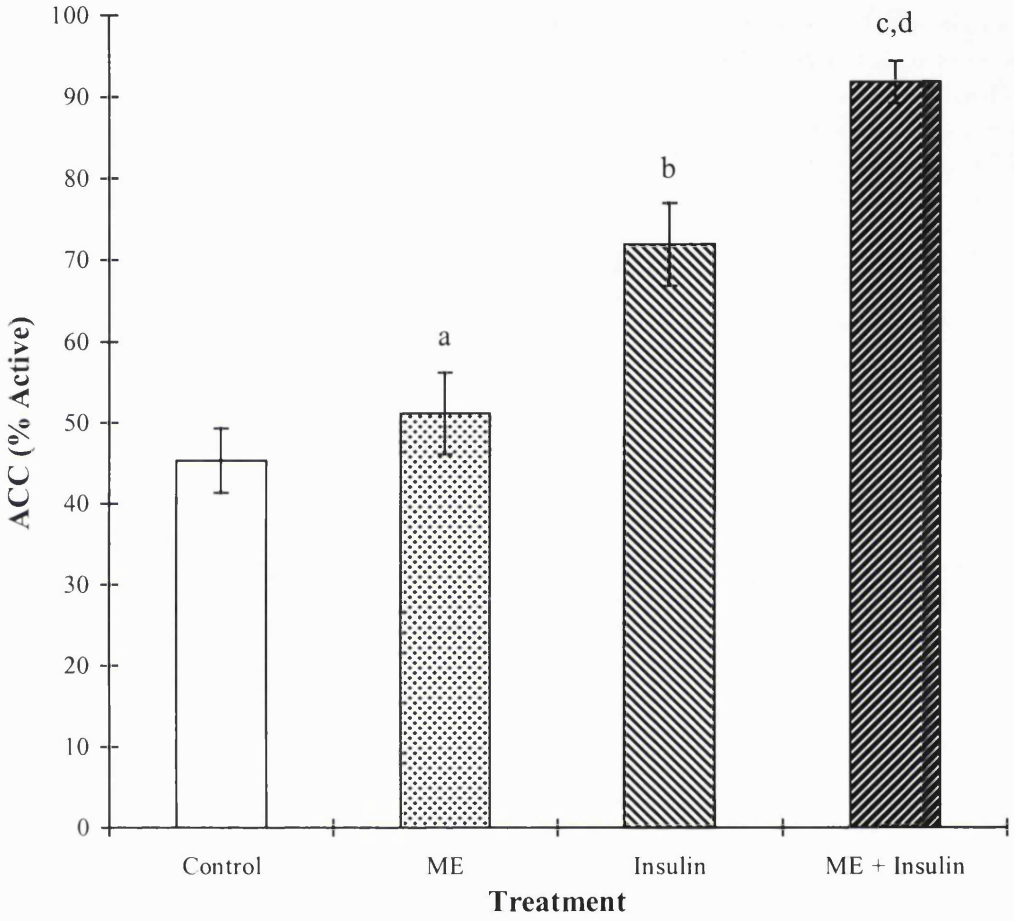
From these results it would appear that the MEs enhance insulin-stimulated lipogenesis by increasing the amount of ACC in its active form, providing more

Fig. 3.20 Effect of Insulin on Acetyl-CoA Carboxylase Activity



Shows the ACC activity in extracts derived from fat pieces which had been incubated in the absence and presence of 100 nM insulin. Results are a percentage of the total ACC activity in the extracts measured in the presence of 20 mM Citrate. Values are means ± SEM for three experiments. a indicates $P<0.025$ compared to control.

Fig. 3.21 Effect of Malarial Extracts on Acetyl-CoA carboxylase Activity



Shows the ACC activity in extracts prepared from fat pieces which had been incubated in 5 ml volumes in the absence and presence of 100 nM insulin, with and without 125 µl of various MEs. Results are given as a percentage of the total ACC activity in the extracts, measured in the presence of 20 mM citrate. Values are means ± SEM for seven experiments.

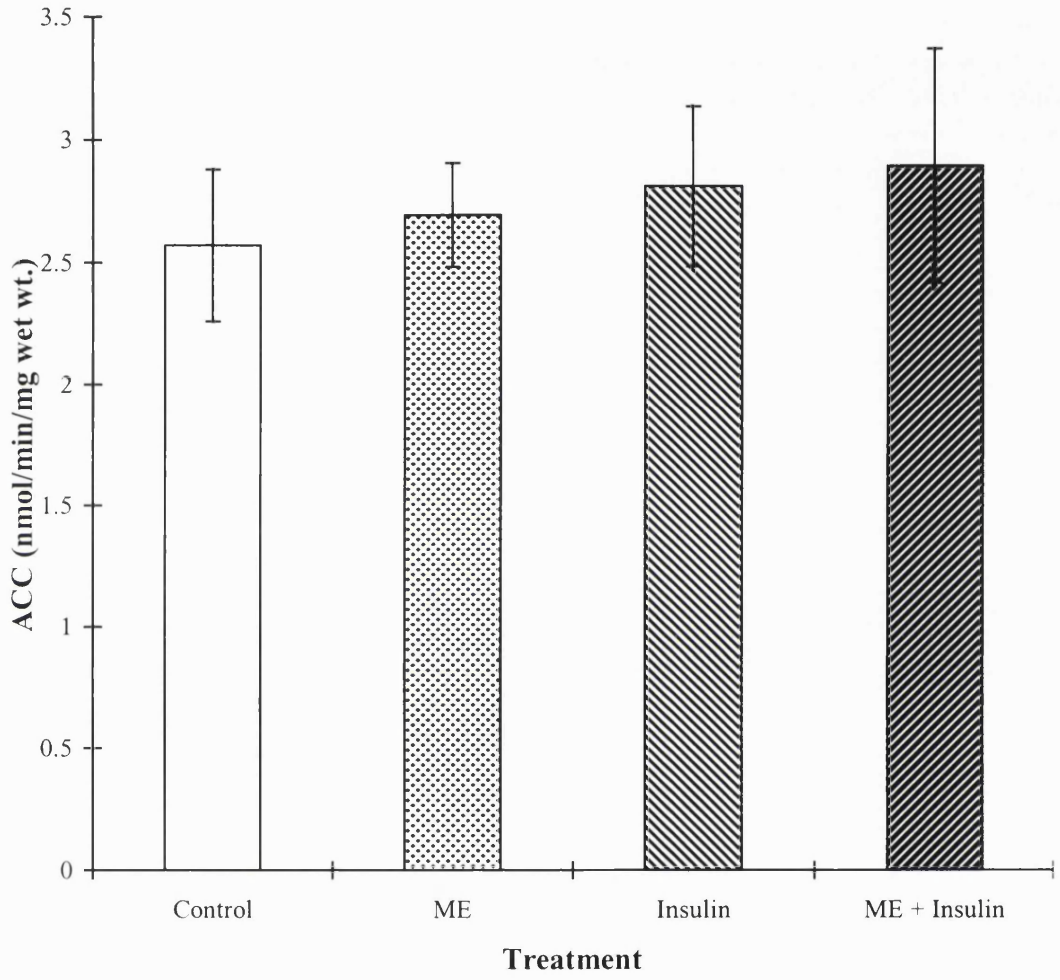
a indicates $P<0.05$ versus control

b indicates $P<0.005$ versus control.

c indicates $P<0.005$ versus incubation with MEs

d indicates $P<0.01$ versus incubation with insulin

Fig. 3.22 Total Acetyl-CoA Carboxylase Activity



Shows the total ACC activity in extracts derived from fat pieces which had been incubated in 5 ml volumes in the absence and presence of 100 nM insulin, with and without 125 µl of various MEs. Total ACC activity was determined by pre-incubating extracts with 20 mM citrate. Results are given as nmol/min/mg wet weight of tissue. Values are means ± SEM for seven experiments.

malonyl-CoA units for fatty acid synthesis. PDH does not appear to play a part in the enhancement of the insulin response by the MEs.

3.4 EFFECT OF MALARIAL EXTRACTS ON GLYCOGEN SYNTHESIS

Having found an effect of the MEs on ACC it was then necessary to decide where in the insulin signalling pathways to look next. Ideally we would have liked to examine the effect of the MEs on the enzymes upstream of ACC. Unfortunately there is very little known about the pathway by which insulin stimulates ACC (see section 1.3.4.3.iii). Activation of ACC by insulin is known to be inhibited by wortmannin, suggesting that this enzyme is downstream of PI3-kinase. Insulin's stimulation of lipogenesis, glucose transport and glycogen synthesis and inhibition of lipolysis are also known to be inhibited by wortmannin suggesting that each of these effects are downstream of PI3-kinase. Although the MEs had been shown to enhance the effects of insulin on lipogenesis via ACC and lipolysis they were not able to enhance glucose transport. It therefore seemed unlikely that the MEs were exerting an effect on PI3-kinase, but it was possible that they were enhancing an element downstream of this enzyme. Consequently it was decided to examine the effects of the MEs on insulin-stimulated glycogen synthesis as far more information was available on the signalling pathways used by insulin to mediate this effect (see section 1.3.4.2), giving more chance of determining any upstream effector(s).

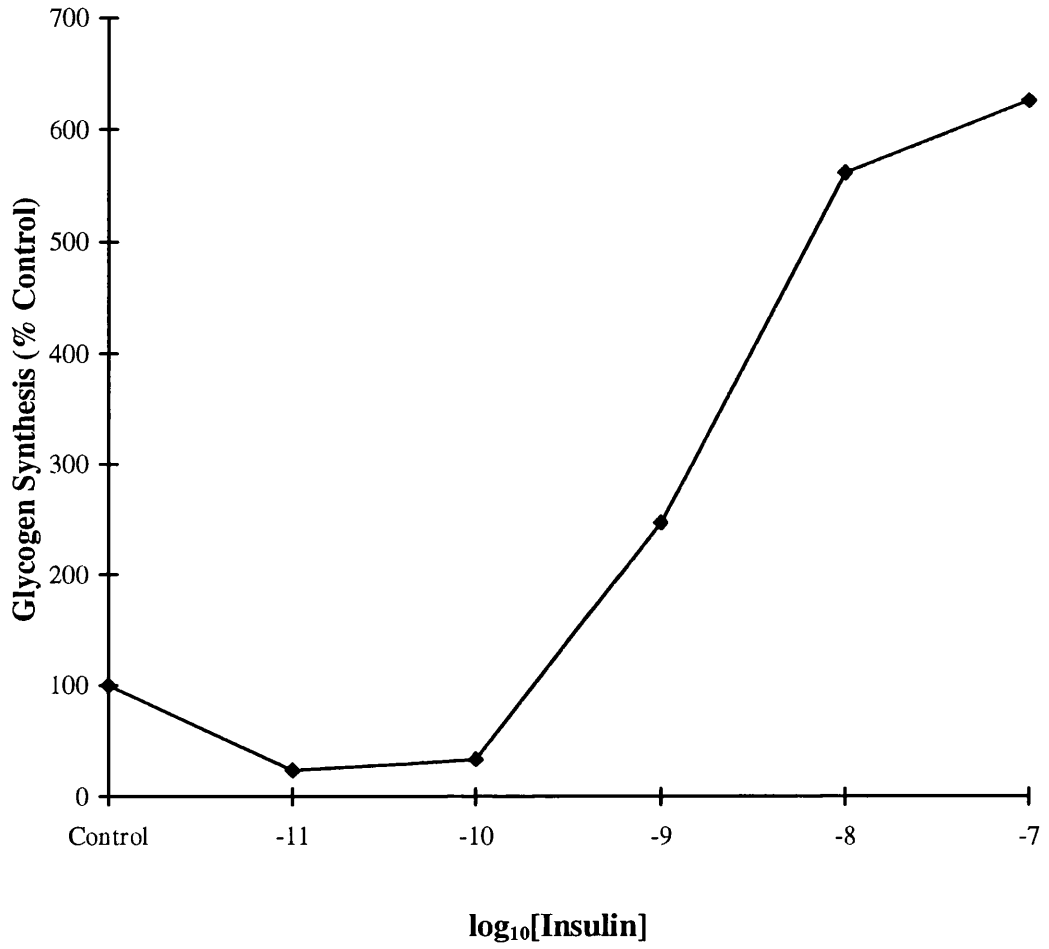
The assay for glycogen synthesis measures the incorporation of [U-^{14}C]glucose into glycogen, which is precipitated on filter paper and measured by scintillation counting. By this stage in the project the adipocyte isolation procedure had been rectified, therefore it was possible to use isolated adipocytes as the test system. As adipose tissue is not considered to be a particularly glycogenic tissue, an insulin dose response curve was established to ensure that glycogen synthesis could be stimulated by a

reasonable extent. From fig 3.23 it can be seen that at the highest insulin concentrations used i.e. 10-100 nM, glycogen synthesis could be stimulated approx. 6-fold in adipocytes.

