β-CELL TROPIN: ITS STRUCTURE, BIOSYNTHESIS AND INSULIN-LIKE ACTION.

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Summary

The hyperglycaemic-obese mouse (ob/ob) has been shown to release a peptide from the pituitary pars intermedia, which stimulates the release of insulin. This peptide, β-cell tropin (BCT), has been characterised by its ability to cross-react with an antiserum raised against ACTH_{17-39} i.e. CLIP. This antiserum has been used as part of a radioimmunoassay to identify and quantify CLIP-like peptides and BCT.

Using Biogel P2/P6 gel filtration columns as a means of peptide separation, tryptic digests of synthetic and native CLIP have produced BCT. The probable tryptic cleavage point on the CLIP molecule is at the C-terminal of lysine-21 which identifies the BCT molecule as ACTH_{22-39}. This has been verified by other workers.

As BCT is part of the ACTH molecule it is probably synthesised from the 31K POMC prohormone. Pulse-labelling studies using the chromatographic methods reverse-phase HPLC and P2/P6 gel filtration have shown that BCT is synthesised in 2h. The results, however, were unable to show if BCT was synthesised from CLIP or POMC.

The effect of native and 'synthetic' BCT on rat adipocytes has also been investigated. BCT stimulated the incorporation of $^3$H, from $^3$H$_2$O, into saponified fatty acids, though CLIP did not. This action was further investigated using the conversion of [1-$^1$C] and [6-$^1$C] glucose into CO$_2$, saponified fatty acids and glyceride-glycerol. The results showed that BCT stimulated the turnover of the pentose phosphate pathway,
fatty acid synthesis and esterification, similar to that obtained with insulin. The action of BCT thus appeared to be similar to that of insulin. BCT, however, did not inhibit noradrenaline stimulated lipolysis, neither did it effect the levels of cAMP. Maximal doses of BCT and insulin, together, showed no additive effect on glucose metabolism suggesting that BCT and insulin act via a rate-limiting step in their action on lipogenesis.

Experiments were carried out with BCT derivatives which showed that the N-val-tyr-pro sequence of BCT was important for its lipogenic activity in adipocytes.
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ABBREVIATIONS.

Ac : acetyl group (CH₃CHO-).
ACC : acetyl-CoA carboxylase.
ACTH : adrenocorticotropin.
BCT : β-cell tropin (ACTH₂₂-₃₉).
cAMP : cyclic 3':5'-adenosine monophosphate.
cGMP : cyclic 3':5'-guanosine monophosphate.
CLIP : corticotropin-like intermediate lobe peptide (ACTH₁₈-₃₉).
E.S.R. : external standard ratio.
HPLC : high pressure liquid chromatography.
IBMX : 3-isobutyl-1-methylxanthine.
IRM : immunoreactive material.
LPE : lipotropin.
α-MSH : α-melanocyte-stimulating hormone (ACTH₁-₁₃).
NA : noradrenaline.
NIL : neurointermediate lobe(s).
PAGE : polyacrylamide gel electrophoresis.
PDE : pyruvate dehydrogenase.
POMC : pro-opiomelanocortin.
RIA : radioimmunoassay.
SDS : sodium dodecyl sulphate.
S.E.M. : standard error of the mean.
TAG : triacylglycerol (triglyceride).

The standard three letter abbreviations for the amino acids were used throughout this thesis and the letter 'K' was used to donate 1,000, usually in reference to the molecular weight of a peptide or protein.
I. INTRODUCTION.
The discovery of 3-cell tropin as an insulin secretagogue

BCT is a novel peptide hormone which has recently been discovered in the obese-hyper-glycaemic (ob/ob) mouse (see Beloff-Chain et al 1980a). However evidence suggests that the occurrence of this peptide is not only associated with the ob/ob mouse. Insulin secretagogue activity, from the pituitary, has been demonstrated in the lean (+/+ ) mouse (Beloff-Chain et al 1975a, 1977b, 1978; Dunmore and Beloff-Chain 1982) and further evidence as to the existence of BCT, in the lean animal, will be presented in this thesis. In addition, lean mice which have been made obese by gold thioglucose treatment or by dietary manipulation produced an increased amount of the pituitary factor which stimulated insulin release (Beloff-Chain et al 1979).

The discovery of BCT came about due to the hypothesis which suggested that the hypersecretion of insulin, by the ob/ob pancreas, was connected with a humoral factor, possibly associated with feeding (Abraham and Beloff-Chain 1971; Beloff-Chain et al 1973).

ACTH, a known insulin secretagogue (Genuth and Lebovitz 1965), was considered as a possible candidate due to the report of Edwardson and Hough (1975) describing elevated ACTH levels in the ob/ob pituitary and plasma. Perifusion of microdissected islets with a physiologically excessive dose of ACTH produced only a small stimulation of insulin release (Beloff-Chain et al 1975a). The same study, meanwhile, reported that perifusate from ob/ob and lean mouse pituitaries stimulated insulin
release from lean mouse islets.

Estimation of the relative amounts of the insulin secretagogue released by the pituitary, demonstrated a gene dosage effect (Beloff-Chain et al 1979). This estimation was achieved by the dilution of pituitary perifusates and the subsequent testing of bioactivity on lean (+/+ ) mouse islets. The results indicated that the ob/ob pituitary released more insulin secretagogue than the heterozygous (ob/+ ) pituitary, which in turn released more than the homozygous lean (+/+ ) mouse pituitary.

The secretion of this pituitary factor has been shown to occur from the neurointermediate lobe of the pituitary gland (Beloff-Chain et al 1977a; Beevor et al 1978). Furthermore CLIP (ACTH_{18-39}), a peptide of the pars intermedia (Scott et al 1973), was implicated due to the ability of antibodies raised against ACTH_{17-39}, to abolish the bioactivity of the pituitary factor (Bogdanovic 1978; Beloff-Chain et al 1980a). In addition, the NIL of the ob/ob mouse synthesised and secreted elevated levels of CLIP compared to the lean mouse NIL (Beloff-Chain et al 1977a; Beevor et al 1978; Edwardson and Donaldson 1979).

However, CLIP was found to have no insulin secretagogue activity (Beloff-Chain et al 1980a). These findings suggested that the pituitary insulin secretagogue was not CLIP but was related to the CLIP molecule.

Gel filtration chromatography of ob/ob NIL perifusate, using Biogel P2/P6 (Scott et al 1976), separated five peaks which cross-reacted with the 'CLIP-antiserum' (Beloff-Chain et al 1980a). Two of these CLIP-like peptides, named 'D' and
'E' had insulin secretagogue activity, although the activity of D was later shown to be due to 'E' contamination (Beloff-Chain et al 1980a).

The active 'E' peak was given the name β-cell tropin (BCT). Experiments to further purify and characterise this peptide have been reported (Beloff-Chain et al 1981). Work that will be described in this thesis, as well as evidence which has been submitted for publication (Beloff-Chain et al 1982b) have provided evidence to suggest that BCT consists of an amino acid sequence identical to ACTH_{22-39} (figure 1).

**I aii Hormonal nature of β-cell tropin**

The release of BCT, from ob/ob and lean (+/+)) mouse pars intermedia, has been detected by its stimulation of insulin release (Beloff-Chain et al 1975a, 1978; Dunmore and Beloff-Chain 1982). The amount of BCT released by the ob/ob pars intermedia is markedly elevated compared to that released by the lean (+/+)) mouse pars intermedia (Beloff-Chain et al 1975a, 1978, 1979; Dunmore and Beloff-Chain 1982). Furthermore, BCT has been demonstrated in the plasma of ob/ob mice (Billingham et al 1982) although plasma from lean (+/+)) mice, diluted 1:10, was apparently devoid of insulin releasing activity.

The response to BCT by the pancreatic islets appears to manifest feedback mechanisms to modulate the effect of BCT. Islets from adult ob/ob mice have been shown to be refractory to the action of BCT (Beloff-Chain et al 1975a; Beloff-Chain and Hawthorn 1976) which may be due to a down-regulation of receptors (Gavin et al 1974; Kahn 1976) and/or decreased
Human CLIP.

\[ \text{N-arg} - \text{pro} - \text{val} - \text{lys} - \text{val} - \text{tyr} \]

\[ \text{ser} - \text{glu} - \text{asp} - \text{glu} - \text{ala} - \text{gly} \]

\[ \text{ala} \]

\[ \text{glu} \]

\[ \text{ala} - \text{phe} - \text{pro} - \text{leu} - \text{glu} - \text{phe-C} \]

* = val in rat and mouse CLIP.

+ = asn according to Browne et al (1981).

The numbers represent the position of the amino acid in ACTH.
responsiveness in response to the high BCT concentration in the circulation.

However, the islets of 3 week old ob/ob mice are slightly hypersensitive to the pituitary insulin secretagogue (Beloff-Chain et al 1979), suggesting that the down-regulation and/or decreased responsiveness develops as the ob/ob mouse matures.

The release of BCT is apparently regulated in response to the nutritional state of the animal; fasting produces a decreased BCT secretion while feeding stimulates BCT release (Beloff-Chain et al 1977b, 1978). The response by the pancreatic islets is also governed by fasting and feeding, since fasting increases the sensitivity of the islets to BCT while feeding has the opposite effect (Beloff-Chain et al 1977b).

BCT stimulation of pancreatic islets produces a rapid monophasic release of insulin, in vitro and in vivo, (Beloff-Chain et al 1975a,b, 1980a,b, 1982a) although the physiological action of BCT upon β-cells may be the potentiation of glucose stimulated insulin release (Beloff-Chain et al 1981, Dunmore and Beloff-Chain 1982).

The obese hyperglycaemic (ob/ob) mouse and β-cell tropin

This genetically transmitted form of obesity was initially identified by Ingalls et al (1950) and is due to an autosomal recessive mutation on chromosome-6 (Coleman 1978). As a model of ob-esity the ob/ob mouse has been extensively studied, yet the primary lesion is still undiscovered (reviews: Bray and York 1971, 1979). The adult ob/ob mouse is readily identifiable due to its excessive body weight, compared to
that of its lean litter mates, entirely accounted for by excessive accumulated fat (Bates et al 1955; Pitts and Hollifield 1963; Westman 1968).

Another characteristic, but variable, syndrome of the ob/ob mouse is hyperglycaemia (Christophe et al 1959; Abraham et al 1971; Westman 1968; Dubuc 1976; Garthwaite et al 1980; Godbole et al 1980). This development appears to occur subsequent to a rise in plasma insulin levels, having been observed at about 4 weeks of age (Dubuc 1976).

Before the plasma glucose becomes elevated, there may be a period of hypoglycaemia (Dubuc 1976). It has been postulated that this may be due to an initial hypersensitivity to insulin, as seen in ob/ob adipocytes (Czech et al 1977), although the timing of these events may not be concurrent.

After weaning, the mice become hyperphagic (Mayer et al 1951, 1954), although this is not the primary reason for the obese syndrome because a restricted diet only partially prevents excessive fat deposition (Alonzo and Maren 1955).

At 3-4 weeks of age, the ob/ob mouse starts to show the increased body weight due to the excessive fat accumulation (Westman 1968; Dubuc 1976; Beloff-Chain et al 1979; Godbole et al 1980). However, due to the difficulty of identifying the preobese ob/ob mouse (i.e. before 3-4 weeks of age) most investigations have used 4 week old mice, missing the onset of the obese syndrome.

Two symptoms characteristic of the obese mouse, which have enabled identification of the preobese mouse, are impaired thermogenesis (Davis and Mayer 1954) and a decreased oxygen consumption (Mayer et al 1952). Because these two symptoms have been reported as occurring as early as 10 days of age in
the ob/ob mouse (Kaplan and Leveille 1974; Trayhurn et al 1977), they can be used to identify the preobese ob/ob mouse from 10 days of age up to 3-4 weeks.

The first detectable signs of lipid accumulation have been reported at 10 days (Thurlby and Trayhurn 1978) which is consistent with the 12-17 days reported by other workers (Joosten and van der Kroon 1974; Dubuc 1976; Godbole et al 1980), but may even occur at an earlier age.

By 4-5 weeks of age the body weight of the ob/ob mouse is significantly elevated compared to their lean litter mates and remains elevated throughout their lifetime (Westman 1968; Garthwaite et al 1980).

Hems et al (1975) found elevated rates of lipogenesis in the ob/ob mouse, in both of the major lipogenic organs, the liver and the adipose tissue, at 1 month and 3 months of age.

Furthermore, in contrast to the lean (+/+ ) mouse in which the majority of lipid synthesis occurs in the liver, in the ob/ob mouse the adipose tissue becomes the major site of lipogenesis (Hems et al 1975). Elevated levels of the lipogenic enzymes have been found in both the liver and adipose tissue of the ob/ob mouse (Bray and York 1971; Martin et al 1973; Lombardo and Menahan 1978; Kaplan and Leveille 1981).

Although the plasma free fatty acid concentration is normal (Abraham et al 1971), or slightly elevated (Elliot et al 1976), the actual turnover of lipid, as measured by the increased glycerol concentration, is increased (Elliot et al 1976). Moreover the response of lipolysis to
noradrenaline appears to be unaltered (Abraham et al 1971; Shepherd et al 1977; Carnie and Smith 1978), even though adenylate cyclase activity may be impaired (Shepherd et al 1977).

Carnie et al (1979) demonstrated an insulin induced antilipolytic action upon noradrenaline stimulated lipolysis in the presence of glucose, in ob/ob adipocytes; although this effect was desensitized compared to the lean (+/+ ) adipocytes. In contrast, Yen and Steinmetz (1972) showed an absence of an insulin effect upon theophylline stimulated lipolysis in ob/ob adipose tissue pieces. This discrepancy in results may be due to the insensitivity, in response to hormones, of the adipose tissue pieces in vitro system.

In addition to hyperinsulinaemia, which will be discussed below, the ob/ob mouse develops various alterations in hormone levels (reviews: Bray and York 1971, 1979; Edwardson and Donaldson 1979; Garthwaite et al 1980).

The secretion of hormones by the pituitary is radically altered, whether this is due directly to the primary lesion or as a secondary consequence is unknown. Yet the pituitary would appear to be an important factor in the aetiology of the obese syndrome, because hypophysectomy of the ob/ob mouse brings about an improvement in many of the symptoms (Herbai 1970).

The pituitary content and rate of secretion of ACTH is excessive in the ob/ob mouse, despite an apparently normal corticotrophin-releasing factor concentration in the ob/ob hypothalamus (Edwardson and Hough 1975). As a consequence of elevated levels of ACTH, the ob/ob mouse gives rise to
increased corticosterone secretion by the adrenals (Nasser 1974; Garthwaite et al 1980) causing secondary complications.

In addition to the increased ACTH synthesis, the ob/ob mouse produces more of the POMC-related peptides (see Ie) compared to their lean litter mates.

Pituitary β-endorphin concentrations are increased in the ob/ob mouse, although this is initially found at 3 months of age (Rossier et al 1979; Garthwaite et al 1980), too late for any effect on the early aetiology of the obese syndrome.

aMSH and CLIP are also synthesised and secreted in greater quantities by the ob/ob (Beloff-Chain et al 1977; Beevor et al 1978; Edwardson and Donaldson 1979).

This excessive synthesis of these POMC related peptides, in both the ob/ob anterior and intermediate lobes, is probably due to the increased synthesis and processing of POMC. This increased synthesis may explain the increased BCT secretion by the ob/ob pars intermedia (Beloff-Chain et al 1975a, 1978, 1979, Dunmore and Beloff-Chain 1982). Since BCT appears to have the amino acid sequence of ACTH_{22-39} (Beloff-Chain et al 1982b), it is probable that this peptide is synthesised by the cleavage of CLIP, or at least from POMC. Thus an increased synthesis and turnover of POMC may result in an increased secretion of BCT.

The increased synthesis of BCT, in the ob/ob mouse, may be a contributing factor to the hyperinsulinaemic syndrome (see Ib). As has already been mentioned (see Ia1), BCT is a potent insulin secretagogue which probably acts, in vivo, by enhancing glucose stimulation of insulin release (Beloff-Chain et al 1980a, b, 1981, 1982a; Dunmore and Beloff-Chain 1982).
However, the longitudinal profile of this hormone, in the ob/ob mouse, is not yet known and thus the relationship of BCT to the onset of hyperinsulinaemia is also unknown.

Various other hormones are secreted in decreased amounts by the ob/ob pituitary. Growth hormone and prolactin are both found in low levels in the pituitary and plasma (Sinha et al 1975), as is the secretion of the sex hormones, follicle-stimulating hormone and luteinizing hormone (Swerdloff et al 1976). Inadequate secretion of follicle-stimulating hormone and luteinizing hormone may explain the atrophic gonads and infertility, which are characteristic of the ob/ob syndrome, as well as the decreased levels of testosterone in the male mouse (Swerdloff et al 1976).

I b Obesity and the role of insulin

The adult obese (ob/ob) mouse has a marked fasting hyperinsulinaemia which develops during the early stages of the animal's life (Westman 1968; Christophe et al 1959; Genuth 1969, Abraham et al 1971; Dubuc 1976; Garthwaite et al 1980). This hypersecretion of insulin has been proposed as the primary cause of the ob/ob syndrome (Genuth 1969), although this is rather controversial.

The onset of hyperinsulinaemia has been reported to be subsequent to the excessive accumulation of lipid (Westman 1968; Joosten and van der Kroon 1974) suggesting that the hyperinsulinaemia is secondary to the obese syndrome. However, other workers have demonstrated hyperinsulinaemia at the same time that lipid accumulation was first apparent, approximately 15 to 17 days (Godbole et al 1980; Dubuc 1976).
Godbole et al (1980) showed that this accumulation of lipid occurred initially in the carcass (adipose), followed later by an increased liver lipogenic rate. In contrast, Kaplan and Leveille (1981) demonstrated an increased liver lipogenic rate at 4 weeks while the adipose lipogenic rate did not increase until 7 weeks. However, this would not explain the greater deposits of fat found in the carcass (adipose) at 10-15 days. (Thurlby and Trayhurn 1978; Godbole et al 1980), nor the increased adipocyte size at 12 days (Joosten and van der Kroon 1974). Furthermore, Hems et al (1975) demonstrated a marked increase in total lipogenic rate in adipose from 1 month old obese (ob/ob) mice, in vivo.

Thus the onset of hyperinsulinaemia and obesity appear to occur concurrently in the ob/ob mouse suggesting that the obesity might be due to the action of the excess insulin. The excessive storage of fat is apparently largely due to an increased lipid synthesis in the adipose tissue, yet the liver may make a significant contribution (Hems et al 1975).

Hyperinsulinaemia is not just a syndrome associated with the obese-hyperglycaemic (ob/ob) mouse model of obesity. Another extensively investigated obese model, the spontaneously obese rat, also has elevated plasma insulin levels (Olefsky and Reaven 1975) although the blood glucose levels are normal. Additionally, the obese human is hyperinsulinaemic, both during periods of fast and after a glucose load (Karam et al 1963). It therefore appears that hyperinsulinaemia is a common syndrome associated with obesity.

As well as the development of hyperinsulinaemia, most forms of obesity also develop an impaired insulin response,
or insulin resistance. The nature of this resistance varies depending on the underlying defect within the tissue. The two basic types of resistance have been described by Kahn (1978, 1980). A decreased sensitivity to insulin is, where at a submaximal insulin concentration, the biological response is decreased, while at supramaximal insulin concentrations a maximal response is produced. This condition is usually due to a decreased receptor number (down-regulation) represented by a rightward shift in the 'dose-response' curve.

A decreased responsiveness to insulin is, where at supramaximal insulin concentrations, the response is submaximal; this defect is of a post-receptor origin. This unresponsiveness to insulin does not necessarily have to be associated with a decreased sensitivity to insulin, although they may often both be manifest within tissues from obese animals. However, in many studies the distinction between these two forms of insulin resistance has not been made.

Administration of an insulin dose to ob/ob mice induces only a small response in blood glucose levels and glucose metabolism, in contrast to the marked response in lean animals (Batt and Mjalhe 1966; Stauffacher and Renold 1969; Chlouverakis and White 1969; Loten et al 1974). This resistance to insulin develops while the animal is young, presumably due to the developing hyperinsulinaemia (Czech et al 1977; Kaplan and Leveille 1981).

The adipose tissue appears to become resistant to insulin at about 4 weeks of age (Stauffacher and Renold 1969; Dubuc 1976) although Kaplan and Leveille (1981) demonstrated insulin resistance in the stimulation of glycogen synthesis, in 3 week
old ob/ob mice.

At 2 to 4 months of age, the ob/ob epididymal fat pad is markedly resistant to the action of insulin upon glucose oxidation (Abraham and Beloff-Chain 1971) although the sensitivity to insulin is partially restored by a restrictive diet.

The obese (ob/ob) mouse also develops insulin resistance in muscle. Both the diaphragm (Abraham and Beloff-Chain 1971) and the soleus muscle (Le Marchand and Freychet 1978) are resistant to insulin's action on glucose metabolism, in the adult obese mouse. Furthermore, in the soleus muscle a defect in basal glucose transport (or phosphorylation) arises which is not reversible by lowering the plasma insulin (Cuendet et al 1976). Le Marchand and Freychet (1978) have demonstrated that the defect in insulin stimulation of glucose metabolism also resides at glucose transport (or phosphorylation), and the stimulation of glycogen synthesis is also depressed.

The development of this insulin resistance in the peripheral tissue may partially contribute to the hyperglycaemic syndrome of the ob/ob mouse, due to the impairment of glucose uptake and utilisation (Cuendet et al 1976; Bray and York 1979).

The ob/ob liver has also been reported as having a slight insulin resistance (Loten et al 1974), however an impaired insulin response upon lipogenesis has not been well documented in this tissue which may be due to the difficulty of showing insulin action upon hepatic lipogenesis in vitro (Hems 1979).
The large 'obese' adipocytes of the spontaneously obese rat are unresponsive to insulin action on glucose metabolism (Di Girolamo et al 1974; Czech 1976a; Olefsky 1976b; Richardson and Czech 1978). These fat cells appear to develop a post-receptor defect within fatty acid synthesis which as a secondary consequence effects the pentose phosphate pathway (Richardson and Czech 1978).

A post-receptor defect to the insulin action on the glucose disposal by peripheral tissues has been identified in human obesity (Kolterman et al 1980; Olefsky 1981). The extent of this post-receptor defect correlates linearly with the fasting plasma insulin levels of the human subjects (Kolterman et al 1980; Olefsky 1981).

The association of insulin resistance to hyperinsulinaemia would seem to be a common characteristic of the obese state. It is probable that the tissues develop a defect to insulin action to prevent over-stimulation by the developing hyperinsulinaemia. This may be especially important in the adipose tissue in order to prevent uncontrolled triglyceride storage (see Czech et al 1977).

If small 'lean' rat adipocytes are incubated in the presence of a physiologically excessive dose of insulin the fat cell down-regulates its insulin receptors and furthermore it develops a post-receptor defect in glucose transport (Marshall and Olefsky 1980; Olefsky 1981). Therefore it would seem that insulin resistance, in the form of desensitisation and unresponsiveness, is a physiological 'defensive' response to hyperinsulinaemia.
The action of β-cell tropin in adipose tissue

The injection of native BCT into the jugular vein of a rat brought about a transient monophasic release of insulin (Beloff-Chain et al. 1982a). Additionally, using the incorporation of \(^{3}H\), from \(^{3}H_2O\), into saponifiable fatty acids (Jungas 1968; Windmueller and Spaeth 1966) as a measure of total fat synthesis, an increase in lipogenesis in the subcutaneous fat was demonstrated after 45 minutes. An increase in the lipogenic rate in the mesenteric fat was also seen but this was not statistically significant. The hepatic lipogenic rate was unaffected by BCT (Beloff-Chain et al. 1982a).

Due to the transient nature of the effect of BCT upon insulin release, the stimulation of lipogenesis was not thought to have been the primary cause of the increased fatty acid synthesis.

To investigate a possible direct action of BCT upon the adipose tissue, collagenase prepared isolated adipocytes (Rodbell 1964) were used. The results showed that native BCT, which had been purified by Biogel P2/P6 gel filtration chromatography (Scott et al. 1976; Beloff-Chain et al. 1980a; 1981) increased fatty acid synthesis (Beloff-Chain 1982a).

The discovery that BCT is probably ACTH\(_{22-39}\) (Beloff-Chain et al. 1982b), made it possible to obtain greater quantities of the peptide using synthetic human CLIP as the starting material (section IIIai). Using this 'synthetic BCT' it was decided to further investigate the insulin-like action upon glucose and lipid metabolism, in the rat adipocyte, as well as performing some preliminary experiments into the mode of action of the peptide.
The existence of a hitherto unidentified lipogenic agent raises the question of its possible significance in obesity, either in association with insulin or due to its own action. The presence of BCT in obesity could possibly result in the stimulation of adipose lipogenesis, even while the tissue is resistant to the action of insulin.

Thus the discovery of a new lipogenic agent, which also has potent insulin secretagogue activity, invokes interesting questions about the control of lipogenesis in the normal and diseased physiological states. This thesis is, in part, an attempt to elucidate the lipogenic role of BCT upon adipose tissue.

I'd Insulin action in adipose tissue

I'd Glucose transport

Glucose is transported into the cell via a plasma membrane protein which acts by facilitating the movement of glucose down its chemical gradient (Crofford and Renold 1965a,b). One of the main actions of insulin upon the adipose tissue, is to stimulate glucose transport (reviews: Czech 1976d, 1980).

It has been proposed that insulin stimulates glucose transport by a redox reaction resulting in the oxidation of specific sulphydryl groups associated with the carrier protein (Czech et al 1974b,c; Czech 1976 b,c,d, 1977). In support of this theory it has been shown that certain reducing agents inhibit insulin activation of glucose transport (Czech et al 1974 a,b; Czech 1976 b,c) and furthermore the oxidising agent H₂O₂ has an insulinomimetic action (Mukherjee and Lynn 1977; May and de Häen 1979 a,b). However, there is little direct evidence to support this theory and neither does this model
explain the kinetic data for glucose transport activation. (Olefsky 1978; Whitesell and Gliemann 1979).

It now appears that the action of insulin upon the glucose transport system increases the number of 'glucose carriers' in the plasma membrane (Wardzala et al 1978). Within the adipocyte, glucose carriers are stored in a 'golgi-microsomal membrane' pool (Suzuki and Kono 1981; Karnieli et al 1981). Insulin stimulation of the adipocyte activates the translocation of these glucose carriers, from the 'golgi pool' to the plasma membrane, increasing the available number of glucose transport sites and thus hexose transport. (Suzuki and Kono 1981; Karnieli et al 1981).

Consistent with this model in the kinetic data showing that insulin action increases the Vmax of glucose transport while the km remains unchanged (Czech 1976 d; Olefsky 1978; Whitesell and Gliemann 1979), which can be explained by an increase in the number of carrier units.

I dii Antilipolytic action

A major action of insulin on the adipose tissue is the inhibition of both basal and hormone stimulated lipolysis (Jungas and Ball 1963; Fain et al 1966). The mode of action by which insulin inhibits lipolysis is as yet unknown.

The levels of cAMP, the secondary messenger responsible for the stimulation of lipolysis, are decreased by insulin action upon catecholamine stimulated fat cells (Butcher et al 1968; Desai et al 1973; Kono and Barham 1973). However the antilipolytic action of insulin and the decrease in cAMP have been shown not to correlate (Siddle and Hales 1974; Schönhöfer
FIGURE 2.

Scheme showing glucose oxidation and incorporation into lipid in adipose tissue.

- Glucose transport and hexokinase
- Glucose-6-P
- Fructose-diphosphate
- Dihydroxyacetone-P ↔ Glyceraldehyde-3-P
- Glycerol-3-P
- Lysophosphatidate
- Phosphatidate
- Lipids
- Diacylglycerol
- Triacylglycerol
- Pyruvate
- PDH → CO₂
- Acetyl-CoA
- ACC
- Fatty Acid Synthetase Complex
- Malonyl-CoA
- Fatty acyl-CoA
- Citrate
- OA
- Succinate
- α-KG
- CO₂
et al 1972). Furthermore, insulin's antilipolytic action has been demonstrated in the absence of any alteration in cAMP levels (Fain and Rosenberg 1972; Khoo et al 1973). Thus it is possible that cAMP may play a role in insulin stimulated antilipolysis although this is probably not of a primary importance.

Insulin may directly inhibit the lipolytic enzyme, hormone sensitive lipase. It appears that insulin can modulate adrenaline stimulated phosphorylation of hormone sensitive lipase which seems to correlate with a decrease in the enzyme's activity (Nilsson et al 1980). However, as yet there is no direct evidence that insulin works by the inhibition of this enzyme.

I diii Glucose metabolism

In the adipose tissue, fatty acid synthesis is stimulated by the action of insulin, which occurs mainly by the metabolism of glucose (Winegrad and Renold 1958a,b; Lynn et al 1960). Fatty acid synthesis can be summarised by:

\[ 8 \text{Acetyl-CoA} + 14\text{NADPH} + 14\text{H}^+ + 7\text{ATP} + \text{H}_2\text{O} \rightarrow \text{palmitic acid (C}_{16}\text{)} + 8\text{CoA} + 14\text{NADP}^+ + 7\text{ADP} + 7\text{Pi} \]

The basic unit utilised by this process is the acetyl-CoA 'C₂ unit' which is mainly produced by glycolysis (figure 2).

The mode of action of insulin upon this process appears to be due to specific enzyme activations. Insulin stimulation of fatty acid synthesis produces a parallel increase in the activities of PDH and ACC (Stansbie et al 1976; Denton et al 1977), two enzymes which control key points linking glycolysis
and fatty acid synthesis (figure 2).

This activation of ACC and PDH is not secondary to insulin stimulation of glucose transport since insulin activation of PDH and ACC occurs in the presence of fructose, whose uptake is only marginally increased by insulin (Denton et al 1977). Thus the stimulation of fatty acid synthesis is not entirely due to an increased glucose metabolism resulting from increased glucose uptake.

As is shown by the above equation, fatty acid synthesis has a large requirement for a reducing agent, in the form of NADPH. Therefore, insulin stimulation of fatty acid synthesis increases the cellular demand for NADPH. To meet this requirement, insulin action also stimulates the turnover of the pentose phosphate pathway (figure 3) (Milstein 1956; Winegrad and Renold 1958 b; Katz et al 1966). The primary function of this pathway is for the provision of NADPH, which in the adipose tissue is mainly used in fatty acid synthesis (Katz et al 1966).

In addition to the increase in fatty acid synthesis, insulin stimulates the incorporation of glucose into glyceride-glycerol, of triacylglycerols (Cahill et al 1959; Bally et al 1960). This reflects an increase in the esterification of coenzyme-A derivatives of fatty acids with glycerol-3-phosphate or dihydroxyacetone phosphate, although glycerol-3-phosphate is the preferred initial acyl acceptor (Dodds et al 1976).

The synthesis of glycerol-3-phosphate and dihydroxyacetone phosphate is predominantly from glucose via the glycolytic pathway. Due to the low glycerol kinase activity in adipose tissue (Shapiro et al 1957; Lynn et al 1960) the glycerol
produced as a by-product of lipolysis is not recycled.

The control of the esterification reaction by insulin is not very well understood. Jason et al (1976) have reported an insulin stimulation of the microsomal enzyme fatty-acyl-CoA ligase, and Sooranna and Saggerson (1975) have provided evidence suggesting that insulin promotes the action of an enzyme involved in the synthesis of TAG. Therefore it is possible that esterification is stimulated by a mechanism similar to the activation of enzymes as seen with fatty acid synthesis, however this has yet to be established.

I div Insulin binding

The initial interaction of insulin with the target cell is that of binding to a specific receptor molecule in the plasma membrane. This event occurs with all the polypeptide hormones which have been investigated, and has been extensively reviewed (Roth 1973; Roth et al 1975; Kahn 1976; Kahn et al 1981).

The measurement of this specific event is normally carried out using a radioligand assay, in which an isotopically labelled hormone is bound to whole cells or membrane preparations. This 'receptor assay' enables kinetic parameters as well as receptor-receptor interactions to be quantified (see reviews above).

Using mono-iodinated insulin ($^{125}$I-insulin), which has full bioactivity (Freychet et al 1971), specific insulin binding with fat cell plasma membranes and isolated adipocytes has been well documented (Freychet et al 1971, Cuatrecasses 1971; Kono and Barham 1971; Gammeltoft and Gliemann 1973;
FIGURE 3.

The Pentose Phosphate Pathway.

\[ \text{glucose-6-}O \quad \text{glucose-6-}O \text{ dehydrogenase} \]

\[ \text{6-}O \text{-glucono-5-lactone} \]

\[ \text{6-}O \text{-gluconate} \]

\[ \text{6-}O \text{-gluconate dehydrogenase} \]

\[ \text{ribose-5-}O \]

\[ \text{epimerase} \]

\[ \text{ribulose-5-}O \]

\[ \text{transketolase} \]

\[ \text{glyceraldehyde-3-}O + \text{sedoheptulose-7-}O \]

\[ \text{triose pool} \]

\[ *= \text{C-1 of glucose.} \]
Gliemann and Vinten 1975). This specific binding of insulin to its receptor, shows saturability, reversibility and temperature dependance (Roth 1973; Kahn 1976).

A simplistic representation of insulin binding is that of a bimolecular equilibrium reaction:

\[
[H] + [R] \xrightarrow{\frac{K_a}{K_d}} [HR]
\]

where \([H]\) is the concentration of free hormone, \([R]\) is the concentration of unoccupied receptors, \([HR]\) is the concentration of hormone-receptor complex and \(K_a\) and \(K_d\) are the rate constants for association and dissociation respectively. (Kahn 1976).

However, this simplistic model does not fully represent insulin binding; mathematical analysis of equilibrium binding data, using a \([HR]/[H]\) v.s. \([HR]\) 'Scatchard' plot, produces a hyperbolic curve suggesting that there are two insulin-receptor populations, one of high affinity for insulin and the other of low affinity (Pollet et al 1977).

An alternative explanation for the hyperbolic Scatchard data is that there are site-site interactions between the receptors, producing a negative co-operative effect (De Meyts et al 1973; 1976). Negative co-operatively is where the formation of an insulin-receptor complex increases the rate of dissociation (\(K_d\)) of the complex.

I e Biosynthesis and secretion of ACTH and related peptides

i The anterior lobe

In the anterior lobe of the pituitary, the synthesis of
ACTH and βLPH occurs by the maturation of a glycoprotein prohormone with an apparent molecular weight of 31K, on SDS PAGE (Mains et al 1977; Eipper et al 1976; Roberts and Herbert 1977 a,b; Nakanishi et al 1977). This prohormone has been given the name pro-opiomelanocortin (POMC).

Synthesis of this protein in a cell-free system, produces a polypeptide with a 28.5K apparent molecular weight (Roberts and Herbert 1977 a,b), although due to nascent glycosylation this product is never found in the cell.

Post-translational modification of the sugar side chains gives rise to two or three forms of the molecule (Roberts and Herbert 1977 a,b; Roberts et al 1978; Crine et al 1979; Loh 1979). The sugar components of these side chains may be important during processing since they are modified as maturation proceeds (Roberts et al 1978).

The maturation of this prohormone occurs in a sequential manner, by a series of specific proteolytic cleavages yielding ACTH-related peptides of apparent molecular weights 20-21K, 13K and 4.5K along with an 11K βLPH-like peptide and a 3.5K β-endorphin-like peptide (Mains et al 1977; Mains and Eipper 1978; Eipper and Mains 1978b; Roberts et al 1978). The sequence of POMC maturation is presented in figure 4.

All of the forms of ACTH are glycosylated, except for the 4.5K ACTH which appears to be the ACTH1-39 peptide (Eipper et al 1976; Eipper and Mains 1978 a). Analysis of the 13K ACTH molecule showed it to be ACTH1-39 which had been glycosylated at a point within the ACTH22-39 region (Eipper and Mains 1977). These two ACTH end products are formed from two different forms
Diagrammatic Representation of POMC Maturation.

$\mathbb{G}$ = carbohydrate side chains.
of POMC, one of which is glycosylated within the ACTH\textsubscript{22-39} region (Roberts et al 1978).

In addition to the ACTH and \beta LPH related peptides, POMC maturation produces a peptide which is not related to either ACTH or \beta LPH. This peptide has an apparent molecular weight of 16-17K, on SDS PAGE, and corresponds to the N-terminal sequence of the prohormone (Mains and Eipper 1978; 1979; Eipper and Mains 1978 a, b; Roberts et al 1978; Gianoulakis et al 1979; Crine et al 1980).

Although this 'N-terminal-POMC' peptide is apparently devoid of bioactivity, various related peptides, usually produced by tryptic digestion of the 16K molecule, have biological activity, associated with steroidogenesis (Pedersen and Brownie 1980; Pedersen et al 1980) and adrenal mitogenesis (Estivariz et al 1982).

The intermediate lobe

The 31K POMC prohormone is found in both the anterior and intermediate lobes of the pituitary and they appear to be identical (Rosa et al 1980). However, in the distinct intermediate lobe, the end-products of POMC maturation are different from those in the anterior lobe. Whereas the anterior lobe produces ACTH and \beta LPH, with some \beta-endorphin, the intermediate lobe produces \alpha MSH, CLIP and \beta-endorphin-like peptides. (Mains and Eipper 1979; Eipper and Mains 1978 b; Scott et al 1976; Crine et al 1978; Smythe and Zakarian 1980).

In the intermediate lobe, ACTH is probably cleaved to produce \alpha MSH (ACTH\textsubscript{1-13}) and CLIP (ACTH\textsubscript{18-39}) (Scott et al 1973, 1974). Although Kricarier et al (1973) have identified ACTH in
the pars intermedia this could be an artefact of the in vitro system used. Rosa et al (1980) noticed that the removal of the intermediate lobe cells from the inhibitory control of dopamine, increased both the content and secretion of 'large ACTH' forms. The intermediate lobe under normal physiological conditions probably contains no ACTH (Scott et al 1976) or only minimal amounts (Gianoulakis et al 1979).

In the intermediate lobe, the processing of βLPH occurs to a greater extent than in the anterior lobe, to produce β-endorphin-like peptides and γ-LPH as the major end-products (Crine et al 1978; Mains and Eipper 1979; Rosa et al 1980; Smythe and Zakarian 1980).

The specific cleavages that are required during the processing of POMC and the intermediate forms, occurs at sites, on the peptides, containing a pair of basic amino acids. (Nakanishi et al 1979; Chretien and Seidah 1981; Loh and Gainer 1982; Loh and Chang 1982). These cleavages are brought about by trypsin-like enzymes; certain peptides are then further modified by carboxypeptidase B-like enzymes (Loh and Gainer 1982; Loh and Chang 1982; Chretien and Seidah 1981).

The reason why the processing of POMC produces different products in the anterior and intermediate lobes is not known. One possibility is that the content of proteases responsible for the dibasic amino acid cleavages is different in the two lobes. Thus, the anterior lobe may contain the proteases necessary for ACTH synthesis whereas the intermediate lobe contains proteases which can produce αMSH and CLIP, these being absent in the anterior lobe.

Loh and co-workers (Loh and Gainer 1982; Loh and Chang
1982) have isolated intermediate lobe secretory granules which when incubated with POMC produced the intermediate ACTH forms, N-terminal POMC, βLPH, a β-endorphin-like peptide and αMSH. Prolonged incubation with the secretory granules did not produce any additional products. This study implies that the secretory granules of the intermediate lobe only contain the necessary proteases for POMC maturation in this tissue. Whether this is also the case with the secretory granules of the anterior lobe is not known.

Using a variety of protease inhibitors, the prohormone converting activity associated with the intermediate lobe secretory granules has been identified as an acid-thiol-arginyl protease(s) (Loh and Gainer 1982; Loh and Chang 1982).

I f Aims and scope of this investigation

The experimental aims of this thesis can be roughly divided into two parts: the biosynthesis of BCT in the ob/ob and +/+ mouse and the action of BCT upon adipose tissue.

For the work on the biosynthesis, the culturing of whole lobes for short periods of time, using the method of Scott et al (1976) was used. This was used in preference to the culturing of isolated pars intermedia cells because the yield of CLIP-like peptides was significantly greater with cultured lobes.

The aim of this work was to investigate the relationship, if any, between the BCT peptide and CLIP; the assumption being that due to its crossreactivity with 'CLIP-antibodies' (Bogdanovic 1978; Beloff-Chain et al 1980 a), BCT was related to CLIP.
This work however reached an impasse until a reliable method of peptide separation, other than Biogel P2/P6 gel filtration (Scott et al. 1976; Beloff-Chain et al. 1980a), was made available. The problem that was found with the Biogel P2/P6 gel filtration was that BCT coeluted with the salt peak, probably due to hydrophobic interaction. The outcome of this was that during incorporation experiments, using isotopically labelled amino acids, BCT could not be separated from the free labelled amino acids.

Various purification methods were tried, including ion exchange chromatography. BCT bound strongly to diethylaminoethyl (DEAE) cellulose, using a volatile triethylamine buffer system (Beloff-Chain et al. 1981). However the concentration and pH of the triethylamine buffer needed to elute the BCT was found to interfere with the 'CLIP-RIA' as well as interact with the peptide.

Affinity chromatography using 'CLIP antibodies' immobilised on a sepharose column enabled BCT to be further purified, although this system was not efficient enough for the very small samples of BCT produced during incorporation experiments.

The problem of desalting BCT was eventually solved using reverse-phase HPLC (Beloff-Chain et al. 1981), which could efficiently handle low BCT concentrations.

The possibility that BCT may have a lipogenic activity upon the adipose tissue in vivo (see Ic) necessitated the investigation of this phenomenon in vitro.

Isolated adipocytes, produced by a modification of the method of Rodbell (1964) were used to investigate the possible lipogenic action of BCT. The adipocyte preparation was chosen.
because of the ease of production of the cells and the reliability of the preparation.

With the discovery that BCT had lipogenic activity (Beloff-Chain et al 1982 a), questions arose about the possibility of further BCT action upon the adipose tissue. This apparent insulinomimetic action of BCT upon isolated adipocytes suggested that BCT may effect other insulin mediated processes. Thus the relationship of BCT activity to insulin activity, in the adipose tissue, was investigated along with some preliminary experiments into BCT's mechanism of action.
II. MATERIALS & METHODS.
II a Animals

i Obese hyperglycaemic (ob/ob) mice

The obese hyperglycaemic (ob/ob) mouse originated in the Jackson Memorial Laboratory, Bar Harbour, Maine, U.S.A. and had been introduced into two mixed colonies (not pure strains) in Birmingham and Edinburgh. Stocks from these two strains were originally used to start the Imperial College colony (Abraham and Beloff-Chain 1971).

The colony was maintained by crossing ob/ob males, on a restricted diet, with heterozygous (ob/+) females. The obese offspring were detected by appearance at about 4 weeks of age and were then separated from their lean litter mates.

ii Homozygous lean (+/+) mice

The homozygous lean colony was originally started by breeding out the ob gene from lean phenotypes (Beloff-Chain et al 1975 b), and was maintained using mice which had been screened for the absence of the ob/ob gene.

To prevent the two colonies, ob/ob and +/+ , from diverging, leans were frequently introduced into the obese colony and screened lean homozygotes, from the obese colony, were introduced into the lean colony.

Only mice between the ages of 8 weeks and 12 weeks were used in these experiments.

iii Sprague-Dawley rats

All rats used in these experiments were male Sprague-Dawley albino rats, weighing between 180 to 220g. These animals were bred in the department; after weaning the male rats were separated from their female littermates.
iv Maintenance of the animals

All the animals were housed in polycarbonate cages lined with sawdust. At all times the animals had free access to food (R & M No.1 expanded diet, Special Diet Services, Witham, Essex) and water. The rooms in which they were kept were maintained at about 22°C with a 12 hour lighting period from 8am to 8pm.

II b Materials

All chemicals were of analytical grade unless otherwise stated.

Mineral salts, glucose, charcoal and mineral acids : B.D.H., Poole, Dorset


Radiochemicals : Amersham International, Amersham, Bucks.


Synthetic ACTH_{34-39} : Bioproducts, Cambridge.


Crystalline bovine albumin : Armour Pharmaceuticals, Eastbourne, Sussex.
Bovine serum albumin -V : Miles, Stoke Poges, Bucks.
Gases : British Industrial Gases, Dagenham, Essex.
SDS-PAGE equipment and chemicals and Biogel P2 and P6 : Biorad, Fullerton, Calif.
Enzymes, insulin, ACTH and : Sigma Chemical Company, Poole, Dorset.
otherwise stated.

The bovine serum albumin was dialysed with six changes of Krebs-Ringer bicarbonate buffer before being aliquoted into lg lots and stored at -20°C.

II c Physiological buffers

The main physiological buffer used was Krebs-Ringer bicarbonate buffer (Krebs and Henseleit 1932) which contained:

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Chemical</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025M</td>
<td>NaHCO₃</td>
</tr>
<tr>
<td>0.118M</td>
<td>NaCl</td>
</tr>
<tr>
<td>0.0012M</td>
<td>MgSO₄·2H₂O</td>
</tr>
<tr>
<td>0.0047M</td>
<td>KCl</td>
</tr>
<tr>
<td>0.0025M</td>
<td>CaCl₂·2H₂O</td>
</tr>
<tr>
<td>0.0012M</td>
<td>KH₂PO₄</td>
</tr>
</tbody>
</table>

Before each experiment, after the addition of the appropriate amount of glucose and albumin, the buffer was thoroughly gassed with 95% O₂:5% CO₂ for at least 30 minutes.

The binding studies were performed using a modification of Hanks buffer (Earle 1943) containing hepes (N-2-hydroxyethylpiperazine-N¹-2-ethanesulphonic acid). This buffer
contained:

- 0.026M NaHCO$_3$
- 0.116M NaCl
- 0.0008M MgSO$_4$.7H$_2$O
- 0.0054M KCl
- 0.0018M CaCl$_2$.2H$_2$O
- 0.001M NaH$_2$PO$_4$.2H$_2$O
- 0.02M Hepes

To this buffer were added 5.5mM glucose and 2% albumin (w/v) and it was then thoroughly gassed with O$_2$ for at least 30 minutes.

**II di Scintillation counting**

All scintillation counting was performed using a Kontron IN (Intertechnique) SL-3000 Scintillation counter.

The scintillation fluid used was produced in the laboratory and consisted of toluene: triton X100 (Rohm Maas, Croyden) 2.25:1 (v/v) with 0.23% (w/v) 2,5- diphenyloxazole (PPO) and 0.0071% (w/v) 1,4 -bis-(5-phenyloxazol-2-yl) benzene (POPOP).

When aqueous samples were being counted, the volume of the sample never exceeded 10% since this caused emulsion collapse.

The only exception to the use of the toluene: triton scintillation cocktail was with the cAMP assay. Here, Atomlite (N.E.N., Soton, Hants) was used due to its high aqueous tolerance.

The efficiency of counting was determined by the external standard method, for both $^3$H and $^{14}$C. Using a known 'dpm' of [{$^{14}$C}] or [{$^3$H}] n-hexadecane, a quench curve was produced by the
$^3\text{H} \& ^{14}\text{C}$ Quench Curves.

FIGURE 5.
sequential addition of acetone as a quenching agent (figure 5). All subsequent efficiency determinations were obtained by relating the E.S.R. of the sample to the appropriate quench curve.

ii Gamma counting

All gamma counting of $^{125}$I was performed on a LKB wallac 1270 Rackgamma II (LKB, Turku, Finland) with an efficiency of approximately 75%.

II e Methods

i Biogel P2/P6 gel filtration

Peptide fractionation was routinely carried out by gel filtration using a Biogel P2 (200-400 mesh) 1.5x30cm column run in series with a Biogel P6 (200-400 mesh) 1.5x90cm column (Scott et al. 1976; Beloff-Chain et al. 1980a). The columns were initially equilibrated with 5% (v/v) acetic acid containing 0.02% (w/v) bovine plasma albumin. Elution of the columns was performed using the same acetic acid buffer with a flow rate of 3ml/hr, at 4°C. Thirty minute fractions were collected.

A maximum sample volume of 5ml was used although a volume of 1 or 2ml was generally loaded onto the column. All samples were centrifuged in a bench centrifuge, before loading, to remove insoluble material.

The void volume ($V_0$) of the columns was determined using Dextran blue and the bed volume ($V_b$) was found using 10uCi of $^3$H$_2$O.

II eii Reverse phase - High Pressure Liquid Chromatography

Samples were applied to a Kontron high pressure liquid
chromatograph with a µBond pack C18 column (25x0.8cm).

Two gradients were routinely used to fractionate BCT and related peptides. The most commonly used was a 30 minute linear gradient of 10% (v/v) to 40% (v/v) propan-1-ol:5% (v/v) acetic acid with a flow rate of 1ml/min at ambient temperature; 1 or 2ml fractions were collected (Beloff-Chain et al 1981).

The second gradient used was a 30 minute concave gradient of 10% (v/v) to 25% (v/v) propan-1-ol:5% (v/v) acetic acid with a flow rate of 2ml/min at ambient temperature; one minute fractions were collected over a 45 minute period.

This gradient was developed to separate the various peptides in a tryptic digest of iodo-CLIP (figure 6) (D Hughes, unpublished data). It became necessary to develop this second gradient because the linear 10%-40% gradient did not adequately separate the iodo-derivatives of BCT and CLIP from their respective parent peptides.

The 10-40% linear gradient was used to purify N-Ac-BCT (Beloff-Chain et al 1981).

II e iii Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis

Separation of the 'void volume' proteins from Biogel P2/P6 columns was by the method of Laemmli (1970). Slab gels of 10-20% acrylamide were produced using a gradient maker, from a stock solution of 30% acrylamide (w/v), 0.8% N,N'-bis-methylene acrylamide (w/v) in 0.375M Tris-HCl pH8.9, 0.1% SDS (w/v). The appropriate dilutions of the acrylamide solution were made using the same buffer. Polymerisation was induced by the addition of 0.025% tetramethylethylenediamine (TEMED) (v/v)
Figure 6

Reverse-phase HPLC separation of CLIP, BCT and their iodo derivatives

CLIP which had been iodinated with $^{127}$I, as previously described, was digested with trypsin at an enzyme:substrate ratio of 1:2. The digest was then fractionated using reverse-phase HPLC with a 30 min concave gradient of 10% (v/v) to 25% (v/v) propan-1-ol:5% (v/v) acetic acid at ambient temperature. A flow rate of 2ml/min was used and 2ml fractions were collected. The elution profile was determined from the absorption at 280nm.