To examine the effects of the MEs on glycogen synthesis, 15 µl of ME was added to the incubation medium of the test samples and 15 µl of PBS was added to the controls. This volume of ME was larger per ml of incubation medium that that used for the lipogenesis assays as the concentration of adipocytes used in this assay was greater i.e. 2/5 fat pad per ml compared to 1/5 fat pad per ml for the lipogenesis assay. The volume of ME used in the glycogen synthesis assay was therefore adjusted accordingly. As with the lipogenesis assays the MEs were assayed in the absence and presence of 10 nM insulin, a concentration which had been shown to be near-maximally effective (fig. 3.23) Fig 3.24 shows the pooled results from the assay of 10 separate ME preparations, derived from both *P. yoelii* and *P. chabaudi* infected mice, namely extracts C, D, E, F, K, L, M, N, O and P from table 3.5. The results are given as a percentage of the [U-^{14}C]glucose incorporated into glycogen in the extracts prepared from adipocytes which had been incubated with insulin alone. In the absence of insulin, the MEs had no effect on glycogen synthesis. In the presence of insulin they markedly enhanced the incorporation of glucose into glycogen, almost doubling the insulin effect. The synergy factor in this case was 1.64.

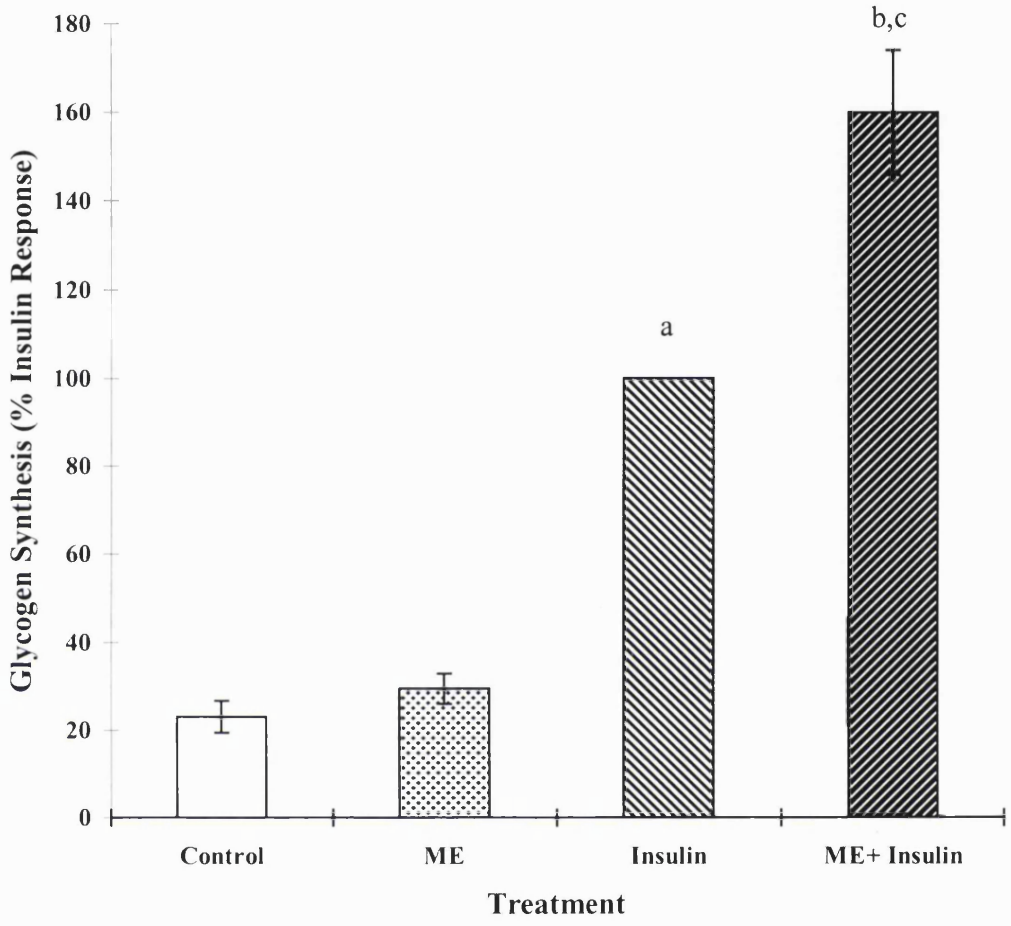
Therefore as well as being able to synergise with insulin to enhance lipogenesis, the MEs were also able to synergise with insulin to enhance glycogen synthesis. As insulin stimulates glycogen synthesis by increasing the activity of the enzyme glycogen synthase, the effect of the MEs on this enzyme was examined next.

Fig. 3.23 Glycogen Synthesis Insulin Dose Response Curve



Shows the incorporation of [^{14}C]glucose into glycogen in isolated adipocytes incubated with the stated concentrations of insulin. Results are given as a percentage of the [^{14}C]glucose incorporated into glycogen in the presence of medium only. n = 1.

Fig. 3.24 Effect of Malarial Extracts on Glycogen Synthesis



Shows the amount of [^{14}C]glucose incorporated into glycogen in adipocytes incubated the absence and presence of 10 nM insulin, with and without 15 µl of various MEs. Results are given as a percentage of the radiolabelled glucose incorporated into glycogen in the presence of insulin alone. The rate of incorporation for the control was 0.0595 + 0.0021 µmol/hr/g dry weight of cells. Values are means ± SEM for the assay of eighteen MEs using nine separate adipocyte preparations. Thus for control and insulin n = 9, and for ME and ME + insulin n = 18.

a indicates $P<0.0005$ versus control.

b indicates $P<0.0005$ versus incubation with MEs

c indicates $P<0.001$ versus incubation with insulin

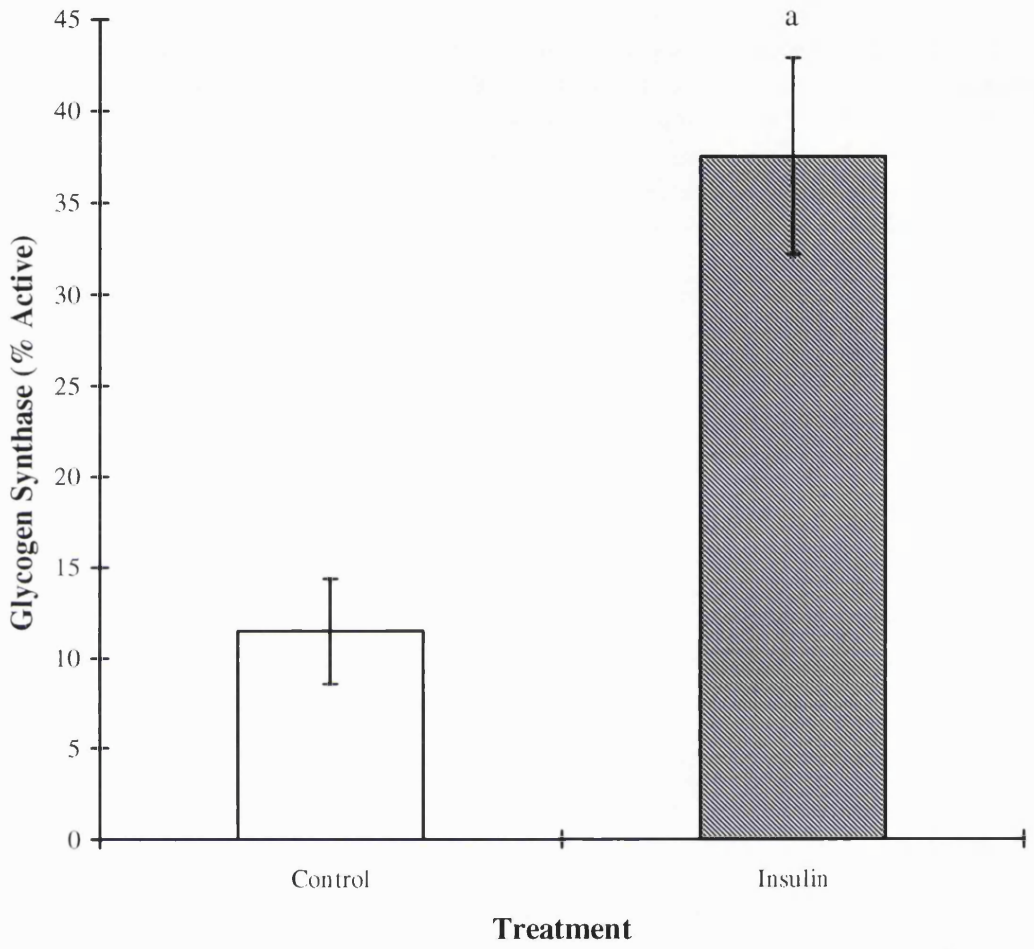
3.4.1 Effect on Glycogen Synthase

Glycogen synthase activity was assayed by measuring the incorporation of UDP[^{14}C]glucose into glycogen. As with the glycogen synthesis assay, isolated adipocytes were used as the test system. Under these conditions, 100 nM insulin could stimulate glycogen synthase activity approx. 3-fold (fig 3.25). Results are given as percentage of the total glycogen synthase activity in the extracts, which is measured in the presence of the allosteric activator G6P. To determine the effect of the MEs on glycogen synthase activity in the absence and presence of 100 nM insulin, 300 µl of ME was added to the incubation medium of the test samples while 300 µl of PBS was added to the controls. The effect of extracts G, H, I, O, P and Q from table 3.5 were tested and the results pooled. While the MEs had no significant insulin mimetic effect, they were able to significantly enhance insulin stimulated glycogen synthase activity (fig. 3.26). This effect had a synergy factor of 1.35. No effect was seen on the total activity of the enzyme under any of the conditions used (fig. 3.27).

These results suggested that the MEs enhance insulin-stimulated glycogen synthesis by increasing the amount of glycogen synthase in its active form. They did not however show whether the increase in glycogen synthase activity was due to direct enhancement of insulin's stimulation of this enzyme, or of an enzyme upstream of glycogen synthase. The fact that the activation of ACC by insulin was also enhanced by the MEs suggested that the they may be acting on a upstream element in the insulin signalling pathways common to both glycogen synthase and ACC.