(Reproduced by permission of D.Hughes).
and 0.01% ammonium persulphate (w/v). The stacking gel was made up of 3% acrylamide (w/v) in 0.125M Tris-HCl pH6.8, 0.1% SDS (w/v), was poured onto the 10-20% gel and polymerised as above.

Dried samples were taken up in 25ul of 0.01M Tris-HCl pH6.8, 20% glycerol (v/v), 1% 2-mercaptoethanol (v/v), 1% SDS (w/v), 0.001% bromophenol blue (w/v) and boiled for 2 minutes. After cooling, appropriate sample volumes were loaded and stacked at 50V. When stacked the gels were run at 150V until the bromophenol blue marker had reached the bottom of the gel. The electrode buffer used was 0.025M Tris-HCl, 0.192M glycine, 0.1% SDS (w/v), 1% agrose (w/v), pH8.3.

The gels were stained in 0.05% Coomassie blue (w/v) in 20% propan-2-ol (v/v), 20% acetic acid (v/v) in water for 3 hours and destained overnight in 10% propan-2-ol (v/v), 10% acetic acid (v/v) in water.

The molecular weights of the stained proteins was determined from marker proteins (Sigma, Poole, Dorset) which were run simultaneously. Then the gel was cut up into 2mm slices and dissolved in 0.5ml Soluene (Packard, Groningen, Holl) overnight. 4.5ml of scintillation cocktail was added and the radioactivity was determined using liquid scintillation counting.

IIe iv Neurointermediate lobe incubations

Neurointermediate lobes of ob/ob and +/+ mice were incubated in an identical manner.

The mice were killed by cervical dislocation followed by decapitation. The top of the skull was removed and the exposed brain was scooped out to reveal the pituitary. After removal of the covering membrane, the pituitary was carefully removed.
and transferred to a petri dish containing media well gassed with 95% \( \text{O}_2:5\% \text{CO}_2 \). Using fine 'watchmakers' forceps, the NIL was carefully teased away from the anterior lobe.

Batches of 5 NIL were placed in 250 ul of medium, in plastic 5ml stoppered tubes and incubated at 37°C for 5 hours (Beloff-Chain et al 1980a). At the end of the incubation, the NIL were removed and the media was pooled and lyophilised.

Generally incubations were carried out using Dulbecco's modification of Eagle's medium or RPMI 1640 (Flow Labs, Paisley, Scotland). However during incubations with isotopically labelled amino acids, Minimal Essential Medium, deficient in the relevant amino acid, (Gibco, Paisley, Scotland) was used.

In order to prepare native BCT, for experiments upon isolated adipocytes, the media from 50 ob/ob NIL was pooled and lyophilised. The BCT was then purified by P2/P6 gel filtration. After a second passage down the P2/P6 column (see II ei) the BCT was aliquoted out into lots of about 50ng and these were dried down under vacuo and stored at -20°C.

IIe v Acetic acid extraction of neurointermediate lobes

NIL lobes were placed into 1ml of glacial acetic acid and homogenised for 1 minute using a Polytron vortex mixer (Vortex mixers, Hampton, Middx). After 1 minute, 1ml of distilled water was added and the NIL were homogenised for a further minute.

The acetic acid extract was then decanted into a siliconised flask and lyophilised.

IIe vi Tryptic digestion of synthetic CLIP

Synthetic CLIP, in 0.005M HCl, was added to 250ul of 0.1M
NH₄HCO₃, pH 8.5. Trypsin treated with diphenyl carbamoyl chloride to inhibit chymotryptic contamination, (7770 BAEE units/mg protein) was dissolved in the same buffer and added to give the required enzyme:substrate ratio. The reaction was incubated at 37°C for 2 hours, after which the reaction was stopped by either placing in a boiling water bath for 3 minutes or by adding 50μl of glacial acetic acid.

The sample was then dried down under vacuo.

For the routine production of 'synthetic BCT', 5μg of CLIP was digested with an enzyme:substrate ratio of 1:5 or 1:2. After digestion the BCT was purified by twice passing it down a P2/P6 gel filtration column (IIe i). The BCT was then aliquoted into 50ng lots which were dried down and stored at -20°C.

It was noticed that BCT which had been kept more than 2 months lost its bioactivity suggesting that the peptide may be labile. Thus new batches of BCT were usually prepared monthly.

BCT produced by this method was used in most experiments using isolated adipocytes and adipose tissue pieces; in the relevant section on the effects of BCT on adipose tissue, the term BCT will refer to tryptically produced ACTH_{22-39} (see above) while the term native BCT will refer to ACTH_{22-39} produced from the incubation of ob/ob NIL (IIe iv).

IIe vii CLIP radioimmunoassay

All measurements of CLIP-like peptides were made using a radioimmunoassay. This assay used an antiserum raised against synthetic CLIP (donated by Dr. J.A. Edwardson, MRC, Newcastle).
100μl samples in 0.05M Na₂HPO₄, pH 7.4 (phosphate buffer) 0.5% 2-mecaptoethanol (v/v), 0.5% albumin (w/v) were incubated overnight at 4°C with 50μl of ¹²⁵I-CLIP (1:1000 dilution) and 50μl of 'CLIP antisera' (1:5000 dilution), in the same buffer.

With each assay a standard curve of 20pg/ml to 20ng/ml CLIP was produced and incubated with ¹²⁵I-CLIP and CLIP antiserum (figure 7).

Separation of the bound and free peptides was by the addition of 3% Norit SX1 charcoal (w/v) in 0.05M phosphate buffer, 80% horse serum -5(v/v), 0.75% dextran (av. m.w. 79,000) and the samples were centrifuged at 3000 rpm for 15 minutes. The supernatant was then aspirated off and the charcoal pellet, containing the free peptide, was counted using the LKB gamma counter.

The concentration of CLIP-like material in the samples was determined with relation to the standard curve.

Throughout this thesis all stated values of BCT are in ng of CLIP immunoactivity.

**The viii Iodination of CLIP**

A modification of the chloramine-T method of Hunter and Greenwood (1962) was used to iodinate CLIP.

0.5mCi of Na¹²⁵I (100mCi/ml in dil. NaOH pH7-11) was placed in an amino acid autoanalyser vial and was neutralised with 10μl of 0.25M KH₂PO₄, pH 7.4. To this was added 1μg CLIP in 5μl of 0.005M HCL. The reaction was started by the addition of 10μl of 2.5mg/ml chloramine T (N chloro-p-toluene sulphanamide, Na) in 0.05M of the phosphate buffer and the vial was agitated for 25 seconds. Termination of the reaction was by the addition of 10μl of 10mg/ml sodium metabisulphite in
A typical CLIP-RIA standard curve.

FIGURE 7.
0.05M phosphate buffer, pH 7.4, followed by dilution of the reaction mixture in 10ml of 0.05M phosphate buffer pH 7.4, 0.5% 2-mercaptoethanol (v/v), 0.25% bovine plasma albumin (w/v).

Purification of the iodinated peptide was carried out by adsorption to Vycor glass. 100mg of Vycor glass (pretreated at 600°C for 1 hour) was added to the iodinated peptide in 10ml of 0.05M phosphate buffer in a plastic tube. The tube was securely capped and the mixture was rotated for at least 35 minutes at 4°C. After rotation, the Vycor glass was centrifuged down at 2000 rpm for 10 minutes and the supernatant was discarded. The Vycor glass was washed with 2ml of distilled water which was discarded after centrifugation at 2000 rpm for 10 minutes.

Elution of the peptide from the Vycor glass was by the addition of ice cold 60% (v/v) acetone in water or with propan-1-ol:5% (v/v) acetic acid, 1:1 (v/v) with a 35 minute period of rotation at 4°C. The Vycor glass was then pelleted by centrifugation and the supernatant containing the iodinated peptide was decanted and stored at 4°C.

The incorporation of iodine by CLIP was about 20% as determined by the radioactivity associated with the 1ml of eluted peptide. However, using an aliquot of 'cold' CLIP the percentage recovery off the Vycor glass was found to be only around 20%. Thus it is assumed that almost total incorporation was achieved by this method.

The \textsuperscript{125}I-CLIP in 60% (v/v) acetone was routinely used at a dilution of 1:1000 in the radioimmunoassay.

\textbf{Ile ix} \textit{Synthesis of iodinated BCT}

\textsuperscript{125}I-BCT was synthesised by the tryptic digestion of
I-CLIP. 2μg of CLIP were iodinated, as above, and eluted from the Vycor glass using propan-1-ol:5% (v/v) acetic acid 1:1 (v/v). After drying down under vacuo, the iodinated peptide was taken up in 250μl of 0.1M NH₄HCO₃ pH 8.5 and digested using trypsin (see IIe vi). The reaction was stopped by the addition of 50μl of glacial acetic acid and the digest was dried down under vacuo.

Purification of the iodo-peptides was by HPLC (see IIe ii). The synthesis of 'cold' ¹²⁷I-BCT was from ¹²⁷I-CLIP as above. CLIP was iodinated with ¹²⁷I in an identical manner to that using ¹²⁵I except that 5μg of CLIP was iodinated and 10μl of 250μM KI (in 0.005M NaOH) was used as the iodine source.

After the iodination reaction had been terminated, the ¹²⁷I-CLIP was desalted using a Sep-Pak C₁₈ cartridge (Waters Assoc., Milford, Mass.). The reaction mixture was slowly drained through the 'Sep-Pak' to allow adsorption of the peptide. The cartridge was then washed through with 20ml of distilled water and elution of the ¹²⁷I-CLIP was with 2ml of propan-1-ol:5% (v/v) acetic acid 1:1 (v/v). The eluted peptide was dried down under vacuo and then digested with trypsin (see IIe vi).

Separation of the iodo-peptides was by HPLC (see IIe ii). Attempts at iodinating purified BCT were made, using the method described above. For reasons unknown, the iodine did not become attached to the tyrosine residue in BCT.

One possibility may be that, due to the hydrophobic nature of the peptide, the BCT did not go readily into solution in the reaction mixture and hence did not become iodinated.

**IIe x Iodination of insulin**

Insulin was iodinated by essentially the same method used
for CLIP. 0.5mCi of Na$^{125}$I (100mCi/ml) was neutralised with 10μl of 0.25M phosphate buffer pH7.4. 2μg of insulin in 5μl of 0.005M HCl was added and the reaction was started by adding 3.5mg/ml chloramine-T in 0.25M phosphate buffer. After 25 seconds, the reaction was stopped with 100μl of 2.5mg/ml sodium metabisulphite in 0.25M phosphate buffer.

The reaction mixture was then applied to a sephadex G25(μw) (Pharmacia, Uppsala, Sweden) column (1.5x30cm), which had been equilibrated with Hanks-hepes buffer, 2.5% albumin (w/v), and the $^{125}$I-insulin was eluted at 1.5ml/min and 1.7ml fractions were collected. The $^{125}$I-insulin peak was the first radioactive peak to be eluted, the free iodine emerged at the salt peak.

This method incorporated 0.4 atoms of iodine per molecule of insulin and this labelled insulin was directly used for binding to isolated adipocytes.

IIe xi N-Acetylation of BCT

1μg of BCT, synthesised by the tryptic cleavage of CLIP, was taken-up in 20μl of distilled water. To this was added 90μl of methanol:acetic anhydride 2:1 (v/v) and the solutions were mixed. The reaction was left for 5 minutes and then terminated by drying down the sample under vaccuo.

Purification of the N-acetylated-BCT was by HPLC (see IIe ii).

IIe xii Preparation of isolated adipocytes

Isolated rat adipocytes were routinely prepared using a variation of the method of Rodbell (1964).

Male Sprague-Dawley rats were stunned by a blow to the
head and killed by cervical dislocation. The epididymal fat
pods were rapidly removed and rinsed in Krebs-Ringer bicarbonate
buffer. The fat pad was then cut into small pieces and placed
in a siliconised 150ml flask, containing 40ml of Krebs-Ringer
bicarbonate buffer (without calcium), 5.5mM glucose and
approximately 10mg/g tissue collagenase (from clostridium
histolyticum; Boehringer, GmbH, Mannheim, W.Germany). After
gassing with 95%O2:5%CO2, the flask was sealed and incubated
in a shaking water bath at 37°C for 1 hour.

At the end of this incubation, the digested tissue was
gently stirred using a plastic rod, to further release the
adipocytes, and the suspension was then filtered through a
nylon mesh to remove undigested lumps of tissue.

The isolated cells were placed in plastic centrifuge
tubes and centrifuged for 30 seconds at 400rpm. The infranatent
containing collagenase was aspirated off and discarded and the
cells were resuspended in Krebs-Ringer bicarbonate buffer
(with calcium), 5.5mM glucose, 4% albumin (w/v). This washing
procedure was repeated twice.

Depending on the purpose for which the isolated adipocytes
were to be used, the concentration of glucose and albumin were
altered as indicated in the results. For the binding studies
the buffer itself was changed and a Hanks-Hepes buffer was
used. When this occurred, the adipocytes were prepared using
the glucose and albumin concentrations cited.

In all experiments, the cells were dispensed using an
automatic pipette with a plastic tip. The orifice of the tip
was enlarged in order to minimise cell damage during dispersion.

Cell numbers were determined using a siliconised Improved
Neubauer Haemocytometer (Gallenkamp, London). From a well suspended adipocyte preparation, at least three counts were performed and the mean value was used.

**Incubation of isolated adipocytes and adipose tissue pieces**

Isolated adipocytes were incubated in a total volume of 1ml in Krebs-Ringer bicarbonate buffer, in the presence or absence of the test substances. The glucose and albumin concentrations were varied depending on the type of incubation. All incubation were carried out in plastic or siliconised glass scintillation vials and were sealed with rubber Suba-seals.

In experiments in which the incorporation of $^3$H, from $^3$H$_2$O, into lipids was measured, the buffer was supplemented with 1mCi/ml $^3$H$_2$O while when glucose metabolism was being investigated [1$^4$C] glucose or [6$^1$C] glucose at 0.1 μCi/μmol. glucose was added. (Rodbell 1964).

The incubations were performed in a shaking water bath at 37°C for 1 hour at 45rpm, unless otherwise stated.

Adipose tissue pieces of approximately 100mg were incubated under identical conditions to that described above, except that 2ml of Krebs-Ringer bicarbonate buffer was used.

**Measurement of [1$^4$C] glucose oxidation**

In experiments in which the oxidation of isotopically labelled glucose was to be measured, 0.5ml plastic tubes were inserted into the incubation vials before they were sealed. Approximately 5 minutes before the incubation was to be terminated, 200μl of hyamine hydroxide solution (1M methylbenzethonium hydroxide in methanol) was injected through
the rubber seal and into the plastic tube. The incubation was terminated by injecting 100μl of 5M H₂SO₄ into the adipocyte preparation and the vials were left overnight at 4°C to enable CO₂ to be absorbed.

For counting, the hyamine hydroxide was decanted out of the tube, and the tube was washed with methanol. The washings and hyamine hydroxide solution were pooled and added to 4.5ml of triton-toluene scintillation cocktail. The radioactivity was determined by liquid scintillation counting.

IIe xv Extraction of lipids

Total lipid extractions, from adipocytes and adipose pieces, were carried out using the method of Folch et al (1957).

Where sulphuric acid had been used to terminate incubations of adipocytes and adipose tissue pieces, this was first neutralised using NaOH aq.

The lipids were extracted from adipocytes by the addition of 20ml of chloroform:methanol 2:1 (v/v). Insoluble material was removed by filtering the extract under vacuo and a further 5ml of the chloroform:methanol was used to wash the incubation vial and filtered material. This was pooled with the filtrate.

In experiments in which lipids were extracted from adipose tissue pieces, the latter were blotted dry on filter paper and were added directly to 20ml of chloroform:methanol and left overnight before filtration.

5ml of 0.1M KCl was added to the tubes which were stoppered and shaken vigorously and then allowed to stand to enable the chloroform and aqueous layers to separate. After
separation, the upper aqueous layer was aspirated off and discarded. This process was then repeated using 5ml of an upper phase of chloroform:methanol:0.1M KCl 3:48:47 (v/v/v).

When the adipocytes or adipose pieces were incubated with $^3$H$_2$O, an extra washing of 5ml of 0.1M KCl was used to ensure total removal of the $^3$H$_2$O.

The remaining chloroform layer, containing the extracted lipid, was then dried down under a stream of nitrogen gas in a 50-60°C water bath.

Cell-free incubation samples were extracted and washed as described above to ensure that the washing of the extract efficiently removed the unincorporated $^3$H$_2$O or $[^{14}$C] glucose. After drying down the chloroform layer was found to contain no $^3$H$_2$O while the $[^{14}$C] glucose content was less than twice background.

Total lipid samples of the extract were obtained by dissolving the lipid in 5ml of chloroform. An aliquot, usually of 1 or 2ml, was removed and placed in a scintillation vial insert to dry down overnight. 5ml of scintillation cocktail was added to the dried material and the samples were counted using liquid scintillation counting.

With the experiments using $^{14}$C labelled glucose the remaining chloroform-lipid solution was dried down for saponification.

**IIe xvi Saponification**

Fatty acids were extracted from lipid extracts as well as adipocyte suspensions by saponification.

When adipocytes were incubated with $^3$H$_2$O, a 500µl aliquot
of the cell suspension was added directly to 2ml of 40% KOH (w/v) and 1.5ml ethanol, in a stoppered boiling tube. With a lipid extract, 5ml of 2M KOH in methanol was added to the dried down lipid. In both cases the saponification was performed in a similar manner, except with the lipid extract of $^{14}$C labelled lipids, methanol was used instead of water to facilitate drying down after fatty acid removal.

The stoppers of the boiling tubes were lightly greased and firmly sealed into the boiling tubes. The tubes were then placed in a 80°C water bath for 3 hours. At the end of this time the tubes were removed from the water bath and allowed to cool. When cool, the silicone grease was wiped off the fittings and 5ml of water/methanol was added.

Unsaponified lipids were removed from the 'soap solution' by adding 10ml of petroleum-ether (bp40-60°C) and shaking vigorously. The layers were allowed to separate and the upper ether layer was aspirated off and discarded.

For the extraction of the saponified fatty acids, the aqueous/methanol layer was acidified with concentrated hydrochloric acid, using phenolphthalein as an indicator. 10ml of petroleum-ether was added to the acidified solution and the tubes were vigorously shaken. After the two layers had separated, the upper ether layer was aspirated off and kept. This procedure was repeated twice, the three ether extractions then being pooled and dried down under a stream of nitrogen.

After drying, the fatty acids were taken up in 5ml of scintillation fluid and the radioactivity was determined by liquid scintillation counting.

The efficiency of extraction of the saponified fatty
acids was determined by carrying out further petroleum-ether extractions of the aqueous layer until the radioactivity associated with these extractions was equivalent to background. From these pooled washings, the initial extraction efficiency was determined as 99±0.2% (4).

IIe xvii Determination of $[^{14}\text{C}]$ glucose incorporation into glyceride-glycerol

The incorporation of $[^{14}\text{C}]$ glucose into glyceride-glycerol was determined by a variation of the method of Denton and Randle (1967). After the saponified fatty acids were removed from the saponified lipid extract, the remaining methanol layer was left containing the glyceride-glycerol.

This methanol layer was filtered under vacuo to remove the KCl precipitated by the addition of the hydrochloric acid. The boiling tube was then washed with methanol and this was added to the filtrate. The filtered precipitate was also washed with methanol to remove any trapped glycerol. The pooled filtrate was then dried down in a 50°C water bath. When dry, the glyceride-glycerol was taken up in 1ml of distilled water and added to 9ml of triton-toluene scintillation cocktail. The radioactivity was determined by liquid scintillation counting.

IIe xviii Calculation of the rate of lipogenesis from $^3\text{H}$ incorporation into fatty acids

The rate of lipogenesis was calculated from the relative total activity (RTA) (Windmueller and Spaeth 1966) which is equivalent to:

$$
\frac{^3\text{H in fatty acids dpm}}{\text{dpm } ^3\text{H}_2\text{O / ug atom H (H}_2\text{O)}}
$$
The specific activity of the $^3\text{H}_2\text{O}$, in dpm/μl, was calculated from a cell-free incubation sample. Using the value of 53M for water, this implied that there were 106 μg atoms H in 1μl of H$_2$O.

The RTA does not allow for the discrimination, during lipogenesis, against $^3\text{H}$ compared with $^1\text{H}$. To correct for this, the discrimination factor of 2.29 (Jungas 1968), for rat tissue in vitro, was adopted.

Finally to convert to μmol C$_2$, the RTA was divided by 4 to account for the 4H:2C ratio. These results were then expressed in relation to $10^6$ cells or per g wet weight of tissue.

2. Calculation of metabolised glucose

The amount of glucose utilised, either by oxidation or incorporation into the parameters measured, was calculated from the known specific activity of the respective $^{14}\text{C}$ atom. All the glucose metabolism results were expressed in nmol C-atom incorporated and this value was corrected to give the results per $10^6$ cells or per g wet weight of tissue.

IIe xix The separation of adipocytes from incubation media by centrifugation with silicone oil

The separation of isolated adipocytes from the incubation media was achieved using the centrifugation method of Gliemann et al (1972), although silicone oil (Olefsky 1978) was used instead of dinonyl phthalate.

100-200μl aliquots of adipocyte suspension were layered upon 100μl of silicone oil (Dow Corning 200/200 cs, relative density = 0.97) (BDH, Poole, Dorset), in plastic Beckman microtubes, and centrifuged for 30 seconds in a Beckman
microfuge (Beckman Inst, Inc., Spinco Div., Palo Alto, Calif.) at 10,000g.

The 0.97 relative density of the silicone oil allows the denser incubation media to pass through the oil on centrifugation, leaving the adipocyte layer above the oil. The cell layer can then be excised with a sharp knife and an aliquot of the incubation media was taken to enable correction for the extracellular water space trapped with the cell layer.

This trapped extracellular water space was determined using \([^{14}C]\) sucrose (Gliemann et al 1972). Identical incubations to those being carried out were performed in 1ml of the incubation buffer, 0.125mM \([^{14}C]\) sucrose (specific activity 8mCi/mM) for 3 minutes. Triplicate 200ul aliquots of adipocyte suspension were separated into the cell layer and incubation buffer as described above. The extracellular water space of the cell layer was calculated from the cpm/ul of an aliquot of the cell-free buffer, which is related to the \([^{14}C]\) sucrose trapped in the cell layer. Sucrose was used since it is neither taken up or metabolised by the adipocyte.

IIe xx Determination of glucose transport using isotopically labelled 2-deoxyglucose

Glucose transport was measured by a variation of the method described by Olefsky (1975) involving the uptake of isotopically labelled 2-deoxyglucose by the adipocytes. Once in the cell, this glucose analogue is phosphorylated but is not metabolised further (Wick et al 1957).

Isolated adipocytes were preincubated, in the presence or absence of the test substances, in 1ml of Krebs-Ringer bicarbonate buffer (without glucose), 2% albumin (w/v) for
30 minutes at 24°C. At the end of this preincubation, a 500ul aliquot of the cell suspension was transferred to 500ul of Krebs-Ringer bicarbonate buffer, 0.25mM 2 deoxy-[1^14C]-D-glucose (specific activity 8mCi/mM) and incubated at 24°C for 3 minutes.

The reaction was terminated by the separation of the cells from the incubation buffer, of a 200ul aliquot, using the silicone oil centrifugation technique. Termination was considered to have occurred when the centrifugation was started. After centrifugation, the excised cell layer and a 50ul aliquot of the cell-free buffer were both added to 5ml of scintillation cocktail and counted by liquid scintillation counting. All readings were performed in triplicate.

The specific activity of the [1^14C] 2-deoxy-glucose solution, in cpm/nmol, was determined from an aliquot of a cell-free sample. From this value, the nmol of 2-deoxy-glucose in the cell layer was determined, after correction for the extracellular water space.

The enzymatic-spectrophotometric determination of glycerol (lipolysis)

The free glycerol concentration in adipocyte suspensions was determined by the method of Weiland (1963). The principle of this assay is based upon the action of two enzymes:

1. \[ \text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{L(-)-glycerol-3}^\text{P} + \text{ADP} \]

2. \[ \text{L(-)-glycerol-3}^\text{P} + \text{NAD}^+ \xrightarrow{\text{GDH}} \text{dihydroxyacetone}^\text{P} + \text{NADH} + \text{H}^+ \]
Glycerokinase (GK) (ATP:glycerol phosphotransferase)  
NAD- dependant glycerophosphate dehydrogenase (GDP)  
(sn-glycerol-3-P :NAD 2-oxidoreductase).

Since the equilibrium of reaction (2) lies to the left, the reaction is carried out at pH9.8 with the dihydroxyacetone-P being trapped by hydrazine, forcing the equilibrium to the right.

The glycerol concentration of the sample is measured by following the increase in A3100, a measure of NADH formation.

1. Deproteinisation of the sample

0.5ml of the adipocyte suspension was added to 50µl of 5M perchloric acid, followed by vigorous whirlmixing to ensure complete protein precipitation. The sample was centrifuged at 3000rpm, for 15 minutes, and the supernatant was aspirated off the pellet and kept. 500µl of 0.5M perchloric acid was then added to wash the pellet. After centrifugation the supernatant was again aspirated off and pooled with the initial supernatant.

Using indicator paper, the pH of the sample was adjusted to 9.95 with 10M KOH and the volume of the sample was made up to 1.25ml with distilled water. The samples were then placed on ice for at least 10 minutes and the precipitated KC104 was separated by centrifugation. The supernatant was removed and stored at -20°C until used in the glycerol assay.

2. Glycerol assay

The reaction was performed in a quartz cuvette (light path = 1cm) into which were added:
1.4ml 0.2M glycine, 1M hydrazine (as hydrazine hydrate), 2mM MgCl₂; pH 9.8  
0.05ml 50mM ATP-Na₂H₂.3H₂O, pH 7  
0.05ml 20mM β-NAD  
0.02ml GDH suspension (50U/mg protein)  
0.5ml deproteinised sample

The contents were mixed and the A₃₄₀ was measured for at least 2 minutes to establish the 'constant creep'. Once a stable lower 'constant creep' value had been obtained, 0.05ml of GK suspension (95 U/mg protein) was added to the cuvette, the solution was mixed and the A₃₄₀ was followed to its peak value. After the A₃₄₀ peak had been reached, the upper 'constant creep' value was followed for at least 2 minutes. To enable these 'creep' values to be ascertained, a spectrophotometer with an attached chart recorder was used for all the 340nm readings.

The A₃₄₀ value corresponding to the glycerol concentration of the sample was calculated by the extrapolation of the two 'creep' values and measuring the difference between these extrapolations at the peak, i.e. ΔA₃₄₀.

At the end of the reaction, a further 0.02ml of GK suspension was added to the cuvette to determine the ΔA₃₄₀ due to the GK enzyme. This value was subtracted from the ΔA₃₄₀ obtained for the sample.

3. Calculation of results

The glycerol concentration of the sample was determined by the calculation:

\[ c = \frac{\Delta A_{340}}{\varepsilon x l} \times \frac{V}{v} \]

where:
c = glycerol concentration in μmol/ml
ε = the extinction coefficient for NADH = 6.22 cm²/μmol
d = light path = 1 cm
V = total sample volume = 2.04 ml
v = sample volume = 0.5 ml

This value gives the concentration of glycerol in the sample, which after correction for the dilution occurring during deproteinisation, was converted to μmol glycerol/10⁶ cells.

IIe xxii cAMP assay

The assay is based on the prelabelling of adenyl nucleotides, using [³H] adenine, in isolated fat cells (Humes et al 1969). After incubation of the adipocytes with the test substances, the cAMP from the cells is purified and the radioactivity associated with it determined.

1. Preincubation of isolated adipocytes with [³H] adenine

4.5 ml of adipocyte suspension, in Krebs-Ringer bicarbonate buffer, 5.5 mM glucose, 2% albumin (w/v), was added to 0.5 ml [8-³H] adenine (1 μCi/ml) in 0.9% NaCl (w/v) in a small siliconised conical flask. The flask was made gas tight with a rubber bung and incubated for 1 hour at 37°C in a shaking water bath.

2. Incubation of [³H] adenine loaded cells

A 100 μl aliquot of the 'adenine preloaded' adipocyte suspension was added to 400 μl of Krebs-Ringer bicarbonate buffer, 5.5 mM glucose, 2% albumin (w/v), 0.125 mM IBMX in a 1.5 ml Eppendorf tube. The suspension was mixed and the tube was incubated at 37°C for 10 minutes. The reaction was terminated by the addition of 50 μl of 5 M perchloric acid
followed by vigorous whirlimixing.

3. Preparation of the cAMP samples

The Eppendorf tube containing the incubated adipocytes and 50μl of 5M perchloric acid was centrifuged at 5000rpm for 10 minutes. A 250μl aliquot of the supernatant was removed and kept. To this sample, 5μl of 0.05% methyl orange (w/v) solution was added. The sample was neutralised using 10M KOH, determined by the colour change of the methyl orange indicator.

Approximately 5000cpm of cyclic ([U^{14}C] adenyl) AMP was added to enable the percentage recovery of the purification method to be established, an equivalent 5000cpm aliquot was diluted in 4ml of 0.1M imidazole-HCl pH7.5, to give the '100% recovery' value.

Finally the sample was made up to a volume of 1ml with 50mM Tris pH7.5.

By adding a sample of cyclic ([U^{14}C] adenyl) AMP to an unlabelled adipocyte incubation, the % recovery of cAMP by this method was determined as 103±2% (5).

4. Purification of [^{3}H] cAMP using Dowex cation exchange resin and aluminium oxide

The isolation of the formed [^{3}H] cAMP was achieved by the method of Krishna et al (1968) which involved the separation of adenine nucleotides using Dowex cation exchange resin. To improve the technique, the modification described by Salomon et al (1974) involving the adsorption of [^{3}H] cAMP to alumina was employed.

Dowex 50 x 4-400 (dry mesh 200-400) (Sigma, Poole, Dorset) was suspended in distilled water and defined. Using an automatic pipette with an enlarged orifice, Dowex suspension
was loaded into a Pasteur pipette, containing a tissue paper plug, to produce a bed volume of 1ml. The syringe-barrel of a 20ml syringe was attached to the top of the column to provide a reservoir.

The Dowex column was then washed with 5ml of 1M HCl followed by 15ml of distilled water. This washing procedure was repeated subsequent to each run.

An alumina column was produced by pouring 0.6g of alumina (neutral, WN3) (Sigma, Poole, Dorset) into a pasteur pipette, in a similar manner to that of the Dowex column. This column was washed, and subsequently regenerated with 10ml of 0.1M imidazole HCl, pH7.5.

The 'cAMP sample' was applied to the Dowex column and allowed to drain through, the displaced liquid was discarded. Next, the column was washed with 2ml of distilled water and the eluant was again discarded.

The bound cAMP was eluted from the Dowex column with 3ml of distilled water and the eluant was drained directly onto the alumina. After this liquid had drained through the alumina column, the adsorbed cAMP was displaced with 4ml of 0.1M imidazole-HCl pH7.5, directly into scintillation vials. 8ml of Atomlite scintillation fluid (NEN Soton, Hants) was added and the samples were counted by liquid scintillation counting using the two channel mode for $^3$H and $^{14}$C.

5. **Identification of the purified product as cAMP**

Thin layer chromatography (t.l.c) using the method of Honegger et al (1977) was used to identify the adenine nucleotides associated with the 3ml eluant from the Dowex column.
Dried down sample of Dowex-eluant were taken up in 25μl of warm water and spotted on a t.l.c plate (Polygram CEL 300 PEI/UV254, Macherey-Nagel, Düren, W.G.). The plate was then chromatographed to the top with 50mM acetic acid, in a t.l.c tank. When finished the plate was removed and dried and the run was repeated using distilled water.

The plate was then run with 0.2M LiCl up to 5.5cm from the origin. Surplus water was then removed from the t.l.c plate and the process was repeated using 1M LiCl up to 8.1cm from the origin. Finally the plate was run to the top with 1.8M LiCl. When dry the plate was scanned to determine the peaks of radioactivity.

The developed t.l.c plate showed that the Dowex-eluant contained one major adenine nucleotide moiety which corresponded to cAMP. The presence of minor contaminating adenine nucleotides was found to be less than 2%.

6. Calculation of cAMP results

Before the [³H] cAMP results could be calculated, the overlap of ¹⁴C counts into the ³H channel had to be ascertained and corrected for. This was done by determining the proportion of ¹⁴C counts, from the '[¹⁴C] cAMP total counts', which appeared in the ³H channel. Once this ¹⁴C overlap proportion had been evaluated, each sample was then corrected for both ³H and ¹⁴C counts.

From the ¹⁴C count, the percentage recovery of the cAMP isolation method was determined and the [³H] cAMP value was altered appropriately.

The data was presented with reference to the 0.1mM IBMX control value, all results being given as a percentage of this.
value.

IIe xxiii Binding of $^{125}$I labelled peptides to isolated adipocytes

1. $^{125}$I-BCT

0.2ng of $^{125}$I-BCT ($\sim 5 \times 10^5$ dpm), in propanol-acetic acid was placed in an incubation vial and dried down under a stream of nitrogen. 1ml of adipocyte suspension in Hanks-hepes buffer, 5.5mM glucose, 2% albumin (w/v) was added and the adipocytes were incubated at 24°C for 30 minutes. At the end of the incubation, 200µl aliquots of cell suspension were centrifuged through silicone oil. The radioactivity associated with the cell layer and an aliquot of the cell-free buffer was determined using the $\gamma$-counter.

The non-specific binding was determined in parallel incubations with an additional 1µg/ml 'cold' BCT. Triplicate readings of all samples were taken.

2. $^{125}$I-insulin

Isolated adipocytes were incubated with 1ng of $^{125}$I-insulin (0.4 atoms I per molecule insulin) in 1ml of Hanks-hepes buffer, 5.5mM glucose, 2% albumin (w/v). The cells were incubated for 1 hour at 24°C. Separation of the adipocyte and incubation medium was as described above.

The non-specific binding was determined by incubating the cells with $^{125}$I-insulin in the presence of 50µM insulin.

Specific binding was determined from the dpm bound after subtraction of the dpm due to non-specific binding.

IIIf Statistical analysis

All results presented in this thesis are mean values
± standard error of the mean (S.E.M.), with the number of observations in parenthesis.

For the determination of significance between two populations, Student's t-test was used. P values of 0.05 or less were considered as meaning that the two populations were significantly different i.e. 95%, or greater, confidence that the two populations were different.
III. RESULTS.
IIIa The structure and biosynthesis of BCT

1 Tryptic digestion of CLIP

The apparent relationship of BCT to the CLIP molecule (Bogdanovic 1978; Beloff-Chain et al 1980a) was investigated by performing a tryptic digest of synthetic CLIP. A range of enzyme:substrate ratios of 1:25, 1:10, 1:5, 1:2 and 1:1 (w/w) were tried and the digests were fractionated using P2/P6 gel filtration.

Figure 8a shows the results of a tryptic digest of 1 μg CLIP using an enzyme:substrate ratio of 1:2 for 2 hours. The digest was found to contain a peptide which eluted in the same position as native BCT. This tryptic peptide was produced by all the enzyme:substrate ratios used, however in both the 1:25 and 1:10 digests only small amounts of the peptide were produced.

A sample of this purified tryptic peptide was added to some purified native BCT and the mixture was fractionated using P2/P6 gel filtration. As shown in figure 8b the tryptic peptide and native BCT eluted as a single peak.

Another sample of the purified tryptic peptide was tested for its insulin secretagogue activity using a pancreas per-fusion technique (Dunmore and Beloff-Chain 1982). Figure 9 shows the action of the tryptic peptide upon insulin release from the perfused pancreas, demonstrating that the peptide stimulated insulin release (S. Dunmore, unpublished data).

Using these two criteria, the results show that tryptic digestion of CLIP yields a peptide probably identical to BCT. The tryptic cleavage point in the CLIP molecule has already
**FIGURE 8.**

(a) CLIP-IRM ng/ml

(b) CLIP-IRM ng/ml

Fraction No.
Biogel P2/P6 fractionation of a tryptic digest of synthetic CLIP

1 μg of synthetic CLIP was treated with trypsin at an enzyme:substrate ratio of 1:2 (w/w) in 0.1M NaHCO₃, pH 3.5 for 2 h at 37°C. The digest was fractionated on a Biogel P2/P6 column and eluted with 5% (v/v) acetic acid, 0.02% (w/v) albumin with a flow rate of 3 ml/h; 30 min fractions were collected. The CLIP-immunoreactivity of each fraction was determined by CLIP-RIA (a).

Insert (b) shows the P2/P6 fractionation of a mixture of samples of native BCT and the tryptic peptide produced from CLIP.
Perfusion of rat pancreas with the CLIP trypptic peptide

A rat pancreas was perfused with 0.7ng/ml (CLIP-IBM) of CLIP trypptic peptide, after purification by P2/P6 gel filtration (figure 8), to test for insulin secretagogue activity. The perfusion was performed using the method of Dunmore and Beloff-Chain (1982) with a flow rate of 4ml/min; 1 minute fractions were collected. Initially the pancreas was perfused with buffer containing 5.5mM glucose to give a basal value. The arrows indicate at what point the pancreas was perfused with trypptic peptide and with 16.7mM glucose, to test the viability of the pancreas. Insulin was measured by RIA. (Reproduced by permission of Dr. S. Dunmore).
been identified as at the C-terminal of lysine-21 (ACTH designation) (Sieber et al 1972), suggesting that BCT is equivalent to ACTH$_{22-39}$ (figure 1).

Running concurrently with, and independently of, these experiments, Beloff-Chain et al (1982b) demonstrated that tryptic digestion of synthetic human CLIP produced a peptide with insulin secretagogue activity which co-eluted with BCT on the Biogel P2/P6 column and on reverse phase EPLC. Using fast atom bombardment mass spectrometry, the molecular weight of the peptide was found to be 1984 daltons which corresponded to that of ACTH$_{22-39}$.

The experiment was repeated using a sample of CLIP obtained from an incubation of ob/ob NIL i.e. peak 'B' (Beloff-Chain et al 1980a). However due to the large amount of albumin associated with the 'B' sample, obtained after P2/P6 fractionation, an enzyme:CLIP ratio of 5:1 (w/w) was used. Figure 10 shows the results of the 'B' digest after fractionation using P2/P6 gel filtration. The digest contained three peptides with CLIP immunoreactivity. Fractionation, on the P2/P6 column, of a sample of undigested 'B' showed that the first peptide eluted represented the undigested CLIP (results not shown) and the last peptide eluted corresponded to BCT.

The nature of the third peptide was not investigated further.

Proteolytic digestion of synthetic CLIP was performed with chymotrypsin (43U/mg protein) using the same conditions as for trypsin. At enzyme:substrate ratios of 1:25 (w/w) and 1:10 (w/w) no digestion of CLIP was detectable.
Figure 10

**Biogel P2/P6 fractionation of a trypsin digest of obese (ob/ob) mouse CLIP.**

A 1μg sample of ob/ob mouse CLIP (B), which had previously been purified by Biogel P2/P6 gel filtration, was digested with trypsin, as described in figure 8, using an enzyme:substrate ratio of 5:1 (w/w). The digest was fractionated using a Biogel P2/P6 column and the CLIP-immunoactive profile was determined by CLIP-RIA.
IIIA ii Comparison of the secretion of CLIP-like peptides in incubations of homozygous lean (+/+ ) and obese (ob/ob) neurointermediate lobes

Fifteen ob/ob NIL were incubated, as previously described, in RPMI 1640 complete medium and the peptides produced were fractionated by P2/P6 gel filtration. This produced the characteristic profile of 5 CLIP-immunoreactive peptides (figure 11) which had previously been reported (Beloff-Chain et al 1980a).

When this procedure was repeated using +/+ NIL, 5 CLIP-immunoreactive peaks were found (figure 12) apparently identical to those produced by the ob/ob NIL. This result, for the first time, directly demonstrates BCT production from neurointermediate lobes of the homozygous lean (+/+ ) mouse.

Quantification of the differences in secretion, by the +/+ NIL and the ob/ob NIL, was achieved by determining the CLIP immunoreactive material under the respective peaks. Table 1 shows the secretion, in ng CLIP-IRM/10 NIL/5 hours, of the 5 CLIP-like peptides from incubations of +/+ and ob/ob NIL. These results show that only the 'A' and 'B' peptides were secreted in significantly greater amounts by the ob/ob NIL under the experimental conditions used.

The ratios of 'E':'B', secreted by the ob/ob NIL and +/+ NIL, were found to be 0.18 and 0.47 respectively.

IIIA iii Pulse-chase experiments to investigate the synthesis of B-cell tropin in ob/ob and +/+ neurointermediate lobes

1. Initial separation using P2/P6 gel filtration

In order to investigate the synthesis of BCT, in the
Figure 11

Separation of the CLIP-like peptides, secreted by obese (ob/ob) mouse neurointermediate lobes during a 5h incubation, by P2/P6 gel filtration

15 NIL from ob/ob mice were incubated, in batches of 5 lobes in 250μl of complete media (RPMI 1640 or DMEM), for 5h at 37°C. At the end of the incubation, the media was pooled, lyophilised, and then reconstituted in 1ml of 5% (v/v) acetic acid for fractionation by P2/P6 gel filtration. The CLIP-immunoreactive profile was determined by CLIP-RIA. The result presented is a typical example of many repeat experiments.
Figure 12

Biogel P2/P6 chromatography of the CLIP-like peptides released by lean (+/+) mouse neurointermediate lobes during a 5h incubation

The experimental procedure was identical to that described in figure 11, except that 15 +/- NIL were used in the incubation. This result represents a typical elution profile of many repeat experiments.
Table 1

Quantification of the CLIP-like peptides released during incubation of neurointermediate lobes from obese (ob/ob) and lean (+/+) mice

CLIP-like peptides released. ng CLIP-IRM/10 lobes/5h.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>+/+</th>
<th>ob/ob</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>192 ± 22 (3)</td>
<td>297 ± 34 (4)*</td>
</tr>
<tr>
<td>B</td>
<td>165 ± 27 (4)</td>
<td>588 ± 223 (4)*</td>
</tr>
<tr>
<td>C</td>
<td>37 ± 7 (4)</td>
<td>69 ± 12 (4)</td>
</tr>
<tr>
<td>D</td>
<td>107 ± 20 (4)</td>
<td>182 ± 32 (4)</td>
</tr>
<tr>
<td>E</td>
<td>78 ± 15 (4)</td>
<td>104 ± 14 (4)</td>
</tr>
</tbody>
</table>

* ob/ob value significantly different from +/+ value, p < 0.05. All other comparisons were not significant.

15 NIL from either obese (ob/ob) or lean (+/+) mice were incubated in complete media for 5h at 37°C. The pooled media was fractionated by P2/P6 gel filtration and the elution profile was determined by CLIP-RIA. The quantity of each peptide was determined from the area under each peak and was expressed as ng of CLIP-immunoreactive material released/10 lobes/5h.
ob/ob NIL, 15 NIL were incubated with 2mCi of $[^3\text{H}]$ phe, in Minimal Essential Medium (without phe), for 2 hours and then transferred to Minimal Essential Medium (with phe) for a further 3 hours. The peptides released into the incubation media, and those extracted from the NIL, were separated using P2/P6 gel filtration.

A constant problem which occurred throughout these experiments in which NIL were incubated with $[^3\text{H}]$ phe, was the unincorporated $[^3\text{H}]$ phe. On the Biogel P2/P6 column this emerged with the 'salt peak' and thus obscured any incorporation of $[^3\text{H}]$ phe which may have occurred in the CLIP-like peptides 'C', 'D' and 'E'. This was particularly a problem with the 2h pulse incubation media which contained the bulk of the 2mCi of $[^3\text{H}]$ phe, and after fractionation on the Biogel P2/P6 column occluded virtually all of the CLIP-like peptides.

An additional problem with the Biogel P2/P6 columns was that the separation of the CLIP-like peptides was not constant; although all 5 CLIP-like peptides were separated out, their elution positions varied as the Biogel P2/P6 column aged. Hence, the identification of the CLIP-like peptides was in relation to the predominant 'B' (CLIP) peak.

Figure 13 shows the release of peptides into the incubation media during the 2h incubation period, with $[^3\text{H}]$ phe. Apparently, the only radioactively labelled material released from the ob/ob neurointermediate lobes eluted with the void volume. Since the 'B' (CLIP) peak was obscured by free $[^3\text{H}]$ phe (see above), the 'B' peak was rerun on Biogel P2/P6 and was found to contain minimal amounts of incorporated $[^3\text{H}]$ phe (results not shown).
Figure 13

P2/P6 fractionation of the peptides released during a 2h pulse incubation of obese (ob/ob) mouse neurointermediate lobes with [3H]phenylalanine

15 ob/ob NIL were incubated with 2mCi of [2,4,5-3H]phenylalanine for 2h at 37°C in minimum essential medium (without phe). At the end of the incubation, the NIL were removed (see figure 15) and the media was lyophilised. The peptides were taken up in 1ml of 5% (v/v) acetic acid and were chromatographed by P2/P6 gel filtration. CLIP-RIA was used to measure the CLIP-immunoreactivity of each fraction and aliquots were taken to determine the 3H content.

The graph shows one of two similar results obtained.
Biogel P2/P6 gel filtration of peptides released by obese (ob/ob) mouse neurointermediate lobes during a 3h chase incubation following a 2h pulse period with [3H] phenylalanine

15 ob/ob NIL were incubated with 2mCi of 3Hphe for 2h, as described in figure 13. Then, the NIL were transferred to minimum essential medium (with 'cold' phe) and were incubated at 37°C for 3h. After lyophilisation, the media was dissolved in 1ml of 5% (v/v) acetic acid and the peptides were separated by P2/P6 gel filtration. The CLIP-immunoreactivity and 3H radioactivity of each was determined. The result presented is typical of three repeat experiments.
After a 3h chase period in the absence of \[^{3}H\] phe, the 'B' (CLIP) peak was labelled showing that the ob/ob NIL's were secreting newly synthesised CLIP. Additionally, various peptides eluting near the void volume peak had incorporated \[^{3}H\] phe, as had a peak eluting adjacent to the free \[^{3}H\] phe peak (figure 14). The identity of the peptides, other than CLIP, was not established although the peptide peak adjacent to the free \[^{3}H\] phe peak (eluted last) was presumed to be \(\alpha\)MSH (Scott et al 1976).

Figure 15 shows the Biogel P2/P6 fractionation of an extract of ob/ob NIL which had been incubated with \[^{3}H\] phe for 2h. The CLIP-immunoreactive profile showed predominant amounts of 'A' and 'B' with no discernable peak of BCT, as had previously been found (J. Morton, unpublished results). The third small peak of CLIP-immunoreactive material did not correspond to 'C' or 'D' (J. Morton, unpublished results). Incorporation of \[^{3}H\] phe into 'B' had occurred and was also seen in the peptides which eluted near the void volume, and in the '\(\alpha\)MSH' peptide. The \[^{3}H\] phe incorporation profile is similar to that for the incubation media after the 3h chase period (figure 14), suggesting that the peptides synthesised in the ob/ob NIL during the 2h pulse period are released into the media during the next 3h.

Extraction of the ob/ob NIL, after the 3h chase period, revealed that the incorporation of \[^{3}H\] phe into 'B' (CLIP) had increased. This increase was also seen with the peptides eluting near the void volume (figure 16). The '\(\alpha\)MSH' peak appeared to have broadened but there apparently had not been an increased \[^{3}H\] phe incorporation to the same extent as into
Figure 15

Separation of an extract of obese (ob/ob) mouse neurointermediate lobes, after a 2h pulse incubation with [3H]phenylalanine, by P2/P6 gel filtration

The 15 ob/ob NIL, which had been incubated with 2μCi of [3H]phe for 2h (figure 13) were extracted in acetic acid and lyophilised. The peptides were taken up in 2ml of 5% (v/v) acetic acid and were chromatographed on the Biogel P2/P6 column. Each fraction was assayed for CLIP-immunoactivity and 3H radioactivity. The result presented is typical of two repeat experiments.
Biogel P2/P6 fractionation of an extract of obese (ob/ob) mouse neurointermediate lobes after a 3h chase incubation following a 2h pulse with $[^3H]phenylalanine$

15 ob/ob NIL were incubated with 2mCi of $^3$Hphe for 2h, followed by a 3h chase period with 'cold' media (figures 13 and 14). At the end of the chase period, the NIL were removed from the media and extracted in acetic acid. The extract was lyophilised and then reconstituted in 2ml of 5% (v/v) acetic acid for separation of the peptides on a P2/P6 column. The CLIP immunoactivity and $^3$H radioactivity were determined for each fraction. The result presented is typical of two repeat experiments.
CLIP. The sharp peak eluting last was due to free [\(^3\)H] phe.

The experiment was repeated using 15 +/+ NIL, for comparison. Figure 17 shows the incubation media after the 2h pulse with [\(^3\)H] phe. As has occurred previously, the free [\(^3\)H] phe obscured any [\(^3\)H] phe incorporation into the CLIP-like peptides. When the 'B' (CLIP) peak was rerun, no labelling was evident (results not shown). Estimations of the CLIP-immunoreactivity under the B peaks produced during the 2h pulse incubation of +/+ and ob/ob NIL (figure 13) give values of 220 and 240ng respectively, showing that slightly more CLIP was released from the ob/ob NIL.

The incubation media after a 3h chase period with +/+ NIL (figure 18) produced similar results to the 3h chase ob/ob NIL incubation media (figure 14), although reduced values of CLIP-immunoreactivity and [\(^3\)H] phe incorporation were found. The amount of CLIP released by +/+ and ob/ob NIL during the 3h chase period was estimated as being 144ng and 372ng respectively, showing a decreased secretion by the +/+ NIL.

Figure 19 shows the extract of the +/+ NIL after a 2h incubation with [\(^3\)H] phe in which various peptides including 'B' have become labelled. The peak adjacent to the free [\(^3\)H] phe peak is thought to be an aberration in [\(^3\)H] phe elution which as a result obscures any incorporation into αMSH.

After a 3h chase period, the extract of the +/+ NIL showed increased incorporation of [\(^3\)H] phe into the peptides, including B (figure 20). As had happened in the ob/ob NIL, the 'αMSH' peak had broadened and the incorporation of [\(^3\)H] phe was not apparently to the same extent as into CLIP.