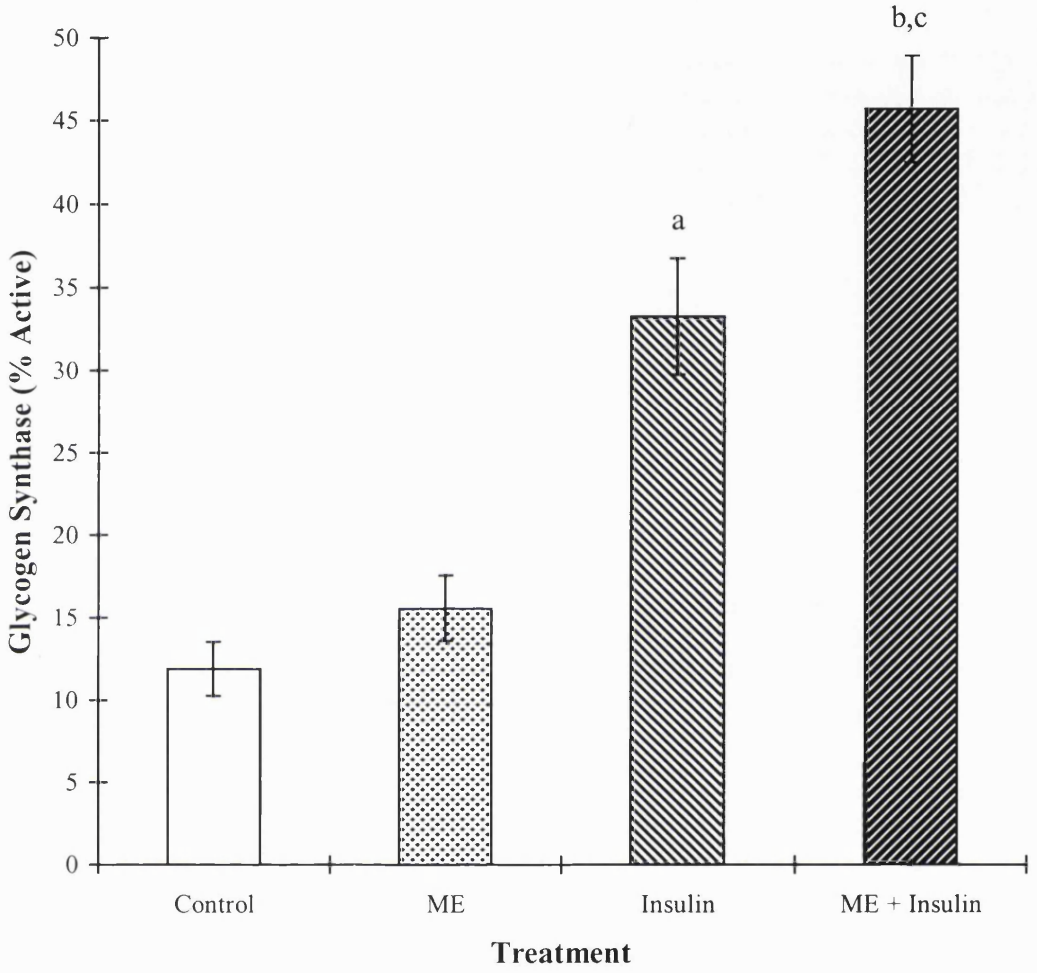
Insulin stimulates glycogen synthase by dephosphorylating the enzyme on certain residues. The phosphorylation of these residues being controlled by the relative

Fig. 3.25 Effect of Insulin on Glycogen Synthase Activity



Shows the glycogen synthase activity in extracts prepared from isolated adipocytes which had been incubated in the absence and presence of 100 nM insulin. Results are given as a percentage of the total glycogen synthase activity in the extracts, determined by including 10 mM G6P in the assay buffer. Values are means ± SEM for three experiments. a indicates $P<0.01$ compared to control.

Fig. 3.26 Effect of Malarial Extracts on Glycogen Synthase Activity



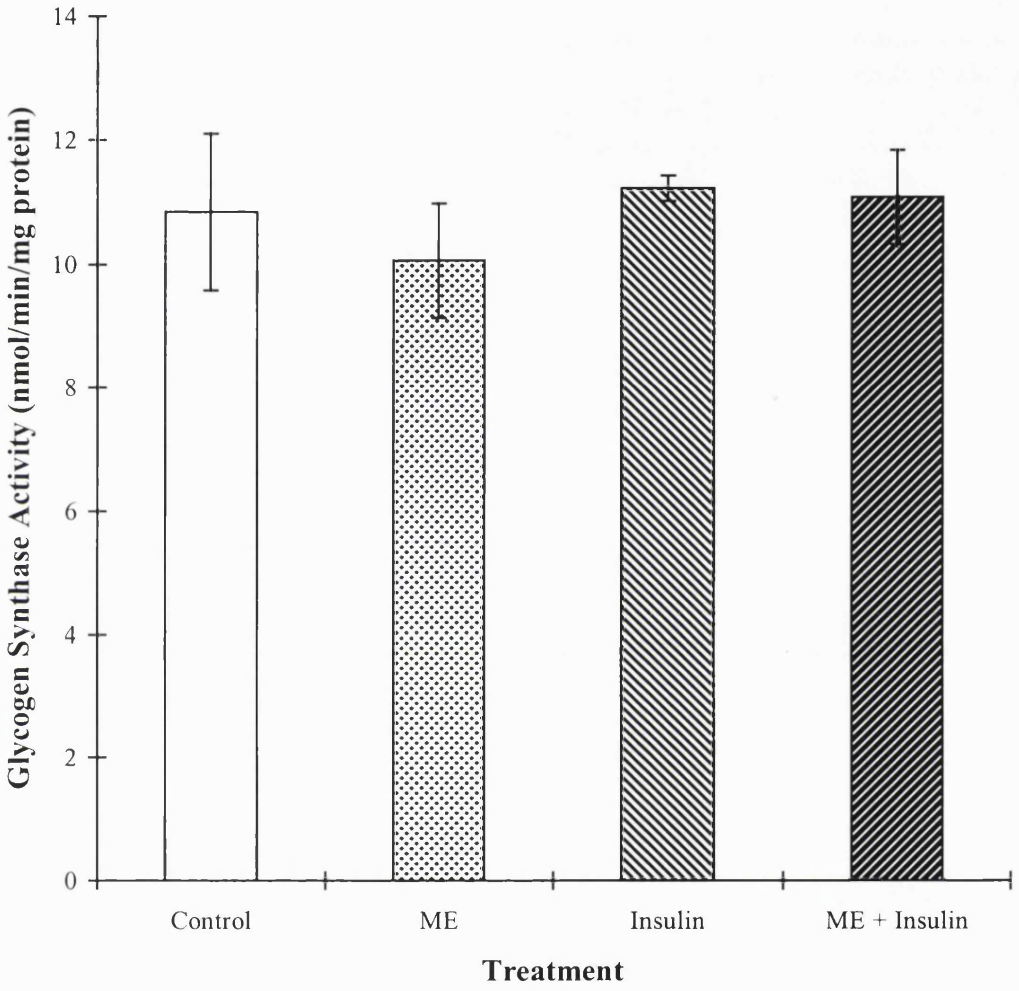
Shows the glycogen synthase activity in extracts prepared from adipocytes which had been incubated in 5 ml volumes in the absence and presence of 100 nM insulin, with and without 300 µl of various MEs. Results are given as a percentage of the total glycogen synthase activity in the extracts. Values are means ± SEM for the assay of nine MEs using six separate adipocyte preparations, thus for control and insulin n = 6, and for ME and ME + insulin n = 9.

a indicates $P<0.0005$ versus control

b indicates $p<0.0005$ versus incubation with MEs

c indicates $P<0.0005$ versus incubation with insulin

Fig. 3.27 Total Glycogen Synthase Activity



Shows the total glycogen synthase activity in extracts derived from isolated adipocytes which had been incubated in 5 ml volumes in the absence and presence of 100 nM insulin, with and without 300 ml of various MEs. Total activity was determined by including 10 mM G6P in the assay buffer. Results are given as nmol/min/mg protein in the extract. Values are means $\pm$ SEM for the assay of eleven MEs using six separate adipocyte preparations. Thus for control and insulin n = 6, and for ME and ME + insulin n = 11.

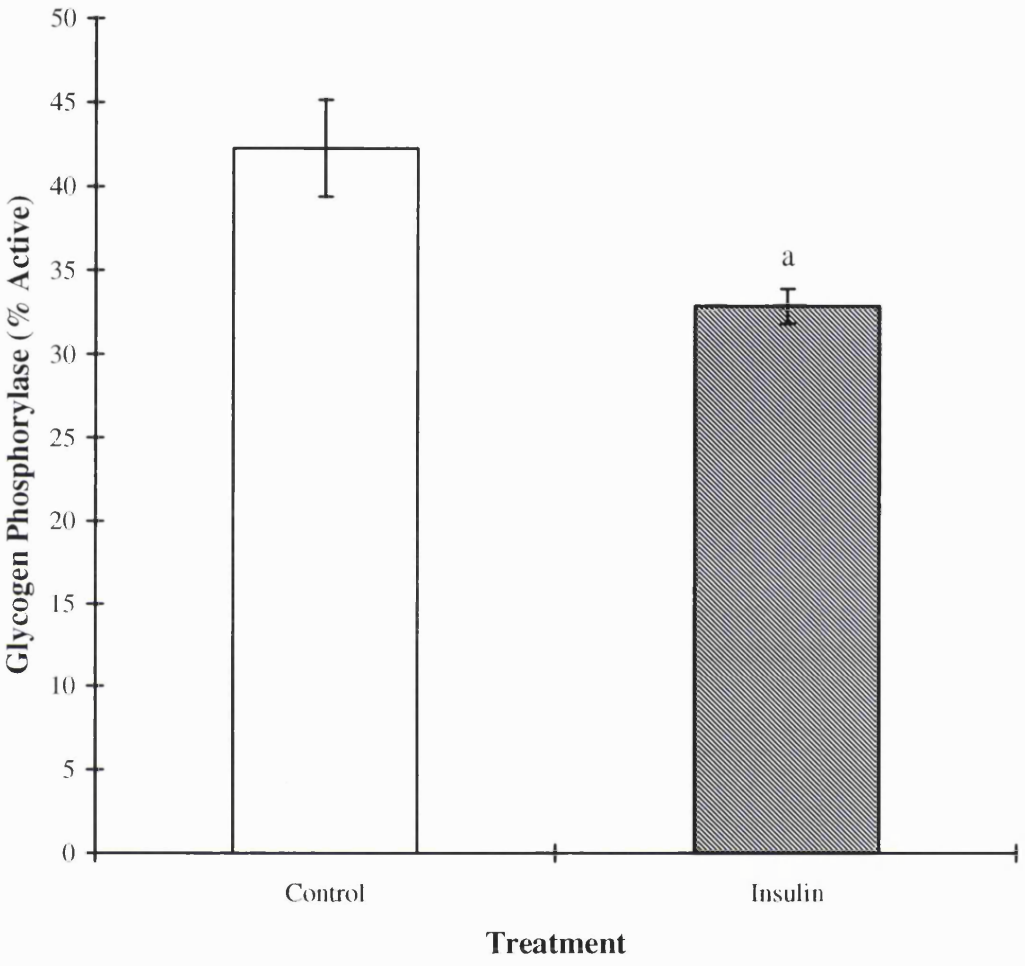
activities of the enzymes GSK-3 and PP-1G. The activation of these enzymes by insulin appears to be mediated via two different pathways i.e. the MAP kinase pathway and the PI3-kinase pathway (see section 1.3.4.2.iv). Therefore it was necessary to determine if either or indeed both of these pathways could be influenced by the MEs.

3.4.2 Effect on Glycogen Phosphorylase

As well as dephosphorylating and activating glycogen synthase, PP-1G can also dephosphorylate and inactivate phosphorylase b kinase, the enzyme which regulates the activation of glycogen phosphorylase (see section 1.3.4.2.ii). Therefore if the MEs were enhancing insulin-stimulated glycogen synthase activity by influencing the activity of PP-1G, they would presumably also enhance insulin's inhibition of glycogen phosphorylase. To test this theory the effects of MEs on glycogen phosphorylase activity were examined.

Although this enzyme actually catalyses the breakdown of glycogen, under the conditions of the assay the reaction was reversed resulting in the production of glycogen. The activity of the enzyme was determined from the amount [^{14}C]glycosyl moiety derived from G1P incorporated into glycogen. As with the glycogen synthase assay isolated adipocytes were used as the test system. In the presence of 100 nM insulin the activity of the enzyme decreased from approx. 40% to approx. 30% of the total glycogen phosphorylase activity in the extracts (fig. 3.28). The total enzyme activity in the extracts was determined by including the allosteric activator AMP in the assay buffer. To determine the effect of MEs on glycogen phosphorylase in the absence and presence of 100 nM insulin, 300 µl of the preparations were added to the

Fig. 3.28 Effect of Insulin on Glycogen Phosphorylase Activity



Shows the activity of glycogen phosphorylase in extracts prepared from adipocytes incubated in the absence and presence of 100 nM insulin. Results are given as a percentage of the total glycogen phosphorylase activity in the extracts determined by adding 2 mM AMP to the assay buffer. Values are the means ± SEM for three experiments. a indicates $p<0.05$ compared to control.

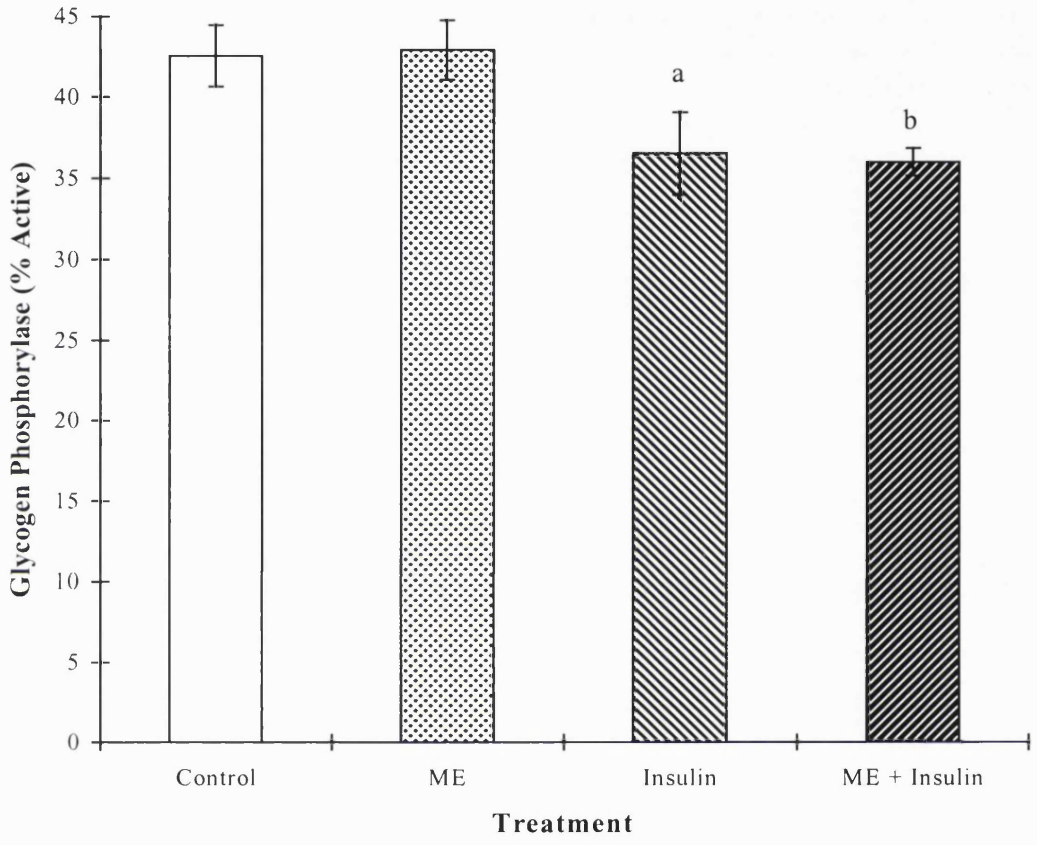
incubation medium of the test samples while 300 µl of PBS was added to the controls. Exactly the same MEs were used as were used for the glycogen synthase assays, i.e. extracts G, H, I, O, P and Q from table 3.5. As with the other assays the results for each extract were pooled. Fig. 3.29 shows that the MEs had no effect on glycogen phosphorylase activity either in the absence or presence of insulin. There was also no significant effect on the total activity of the enzyme under any of the conditions used (fig. 3.20).

These results therefore suggest that the MEs do not influence glycogen synthase activation via the activation of PP-1G. As PP-1G activation by insulin is thought to be mediated via a MAP kinase pathway (see section 1.3.4.2.iv) this also suggests that the MEs do not influence the MAP kinase pathway.

3.4.3 Conclusions

These results show that as well as being able to enhance the ability of insulin to stimulate lipogenesis and inhibit lipolysis, the MEs can also enhance insulin-stimulated glycogen synthesis. This effect appears to due to an increase in the amount of glycogen synthase in its active form. The lack of effect of the MEs on glycogen phosphorylase suggests that this increase in glycogen synthase activity is not the result of an increase in PP-1G activity and therefore is presumably mediated via GSK-3.

Fig. 3.29 Effect of Malarial Extracts on Glycogen Phosphorylase Activity

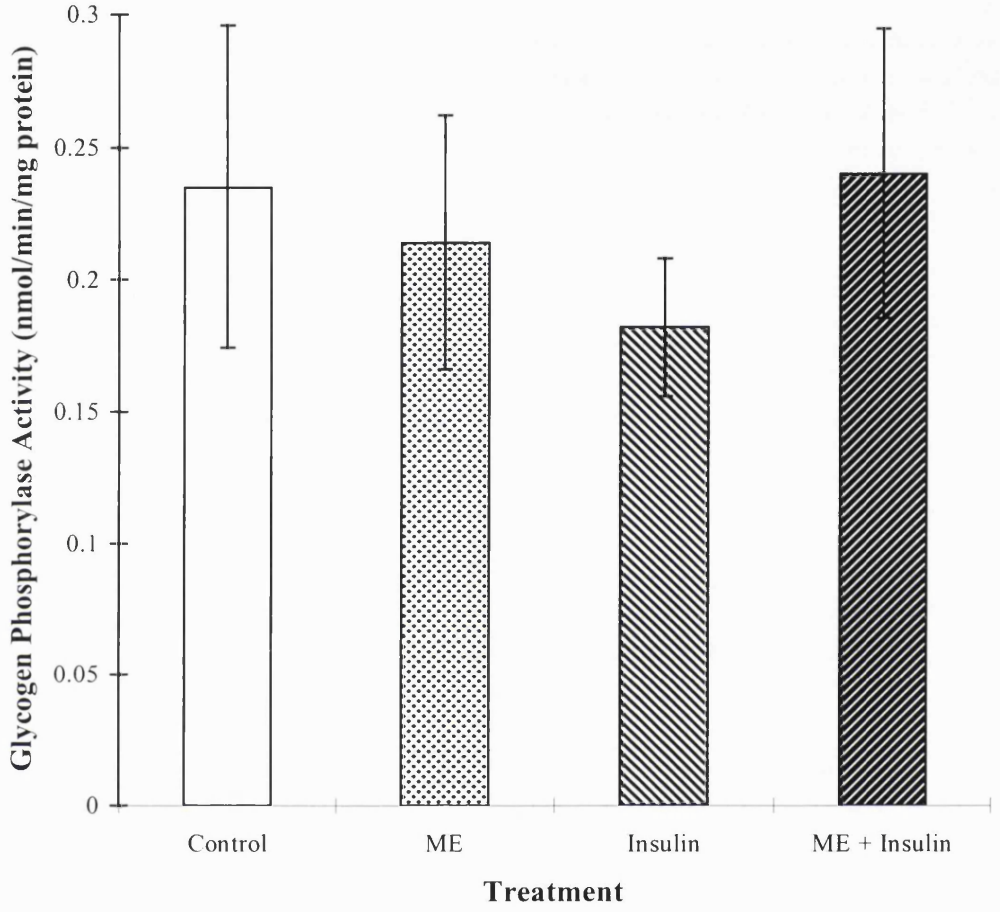


Shows the activity of glycogen phosphorylase in extracts prepared from adipocytes which had been incubated in 5 ml volumes in the absence and presence of 100 nM insulin, with and without 300 µl of various MEs. Activity is given as a percentage of the total glycogen phosphorylase activity in the extracts measured in the presence of AMP. Values are the means ± SEM for the assay of nine MEs using five separate adipocyte preparations. Thus for control and insulin n = 5, and for ME and ME = insulin n = 9.

a indicates $P<0.05$ versus control

b indicates $P<0.005$ versus incubation with MEs

Fig. 3.30 Total Glycogen Phosphorylase Activity



Shows the total glycogen phosphorylase activity in extracts prepared from adipocytes which had been incubated in 5 ml volumes in the absence and presence of 100 nM insulin, with and without 300 µl of various MEs. The total glycogen phosphorylase activity in the extracts was determined by including 2 mM AMP in the assay buffer. Activity is given as nmol/min/mg of protein in the extracts. Values are means $\pm$ SEM for the assay of nine MEs using five separate adipocyte preparations. Thus for control and insulin n = 5, and for ME and ME + insulin n = 9.

3.5 EFFECT OF MALARIAL EXTRACTS ON PI3-KINASE

Although initially PI3-kinase was not thought to be involved in the enhancement of insulin signalling by the MEs due to their inability to enhance glucose transport, all the other evidence which had been collected suggested that this enzyme may be involved after all. The glucose transport assays were in fact carried out at maximal insulin concentrations (100 nM) therefore it is possible that the system was fully activated in the presence of insulin and this was the reason no effect was seen when the MEs were present. The increases in the active forms of both ACC and glycogen synthase in response to the MEs, both of which are inhibited by wortmannin, and the lack of effect of the MEs on PDH and glycogen phosphorylase which are not inhibited by wortmannin pointed to a PI3-kinase mediated pathway being involved. Consequently rather than spending time looking at the effect of the MEs on each of the known points on the PI3-kinase pathway it was decided to look at their effect on the activity of PI3-kinase directly.

As PI3 kinase is activated upon association of the p85 subunit with tyrosine phosphorylated IRS-1, the activity of the enzyme was measured in anti-phosphotyrosine (αPY) immunoprecipitated extracts from adipocyte cell lysates. The PI3-kinase activity in the extracts was determined by measuring the incorporation of radiolabelled phosphate from γ-[^{33}P]-ATP into lipid using phosphatidylinositol as a substrate. The lipid products were extracted and run on a t.l.c. plate to separate them and the amount of PI-3-P product was quantitated using a phospho-imager. To ensure insulin would stimulate the enzyme in this system, cells were first of all incubated in the absence and presence of 100 nM insulin. The results are given as a percentage of

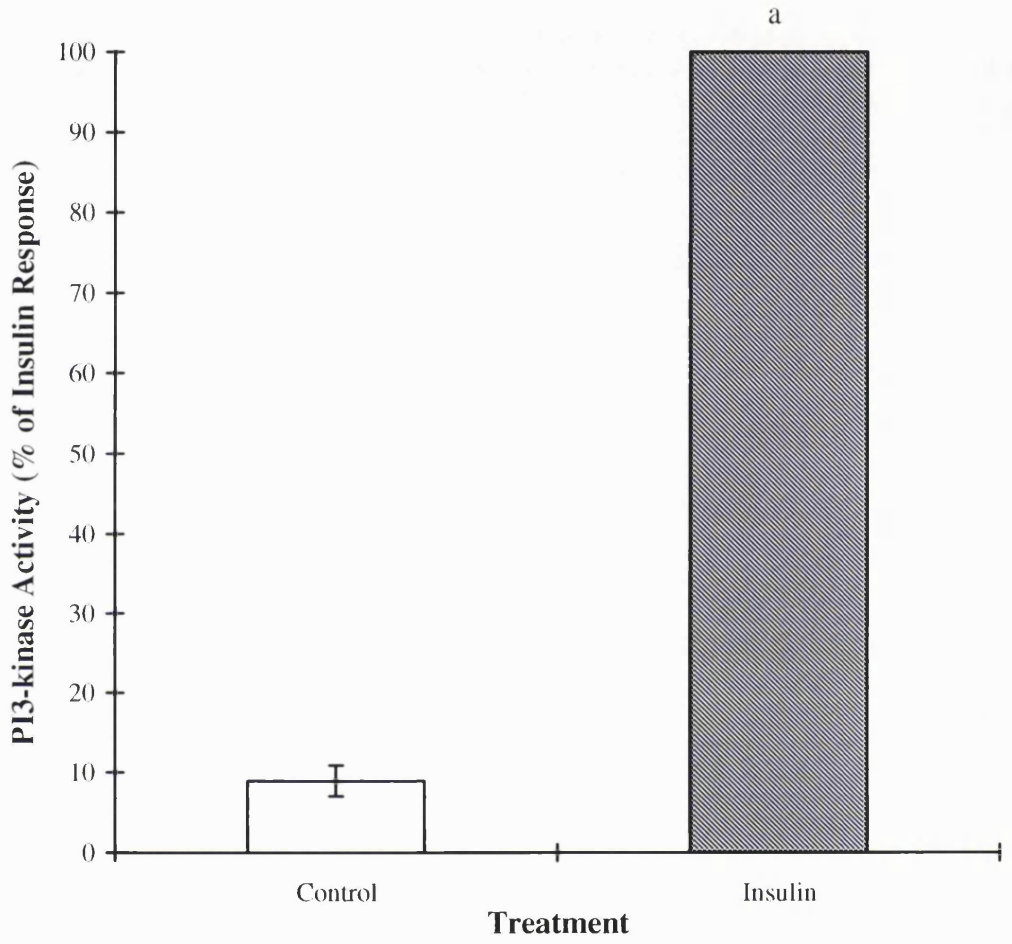
the activity in the extracts treated with insulin alone. As can be seen from figure 3.31 insulin was able to stimulate PI3-kinase activity approx. 10-fold above basal levels. This effect could clearly be seen on the phospho-image pictures (fig 3.32).