The [\(^3\)H] phe incorporation and CLIP-immunoreactivity
CLIP-IRM ng/ml

[3H]-c.p.m. x 10^4 / ml

FIG. 17
Separation of peptides released during an incubation of lean (+/+ mouse neurointermediate lobes, with $[^3\text{H}]$ phenylalanine, by P2/P6 gel filtration

$15^+ / +$ NIL were incubated with $2\text{mCi}$ of $[^3\text{H}]$phe for $2h$ as described in figure 13. The peptides released into the media were fractionated on a Biogel P2/P6 column and the $^3\text{H}$ radioactivity and CLIP-immunoactivity profiles were determined.
Figure 18

Biogel P2/P6 chromatography of the peptides released by lean (+/+ mouse neurointermediate lobes during a 3h chase incubation, following a 2h pulse period with $[^3H]$phenylalanine

After a 2h pulse incubation with 2mCi of $[^3H]$phe, 15 +/+ NIL were incubated for 3h in media containing 'cold' phe (see figures 13 and 14). The peptides released into the media during the 3h chase period were run on a Biogel P2/P6 column and the CLIP-immunoactivity and $^3H$ radioactivity of each sample was determined. The result presented is typical of two repeat experiments.
Figure 19

**Biogel P2/P6 separation of an extract of lean (+/+ ) mouse neurointermediate lobes, after a 2h pulse incubation with \[^3\text{H}\]phenylalanine.**

15 +/+ NIL which had been incubated for 2h with \[^3\text{H}\]phe (see figure 13) were extracted in acetic acid. The peptides of the extract were fractionated by P2/P6 gel filtration and the CLIP-immunoactivity and \(^3\text{H}\) radioactivity profiles were determined.
Figure 26

P2/P6 gel filtration of an extract of lean (+/+) mouse neurointermediate lobes after a 3h chase incubation following a 2h pulse with \[^3H\]phenylalanine.

15 +/- NIL, which had been incubated with 2mCi of \[^3H\]phe followed by a 3h chase incubation without label, were extracted in acetic acid. The peptides from the extract were chromatographed by P2/P6 gel filtration and the CLIP-immunoactivity and \[^3H\] radioactivity of each fraction was determined. The result presented is typical of two repeat experiments.
profiles produced from the extracts of the +/+ NIL were apparently decreased compared to the same parameters of the respective extracts of the ob/ob NIL. However, quantification of the CLIP content of the NIL by estimation from the CLIP peaks was not possible since the concentrations were often too high to be measured by the CLIP-RIA. Hence, comparisons of the CLIP content of ob/ob and +/+ NIL could not be made.

2. Reverse-phase HPLC

After separation using P2/P6 gel filtration (see above), the BCT samples were pooled, dried down and then further purified using reverse-phase HPLC. Reverse-phase HPLC was used since this provided a rapid, efficient system for the removal of unincorporated \(^3\)H phe from samples of BCT which had previously been run on a Biogel P2/P6 column (see If).

It was found that the linear 10-40% propan-1-ol gradient did not sufficiently purify the BCT to be able to measure \(^3\)H phe incorporation, even after a second run. However, if the BCT was purified initially using the linear gradient followed by the concave 10-25% gradient, purification was usually sufficient to see \(^3\)H phe incorporation.

The purification of BCT from 2h pulse media samples, even using the two different propanol gradients, was not sufficient to remove the unincorporated \(^3\)H phe. Hence in all 2h pulse media BCT samples the extent of \(^3\)H incorporation could not be measured since this was obscured by the background \(^3\)H phe.

With the two gradient system, the BCT in extracts prepared from NIL of lean (+/+ ) mice, following a 2h pulse with \(^3\)H phe
FIGURE 21.

(a)

[\textsuperscript{3}H] c.p.m \times 10^{-2}/\text{fraction}  

(b)

[\textsuperscript{3}H] c.p.m \times 10^{-2}/\text{fraction}  

CLIP-IRM ng/ml
Figure 26

**Purification of BCT from extracts of lean (+/+ mouse neurointermediate lobe by reverse-phase HPLC.**

The BCT obtained from the P2/P6 fractionation of extracts of 15 +/+ NIL, after a 2h pulse with $[^3\text{H}]$phe (figure 19) and after a 3h chase incubation (figure 20), was lyophilised and then further purified by reverse-phase HPLC. The BCT sample was initially run with a 30min linear gradient of 10% (v/v) to 40% (v/v) propan-1-ol:5% (v/v) acetic acid at 1ml/min. CLIP-RIA was used to determine the elution position of BCT and after drying down the sample was re-run with a 30min concave gradient of 10% (v/v) to 25% (v/v) propan-1-ol:5% (v/v) acetic acid at 2ml/min; 1ml fractions were collected. The CLIP-immunoreactivity of each fraction was measured by CLIP-RIA, after which the fractions were dried down and the $^3\text{H}$ radioactivity was determined by adding 5ml of scintillation fluid and counting using a liquid scintillation counter.

(a) shows the BCT from an extract of 15 +/+ NIL after a 2h pulse incubation with 2mCi of $[^3\text{H}]$phe (see figure 19).

(b) shows the BCT from an extract of 15 +/+ NIL after a 3h chase incubation following a 2h pulse with $[^3\text{H}]$phe (see figure 20).
and a 3h chase (see figure 19 and 20), was purified. Figure 21a shows that the BCT, from the +/+ NIL after 2h incubation with $[^{3}\text{H}]$ phe, had become labelled demonstrating that 3CT is synthesised within the NIL. Incorporation of $[^{3}\text{H}]$ phe is also seen after a 3h chase, in the absence of $[^{3}\text{H}]$ phe (figure 21b). Furthermore, the specific activity, as estimated from the peaks of CLIP-immunoreactivity and $[^{3}\text{H}]$ phe incorporation, is reduced from approximately 250cpm/ng in the 2h pulse extract to approximately 100cpm/ng in the 3h chase extract.

This procedure was repeated for the peptides released into the incubation media. After the 3h chase, the BCT purified from the incubation media had become labelled with $[^{3}\text{H}]$ phe (figure 22a). The estimated specific activity of this peptide was about 50cpm/ng and was reduced compared to the specific activity of the 2h pulse and 3h chase extracts of +/+ NIL.

Unfortunately, due to the lack of time, purification of BCT from ob/ob NIL was only carried out for that released in the incubation media (see figures 13 and 14). When the BCT from the 3h chase media was purified the results showed that $[^{3}\text{H}]$ phe incorporation had occurred (figure 22b). The estimated specific activity of this $[^{3}\text{H}]$ phe labelled BCT was 80cpm/ng.

The specific activity of the BCT released into the media, after the 3h chase period, was similar for both ob/ob and +/+ NIL, although slightly decreased from the +/+ NIL. Furthermore, the estimated amount of BCT was greater from the ob/ob NIL (~32ng) compared to that from the +/+ NIL (~24ng), although this does not take into account losses at each step in the purification.
Figure 22

Purification of BCT from the 3h chase incubation media of obese (ob/ob) and lean (+/+ ) mouse neurointermediate lobes by reverse-phase HPLC

The experimental procedure was the same as that described in figure 21.

(a) shows the BCT from the 3h chase incubation of 15 +/+ NIL, following a 2h pulse with 2mCi $[^3$H] phe (see figure 18).

(b) shows the BCT obtained from the 3h chase incubation of 15 ob/ob NIL, following a 2h pulse with 2mCi $[^3$H] phe (see figure 14).
IIIa iv Separation of $[^3H]$ phenylalanine labelled proteins of the ob/ob neurointermediate lobe using sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Fifteen ob/ob NIL were incubated with 2mCi of $[^3H]$ phe for 5 hours. From this incubation, both the extract and the incubation media were separately purified using P2/P6 gel filtration, producing profiles of CLIP-immunoreactivity and $[^3H]$ phe incorporation similar to figures 14 and 16 (results not given).

The proteins, of both the incubation media and the extract which eluted in the void volume on the Biogel P2/P6 column, were dried down and separated using SDS PAGE. Figure 23a shows the $[^3H]$ phe labelled proteins of the ob/ob NIL extract, of which there appear to be proteins of apparent molecular weights 31K, 21K, 17K and 14K.

When this was repeated with the incubation media, $[^3H]$ phe proteins with identical apparent molecular weights were seen (figure 23b). Furthermore, the extent to which these proteins were labelled with $[^3H]$ phe, in the incubation media, was less than the proteins from the extract.

When this procedure was applied to the Biogel P2/P6 separations of the pulse-chase experiments (figures 13-20), only minimal amounts of $[^3H]$ phe labelled proteins were seen. Because of the presence of the albumin in the samples, it was
15 ob/ob NIL were incubated with 2mCi of [2,4,6-\textsuperscript{3}H] phenylalanine in minimum essential media (without phe) for 5h at 37°C. Both the media and an acetic acid extract of the lobes were fractionated by P2/P6 gel filtration and the proteins eluting in the void volume were pooled and lyophilised. A sample of these proteins, from both media and extract, were run on 10-20% (w/v) acrylamide Tris-HCl buffered SDS-gels as previously described. Marker proteins were run simultaneously to enable the molecular weight to be determined. Once run, the gels were cut into 2mm slices which were dissolved in 0.5ml of soluene-100. 4.5ml of scintillation cocktail was added and the \textsuperscript{3}H radioactivity was measured by liquid scintillation counting.

(a) shows the 'void volume' proteins obtained from the extract of ob/ob NIL. A sample equivalent to 1 NIL was loaded.

(b) shows the 'void volume' proteins obtained from the incubation media of ob/ob NIL. A sample equivalent to that released by 1 NIL was loaded.
FIGURE 23.

(a) [Graph showing a peak at 31k, 17k, and 14k with corresponding slice numbers.]

(b) [Graph showing a similar pattern to (a) with peaks at 31k, 17k, and 14k.]
found impracticable to increase the sample size since smearing occurred.

III b The effect of β-cell tropin on adipose tissue

i. Determination of cell viability over a 90 minute incubation period

Two metabolic parameters were measured over a period of 90 minutes to ensure that the isolated adipocytes remained viable and did not undergo extensive lysis during incubation. Figure 24 shows the time course of incorporation of $^3$H, from $^3$H$_2$O, into the saponified fatty acids of adipocytes, measured over a 90 minute period. The results show a linear increase in the 30, 60 and 90 minute samples analysed.

The time course of $[^6\text{1}^4\text{C}]$ glucose oxidation by isolated adipocytes is presented in figure 25. As with fatty acid synthesis, $[^6\text{1}^4\text{C}]$ glucose oxidation was linear over the 90 minutes measured.

The fact that these metabolic parameters were maintained at a constant rate, for at least 90 minutes, implies that the isolated adipocytes remained viable without extensive cell lysis.

ii. Determination of the effect of β-cell tropin and insulin on $^3$H, from $^3$H$_2$O, incorporation into saponified fatty acids in adipocytes

The incorporation of $^3$H into saponifiable fatty acids was measured, as previously described, to investigate the effect of native BCT upon fatty acid synthesis.

Beloff-Chain et al (1982a) showed that the injection of
Fatty acid synthesis.

μmol C₂ units/10⁶ cells
Figure 26

**Time course of fatty acid synthesis by isolated rat adipocytes**

Isolated adipocytes were incubated in Krebs-Ringer bicarbonate buffer, 5.5 mM glucose, 4% (w/v) albumin and 1 mCi/ml $^3$H$_2$O at 37°C for 30, 60 and 90 minutes. At the end of the appropriate time, a 500μl aliquot of the adipocyte suspension was saponified in an ethanolic-KOH solution for 3h at 80°C. The unsaponified lipids were removed with 10ml of petroleum-ether (40-60°C), after which the 'soap solution' was acidified and the saponified fatty acids were extracted with 3 washings each of 10ml of petroleum-ether. The $^3$H radioactivity associated with the pool of saponified fatty acid extractions was measured and used to determine fatty acid synthesis. The results were expressed in μmolC$_2$ units/10$^6$ cells; each point being the mean of three results with the bars representing the S:E:M.
FIGURE 25.
Figure 25

**Time course of [6\(^{14}\)C] glucose oxidation by isolated rat adipocytes**

Isolated adipocytes were incubated in 1ml of Krebs-Ringer bicarbonate buffer, 3mM glucose, 4% (w/v) albumin and 0.3μCi [6\(^{14}\)C] glucose at 37°C for 30, 60 and minutes. Each incubation was terminated with 200μl of 5M H\(_2\)SO\(_4\) and the liberated CO\(_2\) was trapped with 200μl of hyamine hydroxide. The results are the mean of three readings with the bars representing the S.E.M.
25ng of BCT into the carotid artery of a rat could elicit insulin secretagogue activity and possibly also lipogenic activity. From this piece of information, it was presumed that 0.5 and 5ng/ml BCT would be a high, if not maximal, concentration of the peptide.

Table 2 gives the results calculated as μmol C<sub>2</sub> units/10<sup>5</sup> cells/h, so that no assumption about the final fatty acid products has to be made. Both 5ng/ml and 0.5ng/ml native BCT were found to significantly stimulate fatty acid synthesis compared to the control values.

In order to be able to compare the observed lipogenic activity of BCT with a known lipogenic agent, the adipocytes were incubated with 100μU/ml insulin. This insulin concentration was assumed to be a maximal stimulatory concentration. Although this fact was not verified by the production of a dose-response curve, various workers have shown that 100μU/ml insulin (±0.7nM or 4ng/ml) is a maximal stimulatory concentration for [1<sup>14</sup>C] glucose oxidation, fatty acid synthesis and glucose transport (Olefsky 1976a, b; Kahn 1976; Cushman et al 1981) in isolated adipocytes.

The effect of 100 μU/ml insulin upon the isolated adipocytes caused a significant stimulation of fatty acid synthesis as determined by <sup>3</sup>H incorporation into saponified fatty acids (table 3).

Comparison of the action of BCT and insulin shows that similar stimulations were obtained for the concentrations used: 100μU/ml insulin produced a stimulation in fatty acid synthesis of 140% while for 5ng/ml and 0.5ng/ml native BCT the values were 125% and 144% respectively. These values, for BCT, were
Table 2

The effect of native BCT upon $^3$H incorporation, from $^3$E$_2$, into saponified fatty acids in isolated adipocytes

<table>
<thead>
<tr>
<th></th>
<th>Fatty acid synthesis</th>
<th>umol C$_2$ units/10$^6$ cells/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.81 ± 0.13 (10)</td>
<td></td>
</tr>
<tr>
<td>5ng/ml BCT</td>
<td>2.63 ± 0.60 (13)*</td>
<td></td>
</tr>
<tr>
<td>0.5ng/ml BCT</td>
<td>1.98 ± 0.34 (5)*</td>
<td></td>
</tr>
</tbody>
</table>

* value is significantly greater than the control, p < 0.01

Table 3

The action of 100uU/ml insulin upon fatty acid synthesis as measured by $^3$H incorporation from $^3$H$_2$O, in saponifiable fatty acids in isolated rat adipocytes

<table>
<thead>
<tr>
<th></th>
<th>Fatty acid synthesis</th>
<th>umol C$_2$ units/10$^6$ cells/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.81 ± 0.12 (12)</td>
<td></td>
</tr>
<tr>
<td>100uU/ml insulin</td>
<td>1.94 ± 0.23 (13)</td>
<td></td>
</tr>
</tbody>
</table>

The insulin result is significantly greater than the control, p < 0.001

Legend on p. 127
Table 4

The effect of ACTH, CLIP and ACTH$_{34-39}$ upon $^3$H incorporation, from $^3$H$_2$O, into saponified fatty acids in isolated rat adipocytes

<table>
<thead>
<tr>
<th></th>
<th>Fatty acid synthesis</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>umol C$_2$ units/10$^5$ cells/h</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.68 ± 0.09 (12)</td>
<td></td>
</tr>
<tr>
<td>0.1 U/ml ACTH</td>
<td>0.79 ± 0.11 (11)</td>
<td></td>
</tr>
<tr>
<td>1µg/ml ACTH$_{34-39}$</td>
<td>0.78 ± 0.05 (7)</td>
<td></td>
</tr>
<tr>
<td>0.5µg/ml CLIP</td>
<td>0.67 ± 0.10 (6)</td>
<td></td>
</tr>
</tbody>
</table>

All results were not significantly different from the control.

Legend on p. 127
Legend to Tables 2, 3 and 4

Isolated adipocytes were incubated in Krebs-Ringer bicarbonate buffer, 5.5mM glucose, 4% (w/v) albumin and lmCi/ml $^3$H$_2$O for 1h at 37°C, in the presence or absence of the test substances, at the concentrations mentioned in the tables. At the end of the incubation a 500ul aliquot of the cell suspension was taken and the lipids were saponified using an ethanolic-KOH solution. The unsaponified lipid was removed by washing the 'soap solution' with 10ml of petroleum-ether (40-60°C), which was then discarded. The aqueous solution containing the saponified fatty acids was then acidified and 3 washings with 10ml of petroleum-ether were used to extract the saponified fatty acids.

The results were expressed in μmol C$_2$ units/10$^6$ cells/h.
not significantly different from the stimulation due to insulin (p>0.2 for 5ng/ml BCT and p>0.5 for 0.5ng/ml BCT).

The finding that stimulation of lipogenesis with native BCT at the two concentrations studied was similar to that obtained in the presence of a maximal concentration of insulin, supports the assumption that 0.5ng/ml of native BCT produces a maximal stimulation of lipogenesis.

iii. Determination of the effect of ACTH and related peptides upon fatty acid synthesis in isolated adipocytes

With the discovery that BCT stimulated lipogenesis in isolated adipocytes, it was of interest to investigate how specific this action was, in relation to the ACTH molecule.

To determine this specificity ACTH and CLIP were tested for possible lipogenic activity by the $^3$H incorporation method, as previously described. In addition to these two peptides, the activity of a synthetic C-terminal ACTH hexapeptide N-ala-phe-pro-leu-glu-phe-c (ACTH$_{34-39}$) on lipogenesis was also measured. At this time the structure of BCT had not been established although it was known to be related to the CLIP molecule. It was thought that a study of the ACTH$_{34-39}$ fragment might provide information as to the sequence of the CLIP molecule which was responsible for BCT lipogenic action.

The results obtained, expressed in umol C$_2/10^6$cells/h, are shown in table 4. These results demonstrate that 0.1 U/ml ACTH, 0.5µg/ml CLIP and 1µg/ml ACTH$_{34-39}$ were devoid of lipogenic activity, having no significant effect upon $^3$H incorporation into saponified fatty acids.

iv. Determination of the lipogenic potency of 3-cell tropin on isolated adipocytes
The preparation of sufficient BCT to produce a dose-response curve, from the NIL of ob/ob mice would have required too many animals and too much time. However, this problem was overcome by the discovery that under suitable conditions BCT was produced by the tryptic cleavage of CLIP, as previously described.

For this dose-response curve the incorporation of $^3$H, from $^3$H$_2$O, into total lipid, as extracted by the method of Folch et al (1957), was used as a measure of lipogenesis. BCT concentrations ranging from 50ng/ml BCT to $5 \times 10^{-4}$ng/ml BCT were tested using isolated adipocytes and the results are presented in figure 26.

The 50% activity concentration of BCT was found to be $5 \times 10^{-2}$ng/ml. If it is assumed that the 'CLIP-antiserum' used in the radioimmunoassay equally recognises CLIP and BCT then the concentrations of BCT determined by the radioimmunoassay would be equivalent to their actual concentrations. It would seem plausible that the 'CLIP-antiserum' equally recognises these two peptides since the purification, using reverse-phase HPLC, of a tryptic digest of CLIP, which showed two equivalent peaks (CLIP and BCT) of absorbance at 280nm, was found to contain equivalent amounts of CLIP and BCT by RIA (G.Taylor, personal communication). Thus, using this assumption, the 50% activity of BCT on lipogenesis was $2.5 \times 10^{-11}$M.

These results also demonstrated that BCT, produced by the tryptic cleavage of CLIP, in addition to native BCT can stimulate lipogenesis. All the BCT concentrations of $5 \times 10^{-2}$ng/ml and above significantly increased the incorporation of $^3$H into total lipid.
FIGURE 26.
Figure 26

Dose-response curve showing the effect of BCT on the incorporation of $^3$H, from $^3$H$_2$O, into total lipid in isolated adipocytes

Isolated adipocytes were incubated in Krebs-Ringer bicarbonate buffer, 5.5mM glucose, 2% (w/v) albumin and 1mCi/ml $^3$H$_2$O at 37°C for 1h, in the presence or absence of 'synthetic' BCT at the concentration stated. After termination of the incubation, the lipids were extracted using chloroform:methanol 2:1 (v/v) and the extract was washed as previously described. An aliquot of the total lipid extract was taken and the $^3$H radioactivity determined by liquid scintillation counting. The results were expressed in umol C$_2$ units/10$^6$cells/h with the number in parenthesis representing the number of observations and the bars representing the S.E.M.

* p<0.001 significantly different from the control
+ p<0.005 significantly different from the control
ϕ p<0.025 significantly different from the control
All other comparisons with the control were not significantly different.
v. Determination of the effect of B-cell tropin, insulin and ACTH<sub>3-39</sub> upon glucose metabolism in isolated adipocytes

It was decided to study in more detail the action of BCT upon isolated adipocytes, by investigating the effect of BCT upon glucose metabolism. This was achieved by incubating adipocytes, in the presence and absence of 5ng/ml BCT, and following the metabolism of specifically labelled [1<sup>14</sup>C] glucose and [6<sup>14</sup>C] glucose molecules. The oxidation and incorporation into total lipid, saponified fatty acids and glyceride-glycerol, of [1<sup>14</sup>C] glucose and [6<sup>14</sup>C] glucose were measured as previously described.

The results of BCT action upon the metabolism of [1<sup>14</sup>C] glucose and [6<sup>14</sup>C] glucose are presented in table 5.

5ng/ml BCT produced a small but significant increase in [1<sup>14</sup>C] glucose oxidation, however, the incorporation of [1<sup>14</sup>C] glucose into total lipid-saponified fatty acids and glyceride-glycerol was not significantly different from the controls.

The oxidation of [6<sup>14</sup>C] glucose was significantly increased by the incubation of the adipocytes with 5ng/ml BCT. A significant stimulation of [6<sup>14</sup>C] glucose incorporation into total lipid, saponified fatty acids and glyceride-glycerol was also seen.

The experiment was similarly repeated using 100μU/ml insulin, for comparison. The results are presented in table 6.

As was seen with 5ng/ml BCT, 100μU/ml insulin significantly increased [1<sup>14</sup>C] glucose oxidation. However the insulin effect on [1<sup>14</sup>C] glucose incorporation into total lipid:saponified
Table 5  The effect of 'synthetic' BCT upon $[1^{14}C]$glucose and $[6^{14}C]$glucose utilization by isolated adipocytes

<table>
<thead>
<tr>
<th></th>
<th>$[^{14}C]$ incorporated into CO₂</th>
<th>$[^{14}C]$ incorporated into total lipid</th>
<th>$[^{14}C]$ incorporated into saponified fatty acids</th>
<th>$[^{14}C]$ incorporated into glyceride-glycerol fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>$[^{14}C]$glucose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>$81.2\pm11.6$ (11)</td>
<td>$58.8\pm9.4$ (9)</td>
<td>$38.3\pm7.6$ (9)</td>
<td>$5.7\pm1.8$ (6)</td>
</tr>
<tr>
<td>5ng/ml BCT</td>
<td>$111.3\pm5.7$ (12)$^\dagger$</td>
<td>$65.5\pm4.6$ (9)</td>
<td>$48.5\pm3.7$ (9)</td>
<td>$9.6\pm1.8$ (6)</td>
</tr>
<tr>
<td><strong>$[^{6}C]$glucose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>$11.2\pm1.0$ (11)</td>
<td>$95.8\pm10.4$ (8)</td>
<td>$77.5\pm7.8$ (8)</td>
<td>$7.0\pm0.9$ (10)</td>
</tr>
<tr>
<td>5ng/ml BCT</td>
<td>$14.5\pm0.8$ (12)$^\dagger$</td>
<td>$129.8\pm7.9$ (9)$^\dagger$</td>
<td>$101.3\pm5.4$ (9)$^\dagger$</td>
<td>$16.0\pm1.2$ (14)$^{*\dagger}$</td>
</tr>
</tbody>
</table>

*See p. 135*
Table 6  The effect of insulin upon $[1^{14}C]$ glucose and $[6^{14}C]$ glucose utilization by isolated adipocytes

<table>
<thead>
<tr>
<th></th>
<th>$1^{14}C$ incorporated into CO$_2$</th>
<th>$1^{14}C$ incorporated into total lipid</th>
<th>$1^{14}C$ incorporated into saponified fatty acids</th>
<th>$1^{14}C$ incorporated into glyceride-glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[1^{14}C]$glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>71.8±8.7 (6)</td>
<td>29.8±5.4 (6)</td>
<td>17.8±2.9 (6)</td>
<td>6.6±1.2 (6)</td>
</tr>
<tr>
<td>100μU/ml insulin</td>
<td>107.5±3.5 (5)*</td>
<td>43.3±12.3 (6)</td>
<td>24.2±7.2 (6)</td>
<td>6.8±2.0 (6)</td>
</tr>
<tr>
<td>$[6^{14}C]$glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.1±0.8 (6)</td>
<td>71.2±16.8 (6)</td>
<td>48.7±11.4 (6)</td>
<td>8.4±1.1 (10)</td>
</tr>
<tr>
<td>100μU/ml insulin</td>
<td>10.4±3.5 (6)</td>
<td>115.7±9.8 (6)$\phi$</td>
<td>83.8±8.2 (6)$\phi$</td>
<td>17.7±1.6 (12)$##$</td>
</tr>
</tbody>
</table>
Table 7  The effect of ACTH$_{34-39}$ upon $[1^{14}C]$glucose and $[6^{14}C]$glucose utilisation by isolated adipocytes

<table>
<thead>
<tr>
<th></th>
<th>$^{14}C$ incorporated into CO$_2$</th>
<th>$^{14}C$ incorporated into total lipid</th>
<th>$^{14}C$ incorporated into saponified fatty acids</th>
<th>$^{14}C$ incorporated into glyceride-glycerol acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[1^{14}C]$glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>86.3±11 (6)</td>
<td>55.3±5.9 (6)</td>
<td>38.2±4.8 (6)</td>
<td>7.0±1.6 (6)</td>
</tr>
<tr>
<td>1µg/ml ACTH$_{34-39}$</td>
<td>82.2±13.6 (5)</td>
<td>53.7±9.7 (5)</td>
<td>36.8±6.7 (5)</td>
<td>10.8±0.2 (5)</td>
</tr>
<tr>
<td>$[6^{14}C]$glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.1±0.5 (6)</td>
<td>78.7±8.0 (5)</td>
<td>70.2±9.6 (6)</td>
<td>7.2±0.2 (5)</td>
</tr>
<tr>
<td>1µg/ml ACTH$_{34-39}$</td>
<td>9.1±0.4 (6)</td>
<td>86.7±7.9 (6)</td>
<td>80.8±8.6 (5)</td>
<td>8.7±1.1 (5)</td>
</tr>
</tbody>
</table>
Legend to Tables 5, 6 and 7

Isolated adipocytes were incubated in 1ml of Krebs-Ringer bicarbonate buffer, 3mM glucose, 4% (w/v) albumin and 0.3μCi \([1^{14}C]\) glucose or \([6^{14}C]\) glucose for 1h at 37°C. The incubations were carried out in the presence or absence of 'synthetic' BCT, insulin or ACTH39 at the concentrations stated in the tables. The reaction was terminated with 100μl of 5M H₂SO₄ and the liberated CO₂ was trapped in hyamine hydroxide as previously described. Chloroform:methanol 2:1(v/v) was used to extract the lipids. After washing the extract, a total lipid aliquot was removed and the remainder of the lipid extract was dried down and saponified. Unsaponified lipids were removed with 10ml of petroleum-ether (40-60°C) and the saponified fatty acids were extracted from the 'soap solution' with petroleum-ether, after it had been acidified. The glyceride-glycerol value was determined from the \(^{14}C\) associated with the aqueous layer after extraction of the saponified fatty acids.

** p<0.001 significantly different from control values
* p<0.005 " " " " " "
+ p<0.025 " " " " " "
φ p<0.05 " " " " " "

All other comparisons were not significantly different from the control values.
fatty acids and glyceride-glycerol, was not significantly different from the control values.

In contrast to the effect of 5ng/ml BCT, $[6^{14}C]$ glucose oxidation was not significantly increased by the action of 100μU/ml insulin. The difference between the results of $[6^{14}C]$ glucose oxidation by BCT and insulin may possibly be due to the small amounts of labelled product being measured. Because these values are so low, small perturbations in the samples could produce proportionally large changes compared to the basal result, and hence give erroneous readings.

100μU/ml insulin significantly stimulated $[6^{14}C]$glucose incorporation into total lipid, into both saponified fatty acids and glyceride-glycerol.

The results given in table 7 show that the peptide ACTH$_{34-39}$, at a concentration of 1μg/ml, had no action on glucose metabolism nor on lipid synthesis from glucose.

vi. The combined effect of β-cell tropin and insulin on $[6^{14}C]$ glucose incorporation into triacylglycerol

Since BCT and insulin were found to have similar effects upon glucose metabolism in isolated adipocytes, it was decided to test their combined action to ascertain whether these effects were additive. The adipocytes were incubated with a 5ng/ml maximal dose (figure 26) and 100μU/ml insulin (assumed to be maximal, see IIIb ii). The incorporation of $[6^{14}C]$ glucose into total lipid, saponified fatty acids and glyceride-glycerol, were measured.

The results of this combination of saturation doses of BCT and insulin are shown in figure 27. When compared with the
Glucose incorporation into total lipid.

nmol C-6 incorporated / $10^6$ cells / h

CONTROL

ACT

INS

ACT + INS

Glucose incorporation into sap. fatty acids.

nmol C-6 incorporated / $10^6$ cells / h

CONTROL

ACT

INS

ACT + INS

Glucose incorporation into gly.-glycerol.

nmol C-6 incorporated / $10^6$ cells / h

CONTROL

ACT

INS

ACT + INS

FIGURE 27.
Figure 27

Histogram to show the effect of BCT and insulin, both separately and combined, on \([6^{14}C]\) glucose incorporation into lipid by isolated adipocytes

The experimental procedure was the same as that described in tables 5-7 except that no results on glucose oxidation were collected. The adipocytes were incubated in the presence or absence of 5ng/ml 'synthetic' BCT and 100μU/ml insulin, both separately and combined. (a) shows the incorporation of 6-C of glucose into total lipid, (b) shows the incorporation of 6-C of glucose into saponified fatty acids and (c) shows the incorporation of 6-C of glucose into glyceride-glycerol. The number of observations are presented in parenthesis and the bars represent the S.E.M.

BCT = 5ng/ml BCT
Ins = 100μU/ml insulin

* p<0.001 significantly different from the control
+ p<0.005 significantly different from the control
Φ p<0.025 significantly different from the control
All other comparisons were not significantly different

see p. 136
control incubations BCT and insulin, both separately and combined, significantly increased $[^{6}^{13}C]$ glucose incorporation into total lipid, both in the saponified fatty acids and glyceride-glycerol moieties. The separate action of BCT and insulin were not significantly different from the combined effect. This demonstrates that there was no additive action of BCT and insulin on lipid synthesis from glucose.

vii. Determination of the effect of B-cell tropin and insulin on pieces of rat epididymal fat pad

Beloff-Chain et al (1980b) have reported that the collagenase treatment used in the isolation of islets of Langerhans may damage the BCT receptors, in this tissue. In order to test if collagenase damaged the BCT effector system in isolated adipocytes and thus reduce the effective response, pieces of rat epididymal fat pad were incubated with BCT. $^{3}H$ incorporation into total lipids (table 8) and the oxidation of $[^{11}C]$ glucose (table 9) were both used as parameters to measure the effect of BCT on adipose tissue pieces.

The results show that BCT did not significantly stimulate either of these metabolic parameters.

Insulin however markedly stimulated both $^{3}H$ incorporation into total lipid and $[^{11}C]$ glucose oxidation.

viii. The effect of B-cell tropin and insulin upon glucose transport in the adipocytes

Following the observed insulin-like action of BCT upon lipogenesis in the adipocyte, it was of interest to test the action of BCT on other insulin stimulated processes. Glucose
Table 8

The effect of 'synthetic' BCT and insulin upon the incorporation of $^3$H, from $^3$H$_2$O, into total lipid by pieces of rat epididymal fat pad

<table>
<thead>
<tr>
<th></th>
<th>Lipogenesis</th>
<th>$\mu$mol C$_2$ units/g wet wt./h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.44 ± 0.09</td>
<td>(5)</td>
</tr>
<tr>
<td>5ng/ml BCT</td>
<td>1.66 ± 0.15</td>
<td>(8)</td>
</tr>
<tr>
<td>100$\mu$U/ml insulin</td>
<td>3.78 ± 0.38</td>
<td>(6)</td>
</tr>
</tbody>
</table>

Control v insulin p<0.001

The value for BCT was not significantly different from that of the control.

Fat pad pieces of approximately 100mg were incubated in 2ml of Krebs-Ringer bicarbonate buffer, 5.5mM glucose, 2% (w/v) albumin, lmCi/ml $^3$H$_2$O in the presence or absence of BCT or insulin for lh at 37°C. At the end of the incubation the fat pad pieces were blotted dry, weighed and the lipids were extracted in chloroform:methanol 2:1 (v/v). After washing, the extract was dried down and an aliquot taken. All results were presented as $\mu$mol C$_2$ units/g wet wt. tissue/h.
Table 9

The action of 'synthetic' BCT and insulin on [1\(^{14}\)C] glucose oxidation by rat epididymal fat pad pieces

<table>
<thead>
<tr>
<th></th>
<th>Glucose oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol l- C converted to CO(_2)/g wet wt./h</td>
</tr>
<tr>
<td>Control</td>
<td>237 ± 18 (14)</td>
</tr>
<tr>
<td>5ng/ml BCT</td>
<td>275 ± 14 (19)</td>
</tr>
<tr>
<td>10ng/ml BCT</td>
<td>298 ± 42 (8)</td>
</tr>
<tr>
<td>100(\mu)U/ml insulin</td>
<td>630 ± 62 (10)</td>
</tr>
</tbody>
</table>

Control v insulin p<0.001. The values for BCT were not significantly different from that of the control.

Fat pad pieces of approximately 100mg were incubated in 2ml of Krebs-Ringer bicarbonate buffer, 3mM glucose, 2% (w/v) albumin, 0.3\(\mu\)Ci/ml [1\(^{14}\)C] glucose for 1h at 37°C, in the presence or absence of 'synthetic' BCT or insulin. The reaction was terminated with 100ml of 5M H\(_2\)SO\(_4\) and the liberated CO\(_2\) was trapped with 200ul of hyamine hydroxide. The results were expressed as nmol l- C converted to CO\(_2\)/g wet wt. tissue/h.
transport has been shown to be markedly stimulated by insulin in the adipocyte (Id.i). Thus it was decided to investigate the action of BCT upon the hexose carrier.

Measurement of glucose transport was carried out by a modification of the method of Olefsky (1975), using 2-deoxyglucose an analogue of glucose which is transported into the cell and phosphorylated but is not metabolised further (Wick et al 1957). The uptake of 2-deoxyglucose has been shown to be linear up to 6 min at 37°C in the absence of glucose; in the presence of glucose the uptake of 2-deoxyglucose decreased markedly (Foley and Glieman 1981). Thus adipocyte incubations were carried out in the absence of glucose.

Figure 28 shows the results of 2-deoxyglucose uptake in nmol/2x10^5 cells/3 mins. A 30 minute sensitization of the adipocytes, to 5ng/ml BCT, produced a significant increase in 2-deoxyglucose uptake over 3 minutes.

The influence of BCT on glucose transport was compared to that of insulin, as had been done in the study of other metabolic parameters. The adipocytes were sensitized by a preincubation with insulin (100µU/ml) for 30 minutes. These insulin sensitized adipocytes significantly stimulated the uptake of 2-deoxyglucose over 3 minutes, and the effect was considerably greater than that of BCT.

ix. Determination of the effect of β-cell tropin and insulin on basal and noradrenaline stimulated lipolysis in isolated adipocytes

The insulin-like effects of the BCT peptide on isolated
Figure 28

**Histogram showing the action of BCT and insulin on 2-deoxyglucose uptake by isolated adipocytes**

Isolated adipocytes were sensitized to 5ng/ml 'synthetic' BCT or 100μU/ml insulin in 1ml of Krebs-Ringer bicarbonate buffer, 2% (w/v) albumin for 30 minutes at 24°C. The control incubations were performed in the bicarbonate buffer without either BCT or insulin. At the end of the 30min preincubation, the adipocytes were transferred to 1ml of Krebs-Ringer bicarbonate buffer, 2% (w/v) albumin containing 0.125mM 2-deoxy-[l-¹⁴C] - glucose (spec. act. ~8mCi/mM) and incubated at 24°C for 3min. Termination of the reaction was by separating the adipocytes from the incubation media by the silicone oil centrifugation technique. The uptake of 2-deoxyglucose was measured from the ¹⁴C radioactivity associated with the cell layer, after correction for the extracellular space as previously described. The results were expressed in nmol of 2-deoxyglucose taken-up/2x10⁵cells/3min with the number of observations presented in parenthesis and the bars representing the S.E.M.

* p<0.005 significantly different from the control
+ p<0.025 significantly different from the control

P.T.O.
2-deoxyglucose uptake

nmol/2 \times 10^5 \text{cells}/3\text{min}

Control
5 ng/ml ACT
100 \mu U/ml Insulin
adipocytes were further investigated by a study of its possible antilipolytic action. This antilipolytic effect of insulin has been widely investigated (see Id ii).

The release of glycerol, as measured by the enzymatic-spectrophotometric assay of Weiland (1963) was taken as an index of lipolysis. This is possible since the glycerol released during the hydrolysis of triacylglycerols is not recycled into new triacylglycerols, to any significant extent (Shapiro et al 1957; Lynn et al 1960).

The possibility that BCT may stimulate futile cycling by stimulating both lipogenesis and lipolysis was examined by studying the effect of BCT upon basal lipolysis. Table 10 shows that 12.5ng/ml and 2.5ng/ml native BCT had no significant effect upon basal glycerol release.

Adipocytes stimulated by $10^{-5}$M noradrenaline were used to investigate the antilipolytic action of insulin and the possible activity of BCT. The glycerol release induced by $10^{-5}$M noradrenaline was markedly increased compared to the controls (table 10).

The addition of 100uU/ml insulin to the noradrenaline stimulated adipocytes produced a marked, although not total, inhibition of glycerol release which was significantly different from the noradrenaline control.

However, the addition of 2.5ng/ml native BCT was ineffective in altering glycerol release induced by noradrenaline. The noradrenaline control and noradrenaline plus BCT were not significantly different (table 10).

The oxidation of noradrenaline during the 30 minute
Table 10

The action of native BCT upon basal and noradrenaline stimulated lipolysis in isolated adipocytes

<table>
<thead>
<tr>
<th>Lipolysis</th>
<th>nmol glycerol/10^6 cells/30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>58 ± 6 (11)</td>
</tr>
<tr>
<td>2.5ng/ml BCT</td>
<td>46 ± 6 (4)</td>
</tr>
<tr>
<td>12.5ng/ml BCT</td>
<td>55 ± 4 (4)</td>
</tr>
<tr>
<td>10^{-5}M Noradrenaline</td>
<td>747 ± 69 (13)</td>
</tr>
<tr>
<td>10^{-5}M NA + 2.5ng/ml BCT</td>
<td>737 ± 60 (5)</td>
</tr>
<tr>
<td>10^{-5}M NA + 100μU/ml insulin</td>
<td>170 ± 31 (11)</td>
</tr>
</tbody>
</table>

Control v NA p<0.001
NA v NA+ insulin p<0.001

The BCT values were not significantly different from their respective basal control or noradrenaline control values.

Isolated adipocytes were incubated in 1ml of Krebs-Ringer bicarbonate buffer, 5.5mM glucose, 2% (w/v) albumin, in the presence or absence of the test substances, for 30 min at 37°C. An aliquot of the cell suspension was then deproteinised and the glycerol concentration was determined by the enzymatic-spectrophotometric assay. All results were presented in nmol glycerol/10^6 cells/30 min.

0.1% (w/v) ascorbate was added to all incubations containing noradrenaline.
Table 11

The response of basal and noradrenaline stimulated lipolysis to 'synthetic' BCT in isolated adipocytes

<table>
<thead>
<tr>
<th>Lipolysis</th>
<th>nmol glycerol/10^6 cells/30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>39 ± 3 (6)</td>
</tr>
<tr>
<td>5ng/ml BCT</td>
<td>38 ± 5 (6)</td>
</tr>
<tr>
<td>10^-5M Noradrenaline</td>
<td>788 ± 29 (6)</td>
</tr>
<tr>
<td>10^-5M NA + 5ng/ml BCT</td>
<td>763 ± 30 (6)</td>
</tr>
</tbody>
</table>

The BCT values were not significantly different from their respective basal or noradrenaline control values.

The conditions and expression of the results was the same as with Table 10, except 'synthetic' BCT was used.
incubation period was prevented by the addition of 0.1% ascorbate. This concentration of ascorbate gave a value of 0.047±0.005 μmol glycerol/10^6 cells/30 min (8) which was not significantly different from basal lipolysis (p>0.1).

The results in table 11 show the effect of 5ng/ml BCT (produced by the tryptic cleavage of CLIP) on basal and noradrenaline stimulated glycerol release. As with the native BCT, the 'synthetic BCT' has no significant lipolytic or antilipolytic activity.

The effect of insulin and B-cell tropin upon basal and noradrenaline stimulated cAMP levels in isolated adipocytes

The absence of any effect of BCT upon lipolysis suggested that cAMP was not involved in the mechanism of action of BCT on the adipose tissue. However since this peptide has a potent insulin secretagogue activity (Ia i) it may act via cAMP in the islets of Langerhans (Sharp 1979). Hence it was decided to investigate the possible effect of BCT upon cAMP levels in the adipocyte; if only to eliminate cAMP as a possible mechanism of action.

cAMP levels were measured after purification as previously described. This method requires the pre-loading of cells with [3H] adenine, which is then subsequently converted to adenine nucleotides, including cAMP.

A time course was produced by incubating an adipocyte suspension in 100μCi/ml [3H] adenine to determine the required period for [3H] adenine uptake. After separation of the cells by silicone oil centrifugation and correcting for trapped intercellular label, the uptake of [3H] adenine was found to
Isolated adipocytes were incubated in Krebs-Ringer bicarbonate buffer, 5.5mM glucose, 2% (w/v) albumin containing 100μCi/ml [8-3H] adenine at 37°C for 30, 60, 90 and 120 min. At the appropriate time, 100μl aliquots of cell suspension were removed and the cells were separated from the incubation media by the silicone oil centrifugation technique. The uptake of [3H] adenine was determined from the 3H radioactivity associated with the cell layer, after correction for the intercellular space. The results are presented in cpm of [3H] adenine taken-up/10^6 cells; each point being the mean of four readings with the bars representing the S.E.M.
FIGURE 29.
be linear over the 2 hour period measured (figure 29). The uptake of $[^3\text{H}]$ adenine had not reached a plateau with the 2 hour period and it was decided to use 1 hour as the standard $[^3\text{H}]$ adenine preincubation time, since a prolonged preincubation period was deemed undesirable.

The optimum incubation period for cAMP production in the $[^3\text{H}]$ adenine preloaded adipocytes was found by producing a time course of cAMP levels in 0.1mM IBMX stimulated adipocytes. Figure 30 shows cAMP levels against time over a 1 hour period. The peak in cAMP levels was found to occur after 10 minutes incubation with 0.1mM IBMX and henceforth 10 minute incubations were routinely used.

The results are presented in relation to the control incubations consisting of 0.1mM IBMX; the mean control value was given the arbitrary 100% value. All incubations were carried out in the presence of 0.1mM IBMX.

Table 12 shows the results of BCT and insulin action upon basal cAMP levels. Neither 10ng/ml BCT nor 100µU/ml insulin significantly altered cAMP levels compared to the control. Since BCT was found to have no effect upon basal cAMP levels, it was decided to investigate the effect of BCT upon the cAMP concentration in noradrenaline stimulated adipocytes. Table 13 shows the effect of incubating noradrenaline stimulated adipocytes with BCT and insulin. $10^{-5}$M noradrenaline was found to significantly stimulate cAMP production and thus was routinely used to elevate the adipocyte cAMP concentration.

10ng/ml BCT did not significantly alter the levels of cAMP in these noradrenaline stimulated cells, compared to the
Time course of cAMP levels in IBMX stimulated adipocytes

$[^3]H$ adenine preloaded adipocytes were incubated in 500µl of Krebs-Ringer bicarbonate buffer, 5.5mM glucose, 2% (w/v) albumin and 0.1mM IBMX for 5, 10, 15, 30 and 60 mins. Zero-time measurements were also taken. The reactions were terminated by deproteinization and a sample of each incubation was then run on a Dowex-alumina column, as previously described, to isolate the cAMP. All results were expressed as a percentage of the zero-time control. The number of observations is presented in parenthesis and the bars represent the S.E.M.

P.T.O.
FIGURE 30.
Table 14

<table>
<thead>
<tr>
<th></th>
<th>cAMP levels</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 15</td>
<td>(5)</td>
</tr>
<tr>
<td>10ng/ml BCT</td>
<td>82 ± 11</td>
<td>(5)</td>
</tr>
<tr>
<td>100μU/ml insulin</td>
<td>94 ± 8</td>
<td>(4)</td>
</tr>
</tbody>
</table>

All comparisons were not significantly different.

[3H] adenine preloaded adipocytes were incubated in a total volume of 500μl of Krebs-Ringer bicarbonate buffer, 5.5mM glucose, 2% (w/v) albumin, 0.1mM IBMX for 10 mins at 37°C in the presence or absence of 'synthetic' BCT or insulin. At the end of the incubation a 250μl aliquot was deproteinized and the cAMP was fractionated from other adenine nucleotides using a Dowex-alumina column as described in the text. All results were expressed as a percentage of the control value.
Table 14.
The effect of 'synthetic' BCT and insulin, both separately and combined, on noradrenaline stimulated cAMP levels in isolated adipocytes

<table>
<thead>
<tr>
<th></th>
<th>cAMP levels</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 6</td>
<td>(15)</td>
</tr>
<tr>
<td>10^{-5}M noradrenaline</td>
<td>333 ± 9</td>
<td>(15)</td>
</tr>
<tr>
<td>10^{-5}M NA + 10ng/ml BCT</td>
<td>316 ± 20</td>
<td>(13)</td>
</tr>
<tr>
<td>10^{-5}M NA + 100μU/ml insulin</td>
<td>297 ± 13</td>
<td>(10)</td>
</tr>
<tr>
<td>10^{-5}M NA + 10ng/ml BCT &amp; 100μU/ml insulin</td>
<td>273 ± 18</td>
<td>(9)</td>
</tr>
</tbody>
</table>

* p < 0.005 significantly different from noradrenaline control
φ p > 0.5 not significantly different from noradrenaline plus insulin value
+ p > 0.4 not significantly different from noradrenaline control

The experiment was carried out as described in table 13, except that [³H]adenine preloaded adipocytes were stimulated with 10^{-5}M noradrenaline to elevate the cAMP levels. 0.1% (w/v) ascorbate was included in all incubations containing noradrenaline. Control incubations in the absence of all test substances, including noradrenaline, were run; all results were presented as a percentage of these controls.
10^{-5}$M noradrenaline controls.

With $100\mu U/ml$ insulin the elevation of cAMP levels by $10^{-5}$M noradrenaline was significantly inhibited. This inhibitory effect by $100\mu U/ml$ insulin on $10^{-5}$M noradrenaline stimulated cAMP levels was not significantly effected by the addition of $10\text{ng/ml}$ BCT.

As with the experiments on lipolysis, $0.1\%$ ascorbate was added to prevent noradrenaline oxidation. The effect of this $0.1\%$ ascorbate ($0.1mM$ IBMX) produced a value for the cAMP levels of $117\pm27\%$ (4) which was not significantly different from the control values.

xi. Determination of the specific binding of $^{125}$I-ß-cell tropin and $^{125}$I-insulin in isolated adipocytes

In view of the observed BCT effects upon isolated adipocytes, it was decided to attempt to demonstrate specific BCT binding and hence demonstrate the presence of BCT-receptors.

Iodo-BCT ($^{125}$I-BCT) was produced and purified for this study as described in 'materials and methods'. The binding assay was performed by incubating $0.2ng/ml$ $^{125}$I-BCT ($\sim5x10^5$ dpm) with isolated adipocytes at $24^\circ C$ for 30 minutes. At the end of the incubation the $^{125}$I-BCT associated with the adipocytes was determined using the silicone oil centrifugation technique followed by $\gamma$-counting.

The binding of $^{125}$I-BCT to isolated adipocytes was found to be indistinguishable from the value obtained for non-specific binding, determined in the presence of $1ug/ml$ 'cold' BCT (results not provided).

Determination of $^{125}$I-BCT associated with the buffer, from
which the cells had been removed, at the end of the incubation showed that $43\pm4\%$ of the $^{125}\text{I}-\text{BCT}$ remained in solution. The apparent loss of $57\%$ of the labelled BCT was attributed to adsorption to the siliconised glass vial due to hydrophobic interaction, reducing the effective concentration of the peptide during the incubation.

The observed effects of BCT upon isolated adipocytes have shown that BCT has an insulinomimetic effect upon lipogenesis and glucose transport whereas it had no effect upon lipolysis. It was thus possible, although rather improbable, that BCT may be a natural partial agonist of insulin, i.e. BCT could possibly interact with the insulin-receptor eliciting, some but not all, insulin mediated events.

In view of the negative results obtained in the attempt to demonstrate the binding of $^{125}\text{I}-\text{BCT}$ to adipocytes, the validity of the assay technique was confirmed using $^{125}\text{I}-\text{insulin}$. Furthermore, the possible influence of BCT and CLIP at high concentrations on insulin binding was measured, to establish whether there was a competitive action.

The specific binding of insulin was determined by using a modification of the method of Gammeltoft and Gliemann (1973). $1\mu\text{g/ml }^{125}\text{I-insulin}$ (0.4 atoms of $^{125}\text{I}$ per molecule of insulin, $\sim5\times10^5\text{dpm}$) was incubated with isolated adipocytes for 1 hour at $24^\circ\text{C}$, in the presence and absence of $1\mu\text{g/ml BCT}$ and $1\mu\text{g/ml CLIP}$. The specific binding of $^{125}\text{I-insulin}$, corrected by the subtraction of the non-specific binding, is presented in table 14, expressed in pg of insulin bound/$10^6\text{cells/h}$.