To determine what effect the MEs would have on the activity of this enzyme, adipocytes were incubated in the absence and presence of insulin, with and without 300 µl of ME preparations. The MEs used in this experiment were I, O, R and S from table 3.5. On their own the MEs did not have any effect on PI3-kinase activity, but in the presence of insulin the MEs increased the activity of the enzyme from approx. 10-fold above basal level to approx. 17-fold, almost doubling the insulin response (fig. 3.33). The synergy factor for this effect was 1.75. Fig. 3.34 shows the phospho-image picture from a t.l.c on which the lipid products of two separate PI3-kinase assays had been run along with those from a recominant PI3-kinase standard.

3.5.1 Conclusions

From these and the previous results it would appear that the malarial extracts are potentiating insulin's stimulation of lipogenesis and glycogen synthesis and inhibition of lipolysis by enhancing the activity of PI3-kinase and enzymes downstream of this component in the insulin signalling pathway.

Fig. 3.31 Effect of Insulin on PI3-kinase Activity

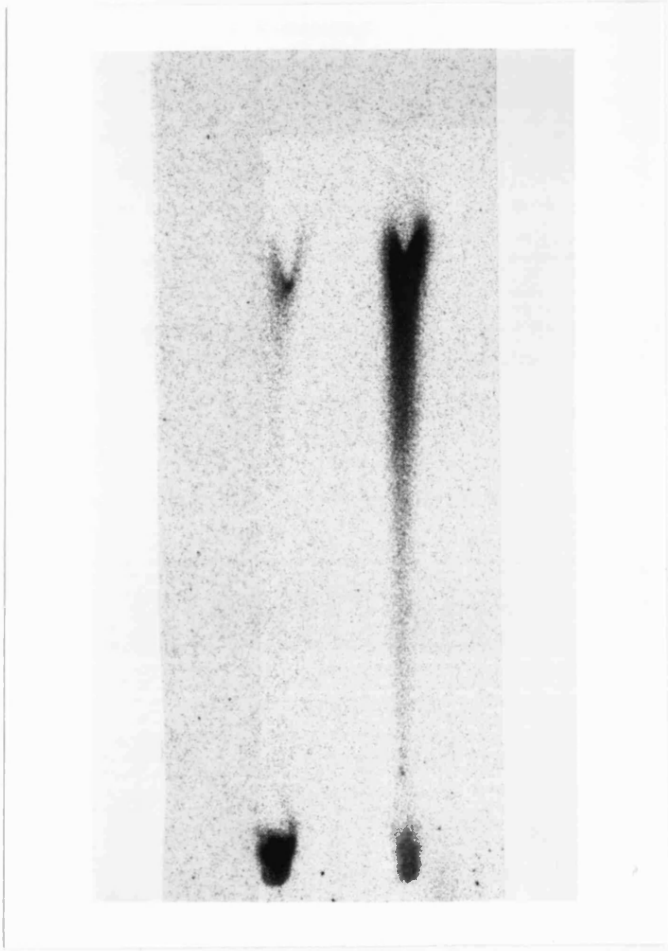


Shows the PI3-kinase activity in anti-phosphotyrosine immunoprecipitable extracts from adipocytes which had been incubated in the absence and presence of 100 nM insulin. Results are given as a percentage of the PI3-kinase activity in the extracts prepared from adipocytes which had been incubated with insulin alone. Values are means ± SEM for three experiments. a indicates $P<0.0005$ when compared to control.

Fig. 3.32 PI3-kinase Phospho-image in the Absence and Presence of Insulin

Lane 1 - Control

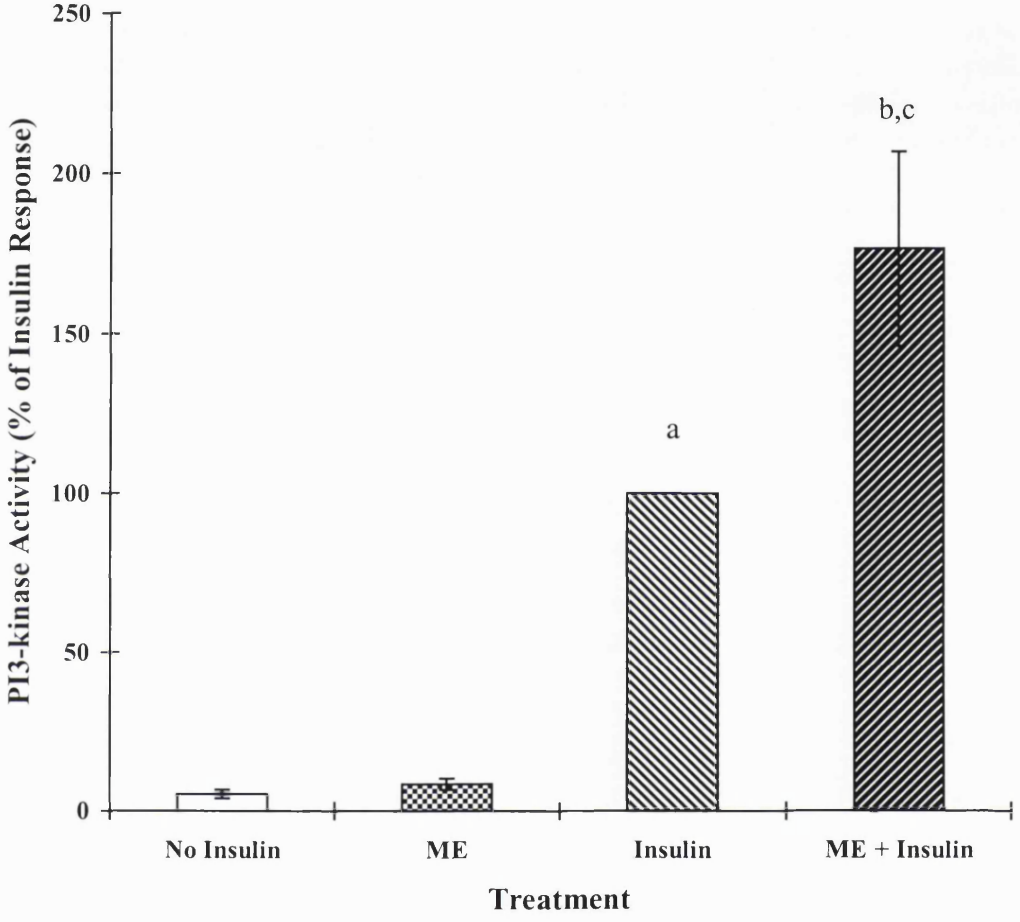
Lane 2 - Insulin



Lane no. **1** **2**

Shows the PI-3-P produced following a PI3-kinase assay on extracts from adipocytes which had been incubated in the absence and presence of insulin. The amount of radiolabelled ATP incorporated into the PI-3-P was determined by drawing equal size boxes round each PI-3-P spot and measuring the radioactivity within. A background measurement was also taken and subtracted from each value.

Fig. 3.33 Effect of Malarial Extracts on PI3-Kinase Activity



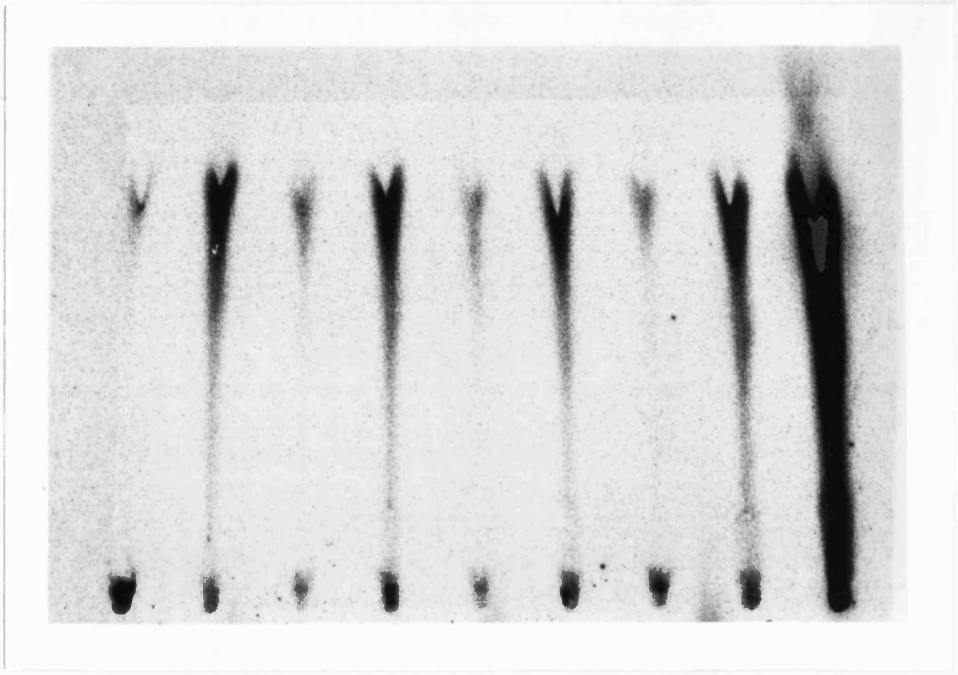
Shows the activity of PI3-kinase in anti-phosphotyrosine immunoprecipitable extracts from adipocytes which had been incubated in 5 ml volumes in the absence and presence of 100 nM insulin, with and without 300 µl of various MEs. The PI3-kinase activity is given as a percentage of the activity in the extracts prepared from adipocytes which had been incubated with insulin alone. Values are the means ± SEM for six experiments.

a indicates $P<0.0005$ versus control

b indicates $P<0.0005$ versus incubation with MEs

c indicates $P<0.05$ versus incubation with insulin

Fig. 3.34 PI3-Kinase Phospho-image in the Absence and Presence of Insulin and/or Malarial Extracts



Lane no.	1	2	3	4	5	6	7	8	9

Lane 1 - Control
Lane 2 - Insulin
Lane 3 - ME
Lane 4 - ME + Insulin
Lane 5 - Control
Lane 6 - Insulin
Lane 7 - ME
Lane 8 - ME + Insulin
Lane 9 - PI-3-P Standard

Shows the PI-3-P produced following two separate PI3-kinase assays, one on extracts derived from adipocytes which had been incubated in the absence and presence of insulin and/or malarial extract I (Lanes 1-4) and the other in the absence and presence of insulin and/or malarial extract S (Lanes 5-8). Lane 9 shows a PI-3-P standard.