Specific $^{125}\text{I-insulin}$ binding to the isolated adipocytes
Table 14

The effect of excess BCT and CLIP on $^{125}$I-insulin binding to isolated adipocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific insulin binding (pg insulin bound/10^6 cells/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>133 ± 2 (5)</td>
</tr>
<tr>
<td>1 ug/ml BCT</td>
<td>125 ± 4 (5)</td>
</tr>
<tr>
<td>1 ug/ml CLIP</td>
<td>132 ± 4 (5)</td>
</tr>
</tbody>
</table>

All comparisons were not significantly different.

Isolated adipocytes were incubated in 1ml of Hanks-hepes buffer, 5.5mM glucose, 2% (w/v) albumin and 2x10^10 M $^{125}$I-insulin at 24°C for 1h. In some of the incubations either 1ug/ml 'synthetic' BCT or CLIP was added. At the end of the incubation, the adipocytes were separated from the Hanks-hepes buffer by the silicone oil centrifugation technique and the $^{125}$I-insulin associated with the cell layer was determined. After correction for the extracellular water space using [U14C] sucrose, the specifically bound $^{125}$I-insulin was found by subtraction of the non-specific binding of $^{125}$I-insulin in the presence of 50uM 'cold' insulin. The results were presented as pg insulin bound/10^6 cells/h.
was demonstrated, as can be seen from the control value in table 14. In addition, 1μg/ml BCT and 1μg/ml CLIP had no significant effect on $^{125}$I-insulin binding.

xii. Determination of the lipogenic activity of derivatives of β-cell tropin in isolated adipocytes

The inability to demonstrate $^{125}$I-BCT binding to isolated adipocytes suggested that the addition of the iodine atom to the tyrosine-23 (ACTH designation) inhibited BCT binding to its receptor.

The relationship of the tyrosine-23 residue and the N-terminal val-tyr-pro tripeptide of BCT, to its bioactivity was studied by examining the lipogenic activity of certain BCT derivatives. Three BCT related peptides were obtained, all of which had a modification of the N-terminal region. $^{127}$I-BCT and N-Ac-BCT were synthesised from "synthetic" BCT, while des-val-tyr-pro-BCT (ACTH$_{25-39}$) had been synthesised de novo (Ciba Geigy UK). These three peptides were tested for lipogenic activity using the incorporation of $^3$H$_2$O, into total lipid, and the results are shown in figure 31. The effect of 5ng/ml BCT was also measured to enable comparison of the results with a maximal stimulatory concentration of BCT.

80ng/ml $^{127}$I-BCT produced a small yet statistically significant stimulation in lipogenesis. This stimulation of 112% was significantly different from the 146% (p<0.05) produced by 5ng/ml BCT.

N-Ac-BCT at 5ng/ml did not produce a significant increase in lipogenesis. However at a higher concentration (50ng/ml) the resultant significant stimulation was comparable with that
Figure 27

**Histogram to show the lipogenic activity of BCT-derivatives upon isolated adipocytes**

Isolated adipocytes were incubated in Krebs-Ringer bicarbonate buffer, 5.5mM glucose, 2% (w/v) albumin and 1mCi/ml $^3$H$_2$O for 1h at 37°C. The incubations were carried out in the presence and absence of $^{125}$I-BCT, N-Ac-BCT or ACTH$_{25-39}$ (des-val-tyr-pro-BCT), at the concentrations stated in the histogram; adipocytes were also incubated with 5ng/ml 'synthetic' BCT for comparison purposes. At the end the incubation, the lipids were extracted using chloroform:methanol 2:1 (v/v) and after washing an aliquot of the extract was taken to determine $^3$H incorporation into total lipid. The results were expressed as µmol C$_2$units/10$^6$ cells/h in total lipid. The numbers in parenthesis represents the number of observations and the bars represent the S.E.M.

* p<0.001 significantly different from the control
+ p<0.01 significantly different from the control

All other comparisons with the control were not significant.
Lipogenesis.

3H incorporation into total lipid

μmol C2 units/10^6 cells/h
produced by 5ng/ml BCT.

Since the ACTH_{25-39} peptide had been stored at -20°C for a long period of time, a sample was chromatographed using P2/P6 gel filtration to determine the integrity of the peptide. The resultant 'CLIP-RIA' profile showed that the peptide had undergone partial hydrolysis (result not shown). Therefore in these studies a high concentration of this peptide (1ug/ml) was used to ensure an adequate concentration of ACTH_{25-39}. However it must be recognised that the peptide preparation probably contained a large number of small related peptides, although none of these would contain the BCT N-terminal N-val-tyr-pro-C tripeptide.

The results given in figure 31 show that this peptide preparation had no lipogenic activity.
IV. DISCUSSION.
IVa  The synthesis and secretion, by neurointermediate lobes of obese (ob/ob) and lean (+/+), mice, of the peptide ACTH_22-39 (β-cell tropin)

The ability of antibodies raised against synthetic human CLIP (ACTH_{17-39}) to be able to abolish the insulin secretagogue activity of BCT suggested that the BCT peptide was related to some part of the CLIP sequence (Bogdanovic 1978; Beloff-Chain et al 1980a). Therefore once the methods for the isolation and purification of BCT had been developed, it seemed reasonable to attempt to establish whether BCT was a fragment of the CLIP molecule by specifically cleaving CLIP with trypsin.

The tryptic digest of synthetic human CLIP produced a peptide fragment which coeluted with native BCT on a Biogel P2/P6 column and also cross-reacted with 'CLIP-antiserum' (figure 8). Thus by these two criteria the tryptic fragment of CLIP corresponded to BCT. However, to confirm this conclusion, the biological activity of the tryptic peptide had to be demonstrated. Using a perfused pancreas technique (Dunmore and Beloff-Chain 1982), the tryptic peptide was found to have insulin secretagogue activity (figure 9). Additionally, the peptide produced by treating CLIP with trypsin was found to stimulate lipogenesis in isolated adipocytes (figure 26) as did the native peptide (table 2). Hence, the chromatographic, biological and antigenic properties provided evidence that BCT was identical with the tryptic fragment of CLIP.

After the completion of this work it was noticed that Sieber et al (1972), during studies on the structure of ACTH, had previously demonstrated that the tryptic digestion of
ACTH produced a peptide fragment ACTH$_{22-39}$. This also showed that trypsin acted upon the c-terminal of lysine-21 (ACTH designation).

Concurrently with this work, but independently, G. Taylor in this department (Beloff-Chain et al 1982b) had carried out a tryptic digestion of human CLIP, and obtained a peptide shown to be identical to mouse pituitary BCT. The predicted structure of this peptide was confirmed by determining the molecular weight using Fast Atom Bombardment (FAB) mass spectrometry. It was shown that the tryptic fragment of CLIP had a molecular weight of 1984 daltons which corresponded to the molecular weight of human ACTH$_{22-39}$.

A tryptic digest of mouse CLIP showed that the natural CLIP peptide also yielded a BCT-like peptide (figure 10).

The relationship of BCT to CLIP having been established, experiments were carried out to investigate the biosynthesis of this peptide. It had previously been shown that POMC in the pituitary pars intermedia was the precursor of CLIP (see Ie ii) and this therefore appears to be the most likely candidate as a precursor of BCT.

Whole NIL were used in pulse-chase experiments to investigate BCT synthesis and its relationship to CLIP and POMC. Due to the very small amounts of BCT produced by the ob/ob and +/- NIL, a long pulse period was deemed necessary to allow a high incorporation of [$^3$H]phe into the precursor. Since the precursor was presumed to be POMC and since it was known that processing of POMC occurs in approximately 2h (Crine et al 1980), it was decided to use a 2h pulse. Both extracts of NIL
and the incubation media were investigated.

In extracts prepared from +/+ and ob/ob NIL, after a 2h pulse with $[^3\text{H}]$phe, labelled CLIP was seen (figures 15 and 19). When the lobes were incubated for a further 3h in the absence of $[^3\text{H}]$phe, the incorporation of $[^3\text{H}]$phe into CLIP increased in extracts of both +/+ and ob/ob NIL (figures 16 and 20). Presumably, this was due to POMC, which had become labelled during the 2h pulse with $[^3\text{H}]$phe, being processed into CLIP and other POMC-related peptides.

When the peptides released into the media were studied, labelled CLIP was apparent in either +/+ or ob/ob NIL, after a 2h incubation with $[^3\text{H}]$phe. However, after a 3h chase period in the absence of $[^3\text{H}]$phe, the ob/ob and +/+ NIL secreted labelled CLIP as well as other radiolabelled peptides (figures 14 and 18). Since the profile of $[^3\text{H}]$phe incorporation in the 3h chase media was similar to that of the 2h pulse extract, for both ob/ob and +/+ NIL, these results suggested that during the 3h chase period the peptides synthesised in the NIL, within the previous 2h, were secreted.

As previously explained, the hydrophobic properties of BCT caused it to be retarded on the Biogel P2/P6 columns and elute with the free amino acids. Therefore, in these experiments it was impossible to ascertain whether BCT was being synthesised. In order to establish the biosynthesis of BCT it was therefore essential to carry out a further step in the purification.

The free $[^3\text{H}]$phe associated with the BCT samples was removed by using reverse-phase HPLC with two different propanol
gradients. In this way it was shown that in the +/+ NIL extract, following a 2h pulse, the BCT had become labelled suggesting that de novo synthesis of BCT can occur within 2h (figure 21a). The extract of +/+ NIL after the 3h chase period also showed $[^3H]$phe incorporation, although the estimated specific activity had decreased compared with the BCT produced during the 2h pulse (figure 21b).

This fall in estimated specific activity may reflect the activity of the incubated NIL; as the incubation progresses, the cell death will increase due to insufficient oxygen diffusion.

An alternative suggestion is that since BCT can be synthesised de novo within 2h, it is possible that BCT is being produced from newly synthesised precursor, using 'cold' phe within the 3h chase period. This would have the effect of diluting the $[^3H]$phe labelled BCT produced from precursor which had been labelled within the 2h pulse.

Figure 22a shows the purified BCT released into the incubation media by +/+ NIL during the 3h chase period. The estimated specific activity associated with this BCT had decreased in comparison with the BCT in +/+ NIL extracts after a 2h pulse with $[^3H]$phe and a 3h chase. Hence, during secretion within the 3h chase period it appears that the BCT loses incorporated $[^3H]$phe. A possible explanation for this is that a carboxypeptidase B-like enzyme acts on the peptide in association with secretion. Since phe is the C-terminal amino acid residue of BCT, then its removal would decrease the specific activity of the peptide. However, this would mean that des-phe-BCT would have to have indistinguishable elution
characteristics, on the Biogel P2/P6 column and on reverse-phase HPLC, as BCT.

As these results are taken from a single experiment due to the lack of time, it is perhaps premature to speculate on the quantitative differences in the specific activities. The main significance of these findings is, however, that they demonstrate for the first time that BCT is synthesised in the NIL in two hours, as measured by the incorporation of $[^3\text{H}]$phe, and some of the labelled precursor remains in the lobe for a further 3h period, whereas part of it is secreted into the incubation medium. Unfortunately due to the lack of time, only the BCT from ob/ob NIL incubation media after the 2h pulse and 3h chase was purified using reverse-phase HPLC, and only the 3h chase media produced positive results (figure 22b). The BCT secreted by ob/ob NIL during the 3h chase period had incorporated $[^3\text{H}]$phe, demonstrating that de novo synthesis had also occurred in the ob/ob NIL. The amount and estimated specific activity of the BCT from ob/ob NIL was slightly greater than the amount and estimated specific activity of the BCT released during the 3h chase period from +/+ NIL. This may reflect the increased BCT synthesis associated with the ob/ob NIL (see Ia), however, statistically viable, quantitative studies would have to be carried out before this could be confirmed.

The results of $[^3\text{H}]$phe incorporation into BCT, at present, can not identify POMC as the BCT precursor or whether CLIP is an intermediate form. The preliminary findings however suggest a difference in the time course of labelling of CLIP and BCT.
Thus, whereas the labelling of BCT in an extract of the +/+ NIL after a 2h pulse period was greater than that following the 3h chase, the labelling of CLIP with $[^3H]$ phe increased in the 3h chase period.

These differences in the pattern of $[^3H]$ phe incorporation into CLIP and BCT may imply that BCT is not synthesised from CLIP, although this does not exclude that BCT is synthesised from POMC. However, in view of the fact that the experiments were only performed once, any conclusions drawn from these results are of a preliminary nature and therefore speculative.

If BCT synthesis has an increased turnover compared to CLIP synthesis, an explanation for this could be that BCT is cleaved from the 21k intermediate ACTH form, after the β-LPH sequence has been detached. However, this would entail the production of a novel intermediate ACTH form consisting of the N-terminal sequence and ACTH$_{1-21}$.

In order to obtain more information as to the precursor of BCT, the proteins separating in the 'void volume' of the Biogel P2/P6 columns were studied by SDS-PAGE, both from an extract of ob/ob NIL and in the incubation media. As can be seen (figure 23) there is apparently no new intermediate form, only proteins of an apparent molecular weight as predicted from the known intermediate forms of POMC (discussed below). Hence, the formation of BCT from a 21k precursor would seem unlikely, but cannot be totally ruled out since the resolution of the 10-20% acrylamide gels may not be sufficient to separate out such an intermediate form.

An alternative but remote possibility is that 3CT is not
synthesised from the 31k prohormone. Recently, Garnier et al (1982) have shown that an 80k neurophysin, which is associated with the hypothalamus and posterior lobe of the pituitary, may contain an ACTH sequence. It is thus a possibility that BCT is not secreted by the pars intermedia but by the neural tissue of the NIL. However this is unlikely since the neurophysins secreted by the posterior pituitary are synthesised in the hypothalamus (Russell et al 1979) whereas figures 22 and 23 indicate that BCT is synthesised in the NIL, and thus presumably in the pars intermedia. Furthermore, other peptides derived from the POMC, eg ACTH, αMSH and β-endorphin have been shown to be elevated in obesity.

Loh and Jenks (1981) have shown that the frog NIL releases POMC-related peptides from a fast and a slow turnover pool, a system which they suggest may facilitate preferential release of certain peptides from the NIL. Hence, it is a possibility that BCT may be synthesised from a fast turnover pool of POMC intermediates in the mouse pars intermedia whereas CLIP may be released from both a fast and a slow pool. It would seem that BCT is not stored to any extent within the NIL and thus presumably is not retained in a slow pool. In the extracts of NIL, both from +/+ and ob/ob mice there is in fact no discernable peak of BCT as measured by CLIP-RIA (figures 15, 16, 19 and 20). The BCT obtained from extracts of +/+ and ob/ob NIL was ascertained from a slightly increased basal CLIP-RIA value eluting at the expected position for BCT on the Biogel P2/P6 column. The BCT from extracts was later found also to elute at an expected position for BCT on
reverse-phase HPLC. Furthermore the fractions when collected and lyophilised demonstrated insulin releasing activity (S. Dunmore, unpublished data).

Larger amounts of BCT were isolated from the medium, following incubation of NIL, than could be obtained from extracts. This suggests that the peptide is cleaved from the precursor just prior to and in association with the process of secretion. The mechanism of control of BCT production and secretion has not been studied, but could be under neural control possibly from the hypothalamus.

Presuming that BCT is cleaved from an ACTH or CLIP intermediate form, then the synthesis would occur by the action of a protease which cleaves at a lys-val bond making this a unique action in the processing of POMC; all the known specific cleavages of POMC to produce the intermediate forms and end products occur at a dibasic amino acid site (Nakanishi et al 1979; Chretien and Seidah 1981). Loh and co-workers have characterised the general prohormone converting activity associated with the intermediate lobe secretory granules as an acid-thiol-arginyl protease, by the inhibitory action of certain protease inhibitors (Loh and Gainer 1982; Loh and Chang 1982). These same workers incubated labelled POMC with the enzymes of the intermediate lobe secretory granules for a prolonged period and in addition to POMC found αMSH, ACTH-intermediate forms, N-terminal POMC, βLPH and βendorphin-like peptides but no other peptides. Since these peptides were characterised by their apparent molecular weight using SDS-PAGE and their ability to cross-react with 'ACTH1-24 antibodies' or
'ßendorphin antibodies', it would have not been possible to identify BCT. Thus these workers, although characterising the prohormone converting activity for the general di-basic amino acid cleavages would not have observed the possible presence of a BCT synthesising protease.

Although prohormones are commonly cleaved at di-basic amino acid sites, it is by no means unusual for the cleavage to occur at different sites. It may be coincidental in view of the fact that neurophysins appear to contain the ACTH sequence (Garnier et al 1982), but neurophysins are detached from a neighbouring glycoprotein by the cleavage at a site containing a single basic amino acid (Brownstein 1982).

Since BCT can be produced by a tryptic cleavage of CLIP and since an NIL extract contains very little BCT, it has been suggested that BCT may be formed by a non-specific trypsin-like enzyme in the incubation medium. However, the demonstration of BCT labelled with $^{3}$H phe in NIL extracts implies that the NIL actually synthesises the peptide. Furthermore, the incubation of NIL with 1mg/ml lima bean trypsin inhibitor did not affect the yield of BCT in the incubation media (results not given) and perifusion of NIL, collecting the perifusate in the presence of a 'cocktail' of protease inhibitors, also had no effect on the yield of BCT (J. Morton, unpublished data). Hence, it would seem that BCT synthesis is due to a specific cleavage.

Post-translational modifications may have an important bearing on BCT synthesis in the NIL. Certain forms of the POMC molecule are glycosylated within the ACTH$_{22-39}$ region (Roberts et al 1978); this could not be the precursor of BCT which has
been shown to be non-glycosylated (J. Morton unpublished data).

Recently a phosphorylated form of CLIP has been isolated from rat NIL. (Browne et al 1981; Bennett et al 1981; 1982). It has been suggested that the phosphorylation of serine-31 (ACTH designation) of CLIP prevents glycosylation of the CLIP peptide (Bennett et al 1981, 1982). However, for this to occur in the mouse NIL during the synthesis of BCT, the BCT would have to be dephosphorylated. As already described native BCT coelutes with synthetic ACTH22-39 on reverse-phase HPLC and on the Biogel P2/P6 column (figure 8; Beloff-Chain et al 1982b) and the addition of a phosphate group would drastically alter the hydrophobicity of the peptide and thus change its elution characteristics.

The reasoning above can also be applied to other post-translational modifications of BCT. If a modification of the peptide was to occur then it would have to be such that the hydrophobicity of the peptide was not altered, or only altered slightly. This immediately rules out N-acetylation since N-Ac-BCT has already been shown to have a different elution profile compared to the native peptide when run on reverse-phase HPLC (Beloff-Chain et al 1981). Furthermore, N-Ac-BCT has a reduced insulin releasing activity (Beloff-Chain et al 1981) and a reduced lipogenic activity (figure 31).

Although BCT may not be N-acetylated, most of the other intermediate lobe peptides can occur as acetylated derivatives. The N-terminal serine of αMSH is acetylated (Harris 1959) and more recently N,O-diacetyl-α-MSH has been isolated from the pars intermedia (Rudman et al 1979; Browne et al 1981). The
β-endorphin-like peptides in the pars intermedia are also mainly N-acetylated (Smythe and Zakarian 1980).

Smythe and co-workers have suggested that N-acetylation of αMSH and β-endorphin-like peptides is a method of bioactivity control in the intermediate lobe (Smythe et al. 1979; Smythe and Zakarian 1980). This hypothesis is based on the fact that N-acetylation increases the melanotropic activity of αMSH (Schwyzer 1977) while inactivating the β-endorphin-like peptides (Geisow et al. 1977; Smythe et al. 1979). However, in view of the fact that des-Ac-αMSH has the same steroidogenic activity on rat adrenal glomerulosa cells as does αMSH (Vinson et al. 1981) it would seem that N-acetylation has a function other than a generalised bioactivity control mechanism.

From the evidence given above, it would seem that BCT is not a typical peptide of the pars intermedia. It is synthesised by an uncommon cleavage involving a single basic amino acid and it does not appear to be stored in the intermediate lobe in any substantial quantity. Furthermore, unlike virtually all the pars intermediate peptides it does not appear to undergo post-translational modification, although there is a possibility that the C-terminal amino acid may be removed during secretion.

Separation of the proteins associated with the void volume of the Biogel P2/P6 column revealed proteins with apparent molecular weights of 31K, 21K, 17K and 14K (figure 23). Despite the fact that these proteins have not been characterised using immunoprecipitation with 'ACTH antibodies' and 'β-endorphin antibodies', it would seem that they are POMC, 21K ACTH, N-terminal POMC and possibly 14KACTH, respectively (figure 4).
Mains and Eipper (1979) have suggested that the 14K peptide isolated from pars intermediate cells may be glycosylated CLIP. However, the 'A' CLIP-like peak found in NIL extracts and incubation media has been shown to bind to concanavalin-A (J. Morton, unpublished data) and hence is believed to be glycosylated CLIP. Since the 'void volume' proteins do not include the 'A' peak then either there are two forms of glycosylated CLIP or the 14K peptide is not glycosylated CLIP.

From the SDS-PAGE of the void volume proteins, from both ob/ob NIL extracts and media, it would seem that the POMC maturation products 11K and 3.5K (figure 4) are absent. This piece of evidence suggests that the peptides labelled with $[^3H]p$he which elute adjacent to the void volume proteins, on the Biogel P2/P6 column, may be these peptides, probably $\beta$-LPH and $\beta$-endorphin.

Unfortunately, due to the lack of time, only an SDS-PAGE of the proteins from ob/ob NIL and media were run. For comparison purposes it had been intended to run an equivalent extract and media sample from +/+ NIL.

Experiments were carried out to compare the BCT release from NIL of ob/ob and +/+ mice into the incubation media. Following a 5h incubation period, the peptides of the media were separated on a Biogel P2/P6 column and the CLIP-like peptides were evaluated. When statistically compared only the A and B peptides were secreted in significantly greater amounts by the ob/ob NIL, the C, D and E peptides released by the ob/ob NIL were not significantly different from those released by the +/+ NIL (table 1). This result is in contrast to the observed insulin secretagogue activity...
released by the ob/ob NIL compared to the +/+ NIL (Beloff-Chain et al 1975a, 1978, 1979; Dunmore and Beloff-Chain 1982).

Furthermore, Billingham et al (1982) demonstrated the presence of BCT in ob/ob plasma but could not demonstrate BCT in +/+ mouse plasma. These results suggest that BCT release from the ob/ob NIL is markedly elevated compared to the +/+ NIL.

A possible explanation for the lack of a significant difference in BCT secretion (table 1) may be due to the invitro incubation method used. Since the incubations were carried out in batches of 5 NIL in 250µl of media, the build-up of secreted peptides may have resulted in a feedback inhibitory effect upon secretion. This would mean that the ob/ob NIL which would be secreting BCT at an elevated rate, would reach an inhibitory concentration of BCT before the +/+ NIL. Thus the +/+ NIL could secrete BCT at a normal rate until it reached the inhibitory concentration, meanwhile, for a proportion of this time the secretion from the ob/ob NIL would be suppressed.

The previous experiments in which the insulin secretagogue activity was compared used perifusions of ob/ob and +/+ NIL which by continually removing the secreted BCT prevents feedback inhibition.

An advantage of the incubation method used has been the identification of BCT in association with the homozygous lean mouse. Previously using perifusions, BCT secretion had been clearly demonstrated by the +/+ NIL, although its presence had been assumed due to the insulin secretagogue activity released by +/+ NIL (Beloff-Chain et al 1975a, 1977b, 1978; Dunmore and Beloff-Chain 1982). The incubation method enabled a longer incubation period to be used with the resultant
increase in BCT collected.

The results in table 1 also showed that the ratio of 'E': 'B' was different for ob/ob NIL (0.18) and +/- NIL (0.47). This may be partly due to the large variation in 'B' secreted by the ob/ob NIL, however, it does demonstrate that BCT secretion is not quantitatively linked with the secretion of CLIP by the same lobes. Hence it would seem that the control mechanisms involved in CLIP secretion may differ slightly from those involved in BCT secretion.

IVb The lipogenic activity of β-cell tropin on the adipose tissue and possible mechanisms of action

The results in table 2 demonstrate that native BCT has lipogenic activity as was suggested by the action of this peptide in vivo (Beloff-Chain et al 1982a). The discovery of this fact opens up a whole new field of endocrine research and is of particular interest since this peptide is related to ACTH: a peptide hormone with lipolytic properties in adipose tissue. (see Fain 1980).

The specificity of the lipogenic action of this ACTH fragment can be seen in table 4, since neither ACTH nor CLIP, the known peptide it most closely resembles, was found to have an effect on lipogenesis in isolated adipocytes. Using the C-terminal hexapeptide ACTH 34-39, it has been shown that the bioactivity does not reside within the free C-terminal sequence. This would seem logical since both ACTH and CLIP have this free C-terminal moiety.

Having identified the ACTH sequence responsible for the lipogenic activity as ACTH 22-39, it was then interesting to
investigate the action in relation to a known lipogenic agent, insulin. Using a maximal concentration of insulin (100μU/ml) for comparison, it was found that 0.5ng/ml native mouse BCT produced a similar stimulation (tables 2 and 3) implying that this concentration of BCT may be maximal. This was corroborated by a dose-response curve (figure 26) showing 0.5ng/ml gave a maximal stimulation of lipogenesis. From this dose-response curve the 50% activity concentration was found to be 2.5x10^{-11}M, presuming that the RIA equally recognises CLIP and BCT. As was mentioned (see IIIb iv), the evidence for the 'CLIP-antiserum' equally recognising the two peptides comes from UV_{280} absorption and RIA data. Additional data in support of this assumption lies with the observed CLIP-immunoactivity of ACTH_{31-39}, suggesting that the major antigenic determinant site(s) reside toward the C-terminal section of CLIP. In view of these two pieces of evidence, it is assumed throughout this thesis that the levels of BCT, as determined by 'CLIP-RIA', are correct. However, since no definite quantitative data has been produced, it is possible that the 'CLIP-antiserum' may preferentially bind to CLIP.