3.6 EFFECT OF MALARIAL EXTRACTS ON EGF SIGNALLING

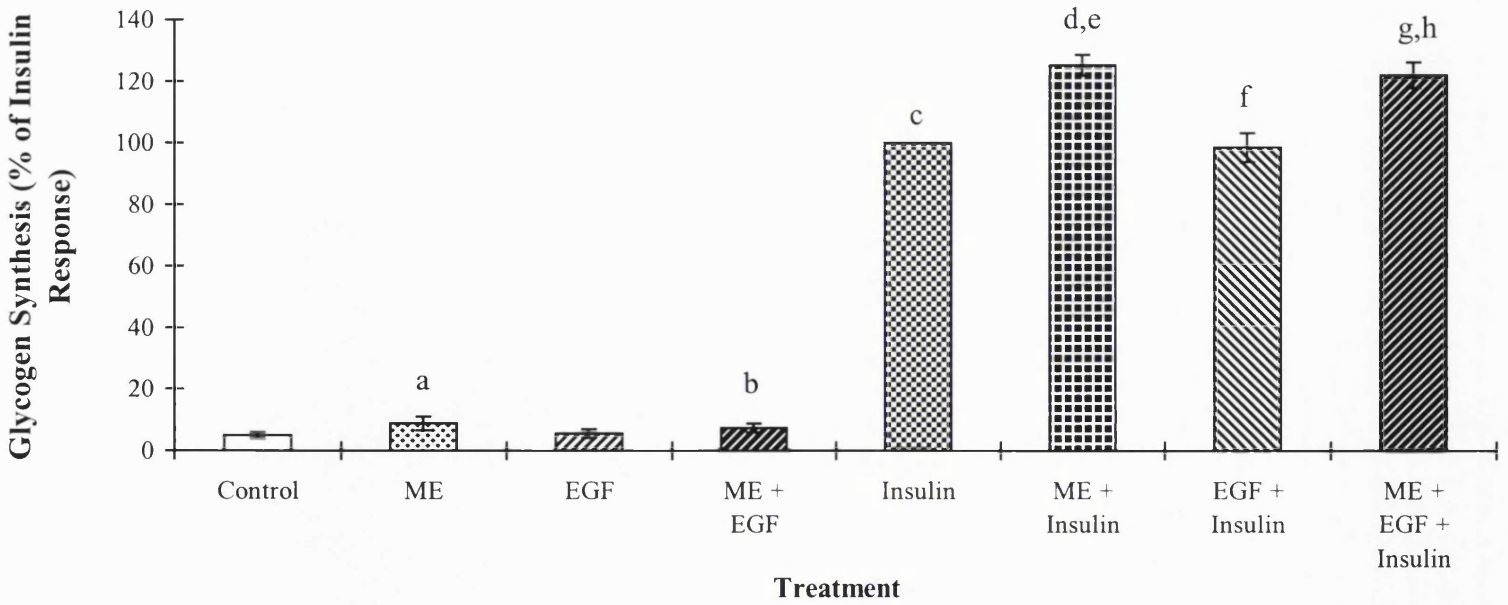
EGF was one of the first polypeptide growth factors to be identified. Like insulin, EGF exerts its effects by binding to specific receptors on the surface of cells. The EGF receptor, like the insulin receptor, has an intrinsic tyrosine kinase in its intracellular domain. Unlike the insulin receptor however the EGF receptor is only a single transmembrane glycoprotein. For the receptor tyrosine kinase to be activated ligand-induced dimerization of the receptors must take place, the idea being that binding of EGF causes a conformational change in the receptor which leads to increased affinity for neighbouring receptors. Dimerization of the receptors, as well as activating the tyrosine kinase, also allows intermolecular autophosphorylation of the receptor on tyrosine residues, similar to the autophosphorylation of the insulin receptor upon insulin binding. However rather than increasing the tyrosine kinase activity of the receptor towards intracellular substrates, as is the case with the insulin receptor, autophosphorylation of the EGF receptor creates specific binding sites for cytoplasmic proteins. The receptor binds to these proteins through their SH2 domains. The majority of the SH2-containing proteins which bind to the EGF receptor are the same as those which bind to IRS-1 upon tyrosine phosphorylation by the insulin receptor. These include Syp, Shc, Grb-2, Nck and in some cell types the p85 subunit of PI3 kinase. The EGF receptor can also bind rasGAP and PLCγ which IRS-1 has not been shown to bind. Binding of these signalling proteins by the activated receptor stimulates EGF-induced signal transduction cascades (for review see Boonstra *et al.*, 1995).

Association of the EGF receptor with Grb-2 allows this adapter molecule to bind to Sos and hence to activate the Ras/MAP kinase pathway using a mechanism similar to that used by insulin. EGF has been shown to stimulate MAP kinase to a similar or greater degree than insulin depending on cell type (Peak *et al.*, 1993; Robinson *et al.*, 1993; Fingar & Birnbaum, 1994a; Lin & Lawrence, 1994). Although as mentioned previously EGF has been shown to activate PI3-kinase in certain cells (Bjorge *et al.*, 1990; Conricode, 1995) there is no evidence to suggest that EGF activates this enzyme in rat adipocytes.

Despite the many apparent similarities between the signalling pathways of insulin and EGF, EGF does not stimulate metabolic responses such as glycogen synthesis and glucose transport in rat adipocytes (Lin & Lawrence, 1994) and only by one group has it been shown to stimulate lipogenesis in these cells (Haystead & Hardie, 1986). We therefore thought it would be interesting to see if the malarial extracts, which can enhance insulin's stimulation of various metabolic responses, could induce EGF to stimulate a metabolic response, namely glycogen synthesis, in rat adipocytes.

For this experiment adipocytes were incubated in the absence and presence of 100 nM EGF, with and without 15 µl of ME, the preparations used being H, O, Q and R from table 3.5. To ensure that the preparations were active, i.e. able to potentiate insulin's effect, the MEs were also assayed in the absence and presence of 10 nM insulin. The results are given as a percentage of the insulin response. As you can see from fig.3.35 EGF did not stimulate glycogen synthesis under the conditions used. The MEs did mimic the effect of insulin on glycogen synthesis in the presence of EGF,

Fig. 3.35 Effect of Malarial Extracts and EGF on Glycogen Synthesis



Shows the incorporation of [^{14}C]glucose into glycogen in extracts prepared from adipocytes incubated in the absence and presence of 100 nM EGF, with and without 15 µl of various MEs and/or 10 nM insulin. Results are given as a percentage of the [^{14}C]glucose incorporated into glycogen in extracts prepared from adipocytes incubated with 10 nM insulin alone. The rate of incorporation for the control was 0.0421 $\pm$ 0.012 µmol/hr/g dry weight of cells.Values are means $\pm$ SEM for the assay of 10 MEs using 6 separate adipocyte preparations.

a indicates P<0.025 versus control
b indicates P<0.025 versus incubation with EGF
c indicates P<0.0005 versus control
d indicates P<0.0005 versus incubation with MEs
e indicates P<0.0005 versus incubation with insulin
f indicates P<0.0005 versus incubation with EGF
g indicates P<0.0005 versus incubation with EGF and MEs
h indicates P< 0.005 versus incubation with EGF and Insulin

but only to the same degree as when no EGF was present. Further, EGF did not have any effect either on the stimulation of glycogen synthesis by insulin or on the enhancement of this effect by the MEs.

3.6.1 Conclusions

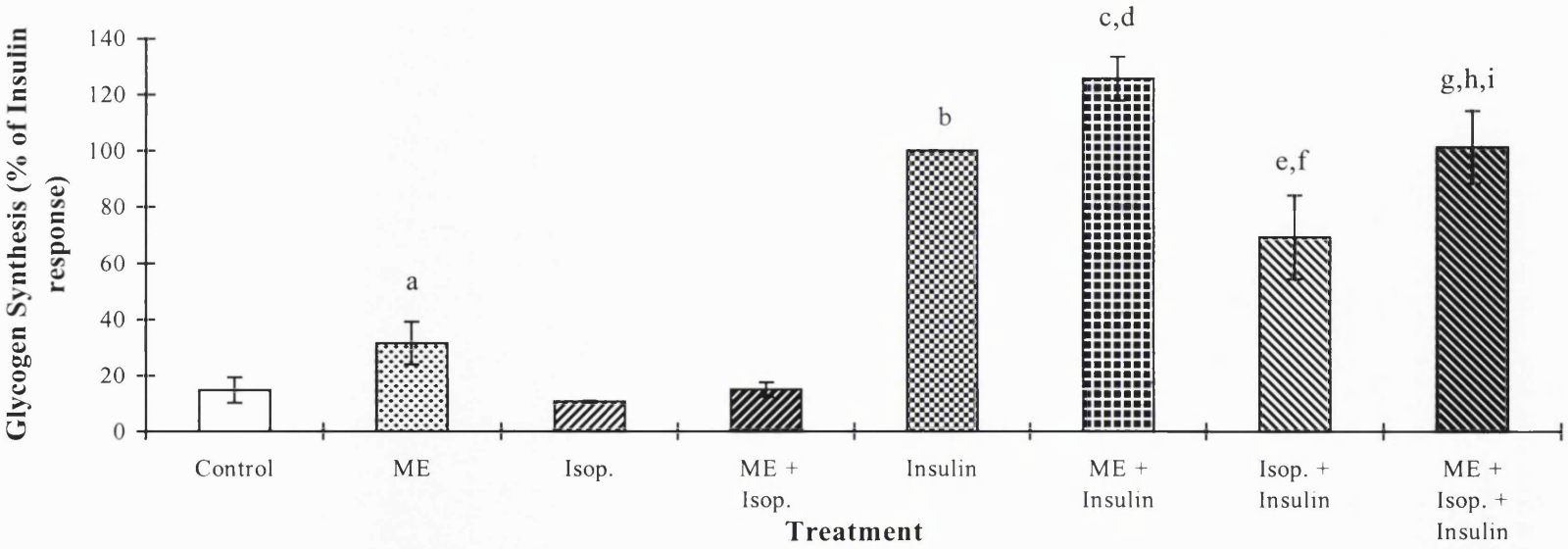
Despite the MEs being able to enhance insulin-stimulated glycogen synthesis, they were unable to induce EGF to stimulate this effect. This result was not really surprising as, as was mentioned previously, EGF has not been shown to activate PI3-kinase in these cells and the results of this thesis suggest that the malarial extracts enhance a PI3-kinase-dependent pathway. However it was an interesting experiment in spite of the negative result.

3.7 EFFECT OF MALARIAL EXTRACTS ON ISOPROTERENOL-INDUCED INSULIN DESENSITISATION

Although a number of substances have been shown to mimic insulin's actions, such as the inositol phosphate glycans mentioned previously very few substances have been shown to potentiate the effects of insulin in the way that the MEs do. One group of compounds which do however exert similar effects are the thiazolidinedione-derived antidiabetic agents. These compounds have been shown potentiate insulin stimulation of PI3-kinase in L6 myotubules (Zhang *et al.*, 1994b) and glycogen synthase in adipose tissue (Berger *et al.*, 1996), which is similar to the effects of the MEs. They are also able to induce hypoglycaemia, as can the MEs. Thiazolidinedione derivatives have also been shown to overcome insulin desensitisation induced by glucose and catecholamines (Berger *et al.*, 1996). To determine whether the MEs could similarly overcome insulin desensitisation their effect on the inhibitory action of isoproterenol upon insulin-stimulated glycogen synthesis was tested.

Adipocytes were incubated in the absence and presence of 10 nM insulin, with and without 30 nM isoproterenol. The effect of the MEs was determined by adding 15 µl of the preparations to the test incubations and 15 µl of PBS to the controls. Extracts I, O,P and R from table 3.5 were tested and the results pooled. The results are given as a percentage of the amount of radiolabelled glucose incorporated into glycogen in the presence of 10 nM insulin. As can be seen from fig. 3.36 isoproterenol had no effect on basal glycogen synthesis but significantly inhibited the amount of glucose incorporated into glycogen in the presence of insulin. In the absence of insulin the MEs were able stimulate glycogen synthesis to a level significantly above basal.

Fig. 3.36 Effect of Malarial Extracts on Isoproterenol-induced Insulin Inhibition



Shows the incorporation of [^{14}C]glucose into glycogen in extracts prepared from adipocytes which had been incubated in the absence and presence of 10 nM insulin, with and without 30 nM isoproterenol and/or 15 µl of various MEs. Results are given as a percentage of the glucose incorporated into glycogen with insulin alone. The rate of incorporation for the control was 0.0768 $\pm$ 0.025 µmol/hr/g dry weight of cells. Values are means $\pm$ SEM for the assay of six MEs using four separate adipocyte preparations.

a indicates $P<0.01$ versus control
b indicates $P<0.0005$ versus control
c indicates $P<0.0005$ versus incubation with MEs
d indicates $P<0.025$ versus incubation with insulin
e indicates $P<0.01$ versus incubation with isoproterenol
f indicates $P<0.05$ versus incubation with insulin
g indicates $P<0.005$ versus incubation with isoproterenol and MEs
h indicates $P<0.005$ versus incubation with isoproterenol and insulin
i indicates $P<0.05$ versus incubation with MEs and insulin

This effect however was inhibited by isoproterenol. In the presence of insulin the MEs enhanced glycogen synthesis to significant extent, an effect which was not inhibited by isoproterenol. The synergy factors for the MEs in the absence and presence of isoproterenol are 1.09 and 1.44 respectively.

3.7.1 Conclusions

These results therefore suggest that the MEs can antagonise the inhibitory action of isoproterenol on insulin-stimulated glycogen synthesis as can the thiazolidinedione derivatives. It was interesting to note that although isoproterenol had no effect on the synergistic properties of the MEs it was able to block the mimetic effect of these preparations, thus improving the synergy factor of the MEs. This finding therefore provides further evidence that the insulin-mimetic effects of the MEs are induced by separate entities to those which potentiate insulin's effects.

3.8 EFFECT OF MALARIAL EXTRACTS ON INSULIN RESISTANCE

Due to their ability to enhance insulin-signalling these malarial extracts, or rather their insulin-enhancing factor(s), might have potential as therapeutic agents for insulin-resistant states. The similarity of their effects to those of known therapeutic agents i.e. the thiazolidinedione compounds (section 3.7) lends further weight to this possibility. Consequently it was decided to have a preliminary look at the effect of the MEs on a model of insulin-resistance to see if these extracts could in fact overcome insulin resistance.

3.8.1. The *ob/ob* Mouse

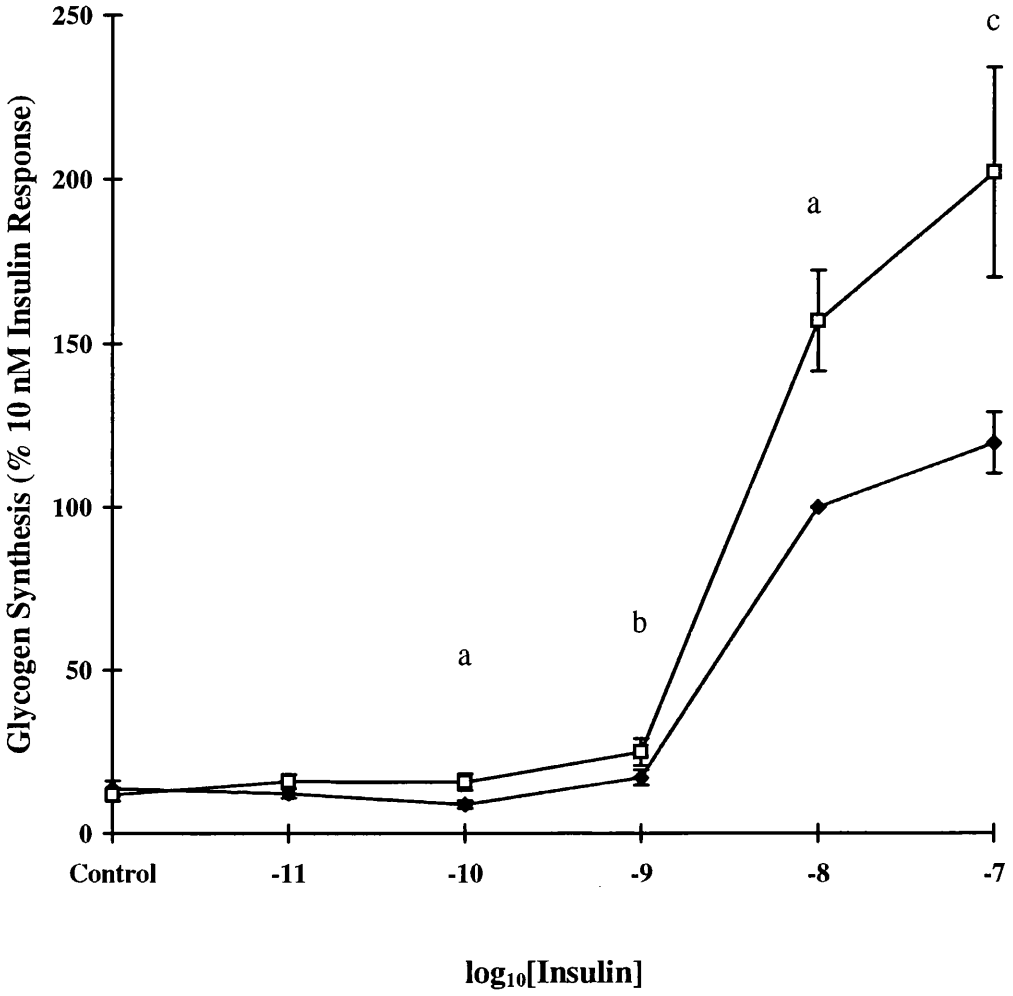
A number of animal models of insulin resistance have been developed, one which is commonly used is the *ob/ob* mouse (Coleman, 1978). Mice with this syndrome display marked obesity, hyperphagia, transient hyperglycaemia, hyperinsulinaemia and are insulin resistant. It is caused by a mutation in the *ob* gene on chromosome 6 which has been established and maintained in the C57BL/6J mouse strain. The *ob* gene encodes a protein of about 16 kDa known as leptin which is expressed in adipose tissue of many mammalian species including mice and humans and is found at high levels in blood. This protein appears to be involved in the regulation of food intake and body weight. How defects in the gene for this protein result in insulin resistance is not fully understood but insulin does induce leptin secretion by isolated adipocytes (Gettys *et al.*, 1996).

ob/ob mice have been shown to have reduced insulin-stimulated IRS-1 phosphorylation (Saad *et al.*, 1992) and PI3-kinase activation (Folli *et al.*, 1993) in

both liver and muscle and are also resistant to insulin-stimulated [^{14}C]glucose incorporation into glycogen in diaphgram muscle *in vivo* (Casieri *et al.*, 1989). As the MEs had been shown to enhance both glycogen synthesis and PI3-kinase activation it seemed possible that they may be able to improve the effects which insulin could exert in these mice. Indeed the thiazolidinedione derivatives have been shown to improve insulin resistance in these animals by increasing insulin action (Chang *et al.*, 1983; Young *et al.*, 1995).

To test whether the MEs could improve the insulin response of these animals their effect on glycogen synthesis in adipocytes derived from the fat deposits of these mice was examined. First of all however the effect of the MEs on adipocytes derived from normal C57BL/6J mice was established (unfortunately no ob/+ lean litter mates were available at the time the experiments were being carried out, therefore normal C57BL/6J had to be used as controls). The incorporation of [U-^{14}C]glucose into glycogen was measured at a range of insulin concentrations in the absence and presence of 15 µl of ME which was added to the incubation medium. ME preparations I, O, R and S from table 3.5 were used and the results were pooled. The results are given as a percentage of the [^{14}C]glucose incorporated into glycogen at 10 nM insulin. As can be seen from fig. 3.37 at 100 nM insulin, glycogen synthesis was stimulated approx. 9 fold above basal. At each insulin concentration, except 10 pM, the MEs significantly enhanced glycogen synthesis, this enhancement was particularly pronounced at the higher insulin concentrations. At 100 nM insulin in the presence of ME glucose incorporation into glycogen was stimulated approx. 17-fold, almost double the level seen in the absence of insulin. This is similar to the effect the MEs

Fig. 3.37 Effect of Malarial Extracts on Glycogen Synthesis in Adipocytes from C57BL/6J Mice (*ob/ob* controls)



Shows the incorporation of [^{14}C]glucose into glycogen in adipocytes isolated from C57BL/6J mice following incubation with the stated concentrations of insulin in the absence (◆) and presence (❑) of various MEs. Results are given as a percentage of the glucose incorporated into glycogen at 10 nM insulin. Values are means ± SEM for eight experiments. a, b and c indicate $P< 0.005$, $P<0.05$ and $P<0.01$ respectively when compared to the incorporation of glucose into glycogen at that particular insulin concentrations in the absence of the MEs.

had on lipogenesis which was also more pronounced at maximal insulin concentrations (Taylor *et al.*, 1992b).

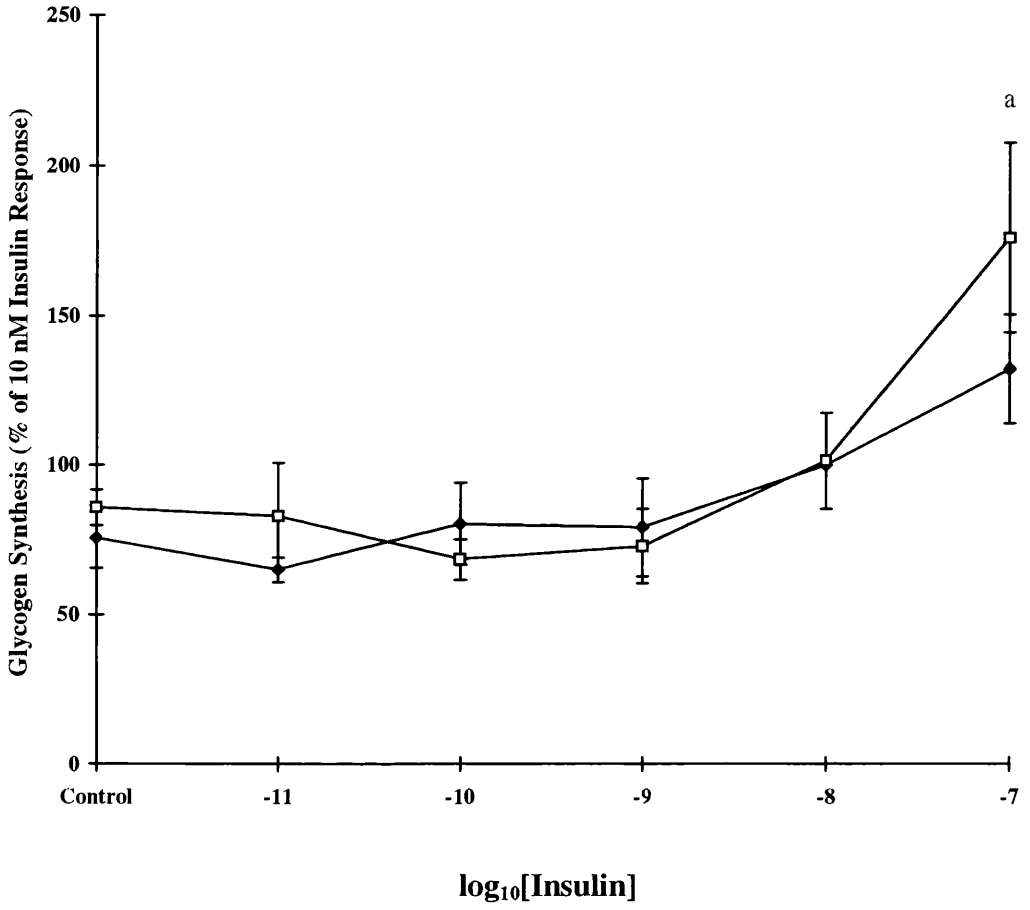
In adipocytes derived from the *ob/ob* mice even at 100 nM insulin [^{14}C]glucose incorporation into glycogen was only 1.5-fold over basal, demonstrating the marked insulin resistance of these mice (fig 3.38). In the presence of MEs (again I, O, R and S from table 3.5), although there was an increase in glycogen synthesis at 100 nM insulin this effect was not significant ($P<0.1$). At all other insulin concentrations the MEs had no effect.

3.8.2 Conclusions

These results suggest that although the MEs appear to be able to antagonise the inhibition of insulin induced by catecholamines, they are not able to antagonise insulin-resistance induced by genetic mutations, at least in this particular animal model. It appears that in order for the MEs to exert their effects there must first be a reasonable level of insulin action. In the *ob/ob* mice there was very little insulin response even at the highest insulin concentrations used which may be why the MEs were unable to exert their effects. Indeed at 100 nM insulin the MEs did induce a slight enhancement of the response but this was not significantly above the insulin response. Perhaps if higher insulin concentrations had been used a more pronounced effect of the MEs may have been seen, however that is merely speculation.

Another possible explanation could be that the main lesion causing insulin-resistance in the *ob/ob* adipocytes is upstream of the site of action of the malarial extracts. This

Fig. 3.38 Effect of Malarial Extracts on Glycogen Synthesis in Adipocytes from *ob/ob* Mice



Shows the incorporation of [^{14}C]glucose into glycogen in adipocytes isolated from *ob/ob* mice following incubation with the stated concentrations of insulin in the absence (◆) and presence (❑) of various MEs. Results are given as a percentage of the glucose incorporated into glycogen when the adipocytes were incubated with 10 nM insulin for one hour. Values are means ± SEM for five experiments. a indicates $p<0.1$ when compared to the incorporation of glucose into glycogen at 100 nM insulin in the absence of MEs.

does not therefore rule out the possibility that the MEs could reverse insulin resistance in other animal models with different lesions.

CHAPTER FOUR:

GENERAL DISCUSSION

4.1 GENERAL DISCUSSION

The purpose of using two strains of *Plasmodium* to prepare the malarial extracts, both of which caused hypoglycaemia during the course of an infection, was to try and establish whether the hypoglycaemia could be related to enhancement of the insulin response. It has clearly been shown in this thesis that extracts derived from both non-lethal *P. chabaudi* and lethal *P. yoelii* infected blood are capable of enhancing insulin's actions and in some cases mimicking them. It is therefore possible that these effects could be involved in the induction of this serious complication of malaria. The enhancement of insulin's actions which have been demonstrated here are relatively minor and would not have a major effect on the whole body disposal of glucose *in vivo* if they were restricted to the adipose tissue. However adipocytes are not the only cells which display insulin receptors and are responsive to insulin. Were the effects which the MEs have displayed in adipose tissue also to be seen in other tissues such as muscle and liver, these properties could play a major part in causing the hypoglycaemia associated with malaria. It would therefore be most interesting to examine the effects of the MEs on other cells.

Unfortunately the initial aim of this project, i.e. to find a method of preparing the malarial extracts which would consistently produce active samples, was not achieved. Of the samples prepared both from the *P.chabaudi* and the *P. yoelii* infected mice, only approximately 40% were actually able to synergise with insulin, a factor which should be borne in mind when considering the results of this project. Despite this however, some interesting observations were made about the insulin enhancing factor (IEF) present in the synergising MEs. It was clearly not destroyed

by pronase treatment suggesting that it is not proteinatious. The IEF does not appear to be lost with the serum during the initial washing of the blood cells suggesting that it is derived either from the parasite residing within the rbc or is tightly bound to the rbc itself. The inability of nrbc extracts to enhance insulin's actions suggests that the IEF is not normally expressed in these cells. The preliminary characterisation of the IEF by Dr Orford suggests that it could be a PI-containing molecule derived from a GPI. As was mentioned previously Schofield and Hackett (1993) managed to purify *a P. falciparum*-derived GPI which could exert similar effects to those which MEs have been shown to exert. Therefore it is possible that the IEF is derived from a similar such GPI. However Schofield and Hackett's GPI was not shown to enhance insulin's actions and it has been demonstrated in this thesis that there is no correlation between the other effects which the MEs exert and their insulin-enhancing abilities. One possible explanation for this discrepancy could be that the entities which cause each of the ME's effects, such as TNF induction, insulin mimicry and insulin synergy, are derived from different cleavage products of a single *Plasmodium* GPI. It is also possible however that the IEF is derived from the actual rbcs which have been altered by the parasite. Parasitized rbcs are known to develop 'knobs' on their surface containing parasite specific antigens through which they adhere to the vascular endothelium (Fujioka & Aikawa, 1996). Also the cell membranes of both parasitized and non-parasitized erythrocytes are thought to undergo changes analogous to cell ageing during the course of the infection (White & Ho, 1992). These changes might therefore produce the IEF in the rbc membrane.

As the methods used to prepare the extracts were relatively crude and non-specific it was not really surprising that a method which would consistently produce active

preparations was not found. However as the origin of the IEF was and still is unknown it would probably have taken three years to examine all the possible methods of releasing the IEF from the cells. As the main aim of the project was to identify the mechanism by which the IEF was enhancing insulin's actions this was not feasible. Purification of the IEF would obviously have been very beneficial to the project but as was explained in section 3.3.2 the partial purification which was achieved by Dr Orford required methods which gave low yield, therefore it was not possible to use this material for further experimentation.

The ability of some of the extracts to both enhance and mimic insulin's actions proved to be a bit of a problem at the beginning of the project as it was difficult to ascertain whether the enhancement of insulin's actions was merely a result of insulin mimicry. However it has clearly been shown from the correlation studies that these two properties are not related to one another. The ability of isoproterenol to suppress the insulin-mimetic effects of the MEs but not the insulin-enhancing effects clearly supports the idea the two separate entities are responsible for these effects. With hindsight it was therefore unnecessary for the mimetic effects of the extracts to be taken into account when determining the synergy factor of the preparations. Had it been known at the beginning of the project that these two properties were separate a great deal more of the MEs would have been considered 'active'. However when dealing with such crude preparations it is perhaps best to be cautious. Although the insulin-mimetic effects of the MEs were more or less dismissed in this project in favour of the insulin-enhancing effects, it would no doubt also be interesting to investigate these effects further.

Despite having to use the crude malarial extracts to investigate where in the insulin signalling network the IEF was exerting its effect some major advances were achieved. Our findings have shown that as well as being able to enhance insulin's effect on lipogenesis and lipolysis (Taylor *et al.*, 1992b) the IEF can also enhance insulin-stimulated glycogen synthesis. Despite adipose tissue being a lipogenic rather than a glycogenic tissue, this effects in most cases appeared to be even more pronounced than the enhancement of lipogenesis. This leads us to hypothesise that this enhancement of insulin-stimulated glycogen synthesis could be even more pronounced in a tissue such as muscle which has a higher propensity to store glucose as glycogen. As was mentioned previously, if this were the case the enhancement of insulin's actions could contribute to the hypoglycaemia associated with malaria. It was also interesting to note that while maximal insulin stimulation of lipogenesis was reached at 1 nM insulin (fig.3.10), even at 10 nM insulin stimulation of glycogen synthesis did not appear to be maximal (fig. 3.22). This may help to explain why, despite adipose tissue obviously having the necessary machinery to produce glycogen, insulin stimulation of this tissue preferentially results in the incorporation of glucose into lipids. It would be interesting to see if this effect was reversed in a glycogenic tissue (e.g. liver) i.e. if the machinery for glycogen synthesis had a lower threshold for insulin than that for lipid synthesis.

As was explained in section 1.3, insulin mediates its downstream effects via enzyme cascades which involve numerous kinases and phosphatases. By starting from the downstream effects which the MEs had been shown to enhance i.e. lipogenesis and glycogen synthesis, and working back along the pathways by which these effects are thought to be mediated I have been able to show that the MEs can enhance the

insulin stimulation of ACC, glycogen synthase and PI3-kinase. They do not however have any effect on PDH or glycogen phosphorylase nor were they able to induce EGF to stimulate glycogen synthesis in adipocytes. Based on the evidence which has been presented by other investigators on the insulin-signalling pathways, these findings suggest that the malarial extracts, or rather the IEF contained in the malarial extracts potentiates insulin's actions by enhancing insulin stimulation of PI3-kinase and hence the enzymes downstream of this component.

While the enhancement of insulin stimulated PI3-kinase activity by the MEs appeared very pronounced it should be noted that the assay procedure used to measure this activity does have its limitations, despite being the commonly adopted method. The radioactive PIP_3 spots on the tlc tend to be elongated making it difficult to determine which area of the spot should actually be counted. Hopefully by measuring the radioactivity of the spots in the way we did i.e. by putting the same size box round each spot and measuring the radioactivity within we will have overcome this problem.

Unfortunately, due lack of time, we were unable to determine the mechanism by which the MEs actually enhance PI3-kinase stimulation. The activity of the enzyme was measured in anti-phosphotyrosine immunoprecipitable extracts and therefore the increased activity is very likely to have been associated with IRS-1/IRS-2. This might suggest that the increase in PI3-kinase activity could be associated with an increase in the tyrosine phosphorylation of IRS-1 by the insulin receptor kinase and possibly an increase in the tyrosine phosphorylation of the receptor itself. However if there was an overall increase in the tyrosine phosphorylation of IRS-1/IRS-2 this

would surely also result in an increase in an enhancement of the Ras/MAP kinase pathway, which from the results shown here does not appear to be the case. Also no effect was seen on the binding of insulin to its receptor in the presence of the MEs, therefore if there was an increase in the tyrosine phosphorylation of the receptor and hence an increase in its tyrosine kinase activity it would have to be via an as yet unknown mechanism.

Another possible explanation could be that the apparently PI-containing IEF can associate with IRS-1's N-terminal PH domain and somehow influence the association of IRS-1 with PI3-kinase. Although this domain is thought to be involved in the membrane localisation of IRS-1 (Pawson, 1995), another PH-containing protein, PKB, has been shown to be activated upon binding of phosphoinositides to its PH domain (Frech *et al.*, 1997; Klippel *et al.*, 1997). The IEF could therefore be exerting a similar effect here.

On the other hand the MEs could be enhancing PI3-kinase activity directly by increasing the association of p110 with p85, inhibiting the serine kinase activity of p110 which is thought to be involved in downregulating the activity of both the PI3-kinase-lipid kinase activity and IRS-1, or acting as an agent to increase the activity of the p110 subunit through an as yet unknown mechanism.

At the present time it is of course impossible to say if one, or indeed any of these possible mechanisms are responsible for the insulin-enhancing effects of the MEs.

Although disappointingly the MEs did not overcome the insulin resistance of the *ob/ob* mice this does not necessarily rule them (or the IEF) out as potential therapeutic agents for insulin-resistant states. It is obvious from the dose response curves both of the normal and the *ob/ob* mice that in order for the IEF to exert its effects there must first be a reasonable insulin response. As was mentioned earlier, the insulin dose response curve for glycogen synthesis appears to be shifted to the right when compared to that for lipogenesis. It is therefore possible that if this experiment had been carried out at the same range of insulin concentrations on lipogenesis, the MEs may have had more of an effect at the higher insulin concentrations. Alternatively had the glycogen synthesis assay been carried out on muscle tissue, as was the case with the thiazolidinediones, the MEs may have had an effect. It may however be that this particular model of insulin resistance is not susceptible to the effects of the MEs, but it does not rule out the possibility that other models may be more responsive. They were after all able to antagonise isoproterenol-induced insulin-resistance, hence the ability of the MEs to enhance the insulin response may depend on the component(s) of the insulin signalling pathway which are actually affected by the insulin-resistance.

As well as having potential as therapeutic agents for insulin-resistant states, the MEs could also be a useful tool in the elucidation of the insulin signalling pathways. The findings of this thesis back-up the evidence already presented which suggests that lipogenesis, inhibition of lipolysis and glycogen synthesis are all mediated by PI3-kinase dependent pathways and in particular that ACC and glycogen synthase, but not PDH or glycogen phosphorylase are downstream of this enzyme. The only discrepancy with the published findings which we have encountered was the inability

of the MEs to enhance insulin-stimulated glucose transport which is also thought to be downstream of PI3-kinase. However as was explained in section 3.5, the glucose transport assays were carried out at maximal insulin concentrations and therefore the system may have been fully stimulated. This being the case, any effect which the MEs may have had would not have been seen. It is however also possible that enhancement of PI3-kinase by the MEs depends on its membrane localisation. It has been suggested that activation of glucose transport by PI3-kinase depends on localisation of the enzyme to the microsomal membranes where the glucose transporters are located (Nave et al., 1996). If the MEs are only able to exert their effects at the plasma membrane this would explain why no effect was seen on glucose transport apparently localised to the microsomal membranes. Alternatively it may be that PI3-kinase mediates its effects on glucose transport via a pathway unaffected by the MEs, although this seems unlikely as an increase in enhancement of PI3-kinase activity would surely affect all of its downstream effectors.

Although the MEs were unable to induce EGF to mimic an insulin response, this does not rule out the possiblilty that they may have an effect on the normal responses of growth factors other than insulin. Given the involvement of PI3-kinase in so many signalling pathways it does not seem unreasonable to hypothesise that the MEs might also be able to enhance the effects of other growth factors and/or cytokines which utilise this enzyme. The mechanism by which the MEs enhance insulin-stimulated PI3-kinase activity has yet of course to be established. If it does involve IRS-1/IRS-2 this would rule out a number of growth factors which bind and activate PI3-kinase directly. However it would be very interesting to look at the effect of the MEs on, for example, IGF-1 as this utilises more or less exactly the same pathways as insulin.

At the present time the possibilities for this malaria-derived insulin enhancing factor seem almost endless. The work which has been presented in this thesis I hope will provide a basis from which further investigation into the properties of this factor and its potential uses can be carried out. Anyone considering research in this area should however take into account how difficult it proved to be to produce malarial extracts containing this factor and would perhaps be advised to concentrate on purifying and characterising the IEF before examining its properties further.

CHAPTER FIVE:

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