When the 50% activity concentrations of BCT and insulin are compared on a molar basis, the observed basis of 2.5x10^{-11}M for BCT is slightly more potent than the value of about 10^{-10}M for insulin action as measured on glucose oxidation and incorporation into lipid in isolated adipocytes (Kahn 1976; Kahn et al 1981; Olefsky 1976b).

The use of ^3H incorporation into fatty acids by labelling the NADP molecules with ^3H (Jungas 1968) enables fatty acid
synthesis to be measured, however this does not reveal any detailed information as to the pathway of metabolism involved. By measuring the fate of isotopically labelled glucose, which in rat adipose tissue is the predominant carbon source for fatty acid synthesis, details of the control of various pathways can be evaluated. The effect of BCT (5ng/ml) on glucose metabolism was therefore examined and compared with the effect of insulin (100μU/ml). The results showed a number of similar effects (tables 5 and 6). It is therefore probable that insulin and BCT act upon a common rate-limiting step in glucose metabolism. The mechanism of action of insulin on glucose metabolism in adipose tissue has been widely investigated, and therefore some of the conclusions reached are discussed as they may ultimately provide a clue for the mechanism of action of BCT as a lipogenic hormone. Unfortunately there was insufficient time to pursue the study of the mechanism of action of BCT at a molecular level, and therefore the hypothesis that it behaves similarly to insulin is speculative. A discussion of the theories proposed for the action of insulin does however suggest how further studies on BCT action in adipose tissue could be pursued.

\[6\textsuperscript{14}C\]glucose incorporation into saponified fatty acids and glyceride-glycerol were stimulated by BCT and insulin (tables 5 and 6). The key enzymes in glucose metabolism responsible for its conversion to saponified fatty acids appear to be PDH and ACC (figure 2). Insulin action on adipose tissue induces a parallel increase in the activities of both of these enzymes (Stansbie et al 1976; Denton et al 1977; Jungas 1971;
It has been suggested that the activation of the mitochondrial enzyme PDH is by an insulin mediated dephosphorylation (Linn et al 1969a,b). Three phosphate sites situated on the α-subunit of the enzyme are all dephosphorylated in response to insulin, whereas protein kinase action in phosphorylating these sites causes deactivation (Hughes and Denton 1976; Denton and Hughes 1978; Hughes et al 1980).

The activation of the enzyme appears to be by PDH-phosphoprotein phosphatase in the mitochondria (Hughes and Denton 1976; Denton and Hughes 1978), but how insulin modulates the activity of this enzyme is not yet fully understood. It has been reported that insulin treatment can activate a phosphoprotein phosphatase in adipose tissue extracts (Mukherjee and Jugas 1975; Sica and Cuatrecasas 1973), however this has been disputed by some workers (Severson et al 1974). Another theory is that insulin acts upon PDH-phosphatase by modulating the levels of Ca$^{2+}$ in the mitochondria; the PDH phosphatase has a requirement for Ca$^{2+}$ and Mg$^{2+}$ (Denton and Hughes 1978; Severson et al 1974; Hughes and Denton 1976). However, no insulin mediated change in mitochondrial Ca$^{2+}$ has been observed (Severson et al 1974, 1976).

ACC has also been shown to be a phosphoprotein (Brownsey et al 1977) although the mechanism of insulin induced activation is different from that of PDH. In contrast to PDH, insulin causes ACC to be phosphorylated while, paradoxically, adrenaline induced deactivation of the enzyme is also by
phosphorylation (Brownsey et al 1979; Denton et al 1981). It has been suggested that insulin and adrenaline mediated phosphorylations occur at different sites; the relative extent of phosphorylation of these two sites dictates the state of activity of the enzyme (Denton et al 1981). A third phosphorylation site is apparently relatively unaffected by these two hormones (Denton et al 1981).

Using \( \left[^{61}\text{C}\right] \) glucose incorporation into glyceride-glycerol as a measure of esterification, then both insulin and BCT can stimulate the triacylglycerol production. Esterification occurs between CoA-thioester derivatives of fatty acids and glycerol-3-phosphate, although dihydroxyacetone-phosphate can also accept the acyl group (Dodds et al 1976).

The ability of insulin to activate esterification has been demonstrated. Sooranna and Saggerson (1975) have shown insulin stimulated incorporation of \( ^{3}\text{H} \) palmitate and \( ^{14}\text{C} \) fructose into TAG.

How this is brought about is poorly understood, although like fatty acid synthesis it may occur by the activation of certain enzymes. Jason et al (1976) have reported insulin activation of fatty acyl-CoA ligase: the enzyme responsible for the production of \( ^{\text{L}}\text{CoA} \) derivative of fatty acids. Furthermore, adrenaline can decrease the activity of two esterification enzymes, glycerol phosphate acyltransferase and phosphatidate phosphohydrolase (Sooranna and Saggerson 1976a; Cheng and Saggerson 1978), while insulin can antagonise these deactivations (Cheng, C.H.K. et al 1980; Saggerson 1979).

Another similarity in the influence of BCT and insulin on glucose metabolism in adipose tissue was shown by the
stimulation of $^{13}C\text{O}_2$ from $[1^{13}C]\text{glucose}$ (tables 5 and 6). The $^{13}C\text{O}_2$ produced is an approximate estimate of the glucose oxidised via the hexose monophosphate pathway.

Unlike fatty acid synthesis and esterification, the effect of insulin on the pentose phosphate pathway is probably indirect. It has been shown that fatty acid synthesis linearly correlates with the activity of glucose-6-phosphate dehydrogenase, the rate controlling enzyme (Katz et al. 1966; Saggerson and Greenbaum 1970). Hence a stimulation of fatty acid synthesis would increase the turnover of the pentose phosphate pathway. This is probably due to the utilization of NADPH by fatty acid synthesis with a resulting fall in the NADPH:NADP$^+$ ratio; this would relax the inhibition of glucose-6-phosphate dehydrogenase by NADPH and thus activate the pathway (Saggerson and Greenbaum 1970; Eggleston and Krebs 1974). Support for this theory has been provided by Richardson and Czech (1978) who showed that the inhibition of fatty acid synthesis in insulin-sensitive small adipocytes, by rotenone, reduced the turnover of the pentose phosphate pathway.

The secondary action of lipogenic agents on the pentose phosphate pathway would explain the observed results for BCT and insulin action on $[1^{13}C]\text{glucose}$ oxidation (tables 5 and 6). Since both BCT and insulin were shown to stimulate fatty acid synthesis, the increase turnover of the pentose phosphate pathway would be as a consequence of that action.

In experiments with $[6^{13}C]\text{glucose}$, no effect on glucose oxidation was observed with insulin although BCT significantly increased $^{13}C\text{CO}_2$ production (tables 5 and 6). This absence of an effect with insulin may be due to the pertubations in the
measurement of small amounts of labelled product, as mentioned above (see IIIb v). This would seem probable since Saggerson and Greenbaum (1970) demonstrated that insulin treated fat pad had an increased AMP concentration which would be expected to increase the glucose flux through glycolysis and the Krebs cycle. A similar interpretation can be applied for the action of BCT on the stimulation \([6^{14}C}\) glucose oxidation (table 5). The resultant increased fatty acid synthesis due to BCT would utilize ATP causing an increase in the cellular concentration of ADP and AMP and hence activate phosphofructokinase. The increased \([6^{14}C]\) glucose oxidation would thus be as a result of the increased flow of glucose through glycolysis and the Krebs cycle.

When the action of 5ng/ml BCT and 100μU/ml insulin on \([1^{14}C]\) glucose incorporation into total lipid, saponified fatty acids and glyceride-glycerol, in isolated adipocytes, was examined (tables 5 and 6) then neither of these hormones had a significant effect. This may be due to a dilution of the triose phosphate pool due to the increased turnover of the pentose phosphate pathway (figure 3). After the specific decarboxylation of the C-1 atom of \([1^{14}C]\) glucose, the intermediates processed by the pentose phosphate pathway would be unlabelled. Hence the glyceraldehyde-3-phosphate, an end-product of this pathway, entering the triose-phosphate pool would be 'cold', reducing the specific activity of the pool and ultimately the specific activity of the triglyceride moieties.

The measured effects of BCT and insulin in this thesis, though significant, are low compared to the observed effects
reported by many workers for insulin. This is thought to be due to the presence of free fatty acids associated with the albumin used in the incubations; the albumin had been dialysed with Krebs-Ringer bicarbonate buffer to remove impurities, however, it was not defatted. The presence of free fatty acids in the incubation of isolated adipocytes would inhibit fatty acid synthesis due to an inhibitory effect on ACC and PDH (Sooranna and Saggerson 1975, 1976b, 1979; Halestrap and Denton 1973, 1974). An inhibited fatty acid synthesis may be a contributing factor to the negative results obtained in measuring the stimulatory action on \([l^{14}C]\) glucose incorporation into lipid.

Despite the observed effects of BCT in adipocytes, there was a possibility that collagenase treatment may have damaged the BCT-receptors with a resulting rightward shift in the dose-response curve; the BCT-receptors on pancreatic islets may be damaged by collagenase (Beloff-Chain et al 1980b). Therefore, some experiments were carried out with adipose tissue pieces. The latter were incubated with 5ng/ml BCT and the resulting \([l^{14}C]\) glucose oxidation and \(^3\)H incorporation into total lipid was measured (tables 8 and 9). Control experiments with insulin showed a marked stimulation of both these parameters (tables 8 and 9) as had been reported by other investigators (see Id iii). BCT however had no action in adipose pieces. This result could be due to the inability of the peptide to penetrate the adipose tissue mass. The hydrophobic nature of BCT could result in a high level of non-specific binding and hence reduce the penetration into the tissue.
A more plausible explanation for the absence of a 2C37-effect on adipose tissue pieces is that 3CT is more prone to the action of peptidases than is the larger insulin molecule and hence is rapidly broken down. In the isolated adipocyte preparation, the presence of proteolytic enzymes would be less of a problem since dispersal of the cells would allow these enzymes to be removed when the adipocytes were washed.

As has been mentioned, insulin appears to act upon glucose transport by stimulating the translocation of glucose carriers from a golgi-microsomal membrane pool to the adipocyte plasma membrane (Kono and Suzuki 1981; Karnieli et al 1981). The increase in plasma membrane glucose carriers has the effect of increasing the Vmax of glucose transport. The mechanism of insulin action in translocating the glucose carrier proteins is unknown.

In order to ascertain whether BCT influenced glucose transport, experiments were carried out in which the uptake of 2-deoxyglucose in adipocytes was measured. BCT had a significant effect but this was small in comparison to the action of insulin (figure 28).

If it is presumed that BCT acts upon the glucose transport system in an analogous manner to that of insulin, then the interfacing of the 'BCT-signal' to this system must be inefficient compared to that of insulin. On the other hand, BCT may act in a different manner to insulin, possibly in a way similar to that of the redox reagents (Czech et al 1974 a, b; Czech 1976d, b, c), by effecting the redox state of certain sulphydryl groups.
It has been suggested that insulin may control glucose metabolism in the adipocytes by controlling glucose transport (Kather et al 1972; Cushman et al 1981). This is based on the linear relationship of glucose transport and the pentose phosphate pathway or fatty acid synthesis (Kather et al 1972). This theory does not take into account the specific activation of certain enzymes by insulin.

In experiments using fructose, whose uptake is only minimally effected by insulin, Denton et al (1977) demonstrated that PDH and ACC were activated by insulin in rat epididymal fat pad. Furthermore, insulin was shown to stimulate \([\text{[^{13}C]}\) fructose incorporation into triacylglycerol in isolated adipocytes (Sooranna and Saggerson 1975).

The results produced for \([{^{14}C}]\) glucose incorporation into total lipid, saponified fatty acid and glyceride-glycerol (table 5) indicated preferential pathway activation. It would appear probable that BCT, which only has a weak effect on glucose transport, must activate the appropriate enzymes for lipogenesis, and thus direct glucose utilization.

When maximal concentration of BCT and insulin were tested together on isolated adipocytes, the incorporation of \([{^{14}C}]\) glucose into lipid was not significantly different from the action of the individual peptides at maximal concentration (figure 27). This would suggest that the mechanism of action of BCT and insulin has a common rate-limiting step.

Presuming that BCT acts upon fatty acid synthesis and esterification in a similar manner to insulin, the common site of action is unlikely to be at the enzyme level since the
activation-deactivation mechanisms of the enzymes themselves are apparently different. This means that the common step of insulin and BCT action on lipogenesis is either the release of a secondary messenger or in a cascade reaction needed to amplify the hormonal 'signal'. However, neither the insulin secondary messenger nor the intervening steps of the cascade reaction are known.

It is improbable that both insulin and BCT have an identical secondary messenger since BCT only elicits a weak response to glucose transport and has no antilipolytic effect, both of which are highly responsive to insulin. The remote possibility that BCT was a partial agonist of insulin was eliminated since excess BCT had no effect on $^{125}$I-insulin binding to isolated adipocytes (table 14).

From the results in tables 12 and 13 it can be seen that BCT has no effect on cAMP levels in isolated adipocytes. cAMP was not expected to be involved in BCT action since it was demonstrated that BCT had no lipolytic or antilipolytic action (tables 10 and 11).

Insulin has been reported as causing a decrease in catecholamine elevated cAMP levels in adipose tissue (Butcher et al 1968; Desai et al 1973; Kono and Barham 1973), which was also seen in this study (table 13). The lowering of cAMP levels may be associated with the antilipolytic effect of insulin since insulin does not antagonize dibutyryl-cAMP induced lipolysis (Blecher et al 1968; Fain and Rosenburg 1972). However, the antilipolytic action of insulin does not, according to some investigators, correlate with the decreased cAMP levels (Siddle and Hales 1974) and there are reports that
insulin induced anti-lipolysis can occur without any change in cAMP concentrations (Fain and Rosenberg 1972; Khoo et al 1973). Hence, cAMP is probably of secondary importance to the antilipolytic action of insulin.

Since cAMP does not appear to be the secondary messenger for either insulin or BCT then other mechanisms must be considered. Despite the assumption that BCT and insulin probably have a different secondary messenger, there must be some similarities in their common action. Thus, mechanisms of action which are not appropriate for insulin are unlikely to apply to BCT either.

Recently much work has been carried out on the role of Ca\(^{2+}\) as a secondary messenger (review Berridge 1980). There is a lot of evidence to suggest that the coupling of the agonist's signal to the gating of Ca\(^{2+}\) works by the stimulation of phospholipase-C on the inside of the plasma membrane, which results in a breakdown of the phospholipid phosphatidyl-inositol (Michell 1979; Berridge 1980). This breakdown of phosphatidyl-inositol is thought to be linked with Ca\(^{2+}\) gating into the cell (Michell et al 1977; Michell 1979).

Insulin treated adipocytes have been shown to increase \(^{32}\)Pi incorporation into phospholipids and especially phosphatidyl-inositol, suggesting a preferential increase in the turnover of this phospholipid (De Torrontegui and Berthet 1966). Stein and Hales (1974) also demonstrated an insulin induced incorporation of \(^{32}\)Pi into phospholipids, however this was attributed to an increase in the specific activity of ATP.

Recently, Garcia-Sainz and Fain (1980) have shown that
under conditions where no increase of $^{32}$P labelling of phosphatidyl-inositol was seen in isolated adipocytes, the insulin action on [1$^{14}$C] glucose oxidation and inhibition of lipolysis were normal. This data appears to preclude the possibility that insulin acts by stimulating phospholipase-C and phosphatidyl-inositol, and hence Ca$^{2+}$ gating. Furthermore, a Ca$^{2+}$ gating, alpha-1 adrenergic response has been demonstrated in the rat adipocyte, however its only cellular action was the inhibition of glycogen synthetase (Lawrence and Larner 1978). Hence, in the adipose tissue, Ca$^{2+}$ may have an antagonistic action to that of insulin. This would seem probable since insulin has been shown to increase Ca$^{2+}$ sequestration by adipocyte membranes (McDonald et al 1976) while cellular depletion of Ca$^{2+}$ from adipocytes inhibits lipolysis (Efendic et al 1970).

Many agonists which facilitate Ca$^{2+}$ gating also stimulate guanylate cyclase (Berridge 1980). Although insulin does not stimulate Ca$^{2+}$ gating, it has been shown to stimulate cGMP production in adipocytes (Illiano et al 1973). This, however, can be discounted as a mechanism of insulin action since noradrenaline, carbachol (which has no action in adipocytes) and the calcium ionophore A23187 all increase cGMP levels in adipocytes (Fain and Butcher 1976). Furthermore, adipocytes incubated with insulin in Ca$^{2+}$ free buffer had an undiminished antilipolytic response despite a decreased cGMP concentration (Fain and Butcher 1976).

Denton et al (1981) have recently put forward the hypothesis that insulin acts upon the adipocyte to stimulate the action of a cyclic nucleotide and Ca$^{2+}$ independant protein
kinase. Once activated this protein kinase would phosphorylate specific proteins to activate and deactivate various metabolic processes. To explain the specific dephosphorylation of PEH, the theory envisages the existence of a phosphoprotein phosphatase activator which is activated by the protein kinase (Denton et al 1981).

The immediate activation of a protein kinase, by a hormone binding to its receptor, is not a novel concept. Epidermal growth factor acts upon A431-epidermoid carcinoma cells to activate a kinase associated with the receptor, and the kinase phosphorylates the receptor and certain endogenous proteins (Carpenter et al 1978, 1979; Cohen et al 1980).

In support of this hypothesis, Brownsey et al (1981) have discovered a protein kinase associated with fat pad plasma membranes. This protein kinase stimulated ACC activity by phosphorylation, in a reaction independant of cAMP and Ca$^{2+}$ (Brownsey et al 1981). Furthermore, high speed supernatant fractions of adipocytes exposed to insulin were found to have an increased protein kinase activity capable of phosphorylating ACC and ATP–citrate lyase (Brownsey et al 1982).

In a reaction which may be related to the above protein kinase hypothesis, insulin treatment of liver plasma membranes, heptoma cells and 1M-9 lymphocytes, stimulated the phosphorylation of the 95K $\beta$-subunit of the insulin receptor, in a specific and dose dependant manner (Kasuga et al 1982a,b). Hence it appears that one of the initial acts of insulin, after binding to its receptor, is the stimulation of a protein kinase. Whether this kinase phosphorylates proteins other than the insulin-receptor is not known, however only the $\beta$–
subunit appeared to labelled in intact cells (Kasuga et al 1982b).

Whether the activation of the above mentioned protein kinases is responsible for transmitting the insulin signal is unknown. It may be that these protein kinases are activated as a secondary event in the cascade, possibly analogous to the cAMP activation of cAMP dependant protein kinase.

Another recent theory of insulin action involves the production of a peptide secondary messenger by a plasma membrane mediated event. Various putative peptidergic secondary messengers have recently been reported. Larner and co-workers have isolated from an insulin treated muscle extract an oligoglycopeptide(s) of molecular weight 1-1.5K (Larner et al 1979, 1981; Cheng, K. et al 1980). The isolated active fraction stimulated phosphoprotein phosphatase and inhibited cAMP dependant protein kinase (Larner et al 1979) and furthermore this factor was also active in stimulating PDH activity in an adipocyte mitochondria-plasma membrane broken cell system (Jarett and Seals 1979).

A similar, if not identical, peptide has been isolated from insulin treated adipocyte plasma membranes (Kiechle et al 1981). This peptide also stimulate PDH activity in the adipocyte broken cell system by the action of a phosphatase (Kiechle et al 1981).

Seals and Czech (1980, 1981) have also reported a putative insulin secondary messenger, isolated from insulin stimulated adipocyte plasma membranes. This peptide also increased PDH activity in the 'broken cell assay', however the peptide was reported to be of a molecular weight of 2-4K (Seals and Czech
The differences in these putative secondary messenger peptides may be due to methodology, however, on the other hand insulin may initiate the synthesis of more than one peptidergic secondary messenger. If this latter suggestion is correct then it is possible that BCT may specifically work via the action of one of these peptides. However, this is speculative since this hypothesis has yet to be fully proven for insulin.

Larner et al (1981) have proposed that the secondary messenger peptide of insulin is synthesised by the proteolytic cleavage of a precursor on the external surface of the plasma membrane; this peptide is then internalised by an event associated with insulin-receptor aggregation.

The insulinomimetic action of trypsin has been cited in support of this theory as has the fact that a sufficiently high concentration of tosyl-lysine chloromethyleneketone, a protease inhibitor, can inhibit insulin action without inhibiting \[ ^{125}\text{I}\]-insulin binding (Larner et al 1981).

One weakness with this theory is that a single proteolytic cleavage would produce one molecule of peptide per insulin-receptor event. Stoichiometrically, this would not be sufficient for biological activity (Denton et al 1981) unless multiple copies of the peptide were produced or alternatively that this was the first step in a cascade reaction.

Either of these last two mechanisms may ultimately explain insulin's action, although further research has yet to be carried out. This lack of knowledge about insulin's control of
adipose tissue metabolism has made it difficult to speculate on a possible mechanism for BCT's action on lipogenesis.

**IVc The relationship of the structure of 3-cell tracin to its biological activity in adipocytes**

In order to study the specific binding properties of BCT with the adipocytes, \(^{125}\text{I}-\text{BCT}\) was prepared by the tryptic cleavage of \(^{125}\text{I}-\text{CLIP}\). Unfortunately, the \(^{125}\text{I}-\text{BCT}\) binding observed was indistinguishable from the non-specific binding measured in the presence of 1 μg/ml BCT.

It is possible that the adipocyte contains very few BCT-receptors and that the binding assay used was unable to distinguish a low number of sites from the non-specific binding.

An alternative explanation for the inability to demonstrate specific \(^{125}\text{I}-\text{BCT}\) binding, is that the addition of an iodine atom to the tyrosine-23 (ACTH designation) of BCT may prevent the peptide binding to the receptor. The addition of the iodine atom to the tyrosine residue may prevent the molecule binding with the BCT-receptor due to steric hinderance, or alternatively the iodine may alter the pKa of the phenolic group of tyrosine which could also prevent binding to the receptor.

To test the possibility that the addition of the iodine atom to the tyrosine residue was responsible for the absence of binding; 'cold' \(^{127}\text{I}-\text{BCT}\) was produced as previously described. When the lipogenic activity of this peptide was examined using isolated adipocytes, a small but significant stimulation of lipogenesis was observed. Even in the presence
of a high concentration of $^{127}$I-BCT (80ng/ml) the response was
significantly less than that obtained with 5ng/ml BCT
suggesting that the addition of the iodine atom does reduce
the peptide's lipogenic activity (figure 31).

It may be coincidental, but the iodination of sMSH on
the tyrosine, which as in BCT is the second residue from the
N-terminus, abolishes the steroidogenic activity of the
molecule (A. Bateman, personal communication). Hence it is
probable that these tyrosine residues play an essential part
in the bioactivity of these peptides.

The decreased bioactivity of iodo-BCT, presumably will
be reflected in the reduction of specific binding to the
BCT-receptors in isolated adipocytes, and hence may explain
why specific $^{125}$I-BCT binding was not seen.

An important observation that was made in the $^{125}$I-BCT
binding studies was that 57% of the iodo-BCT bound to the
siliconized incubation vial. Although iodo-BCT will be slightly
more hydrophobic than BCT, it must be recognised that during
adipocyte incubations a proportion of the peptide could have
bound to the incubation vial and reduced the effective
concentration of the BCT in lh incubation media. Because the
concentrations stated in this thesis represent the amount of
BCT added at the beginning of the incubation, they will
probably not represent the effective concentration during the
incubation. Since a maximal concentration of BCT was routinely
used this will probably not unduly effect the results. However,
with the dose-response curve, it is probable that the effective
50% activity concentration is lower than that determined.
N-Ac-BCT has a reduced insulin secretagogue activity compared to the native peptide (Beloff-Chain et al 1981) implying that the N-terminus of the BCT peptide is important for bioactivity on islets. In this thesis it has been shown that 1ug/ml C-terminal hexapeptide ACTH_{3-39} is devoid of lipogenic activity (tables 4 and 7) and ^{125}I-BCT has a reduced lipogenic activity. These results also suggest that the N-terminus of BCT is important for its biological activity on the adipocytes. Using des-val-tyr-pro-BCT and N-Ac-BCT, the importance of the N-terminus of BCT to the lipogenic activity of the molecule was further investigated. The absence of a response to des-val-tyr-pro-BCT, on isolated adipocytes demonstrated that the val-tyr-pro sequence was essential for the lipogenic activity of the peptide.

The addition of an acetyl group to the N-terminus of the BCT peptide caused a reduction in its lipogenic activity. The extent to which the activity was reduced is unknown and must await the production of a dose-response curve. However at 5ng/ml NAc-BCT did not significantly stimulate lipogenesis, while at 50ng/ml NAc-BCT the stimulation was comparable with that produced by 5ng/ml BCT.

From these preliminary experiments, it can be seen that the tripeptide sequence val-tyr-pro at N-terminus of the BCT peptide is essential for the stimulation of lipogenesis in isolated adipocytes. This tripeptide sequence presumably requires the free N-terminal valine residue since acetylation of this residue reduces bioactivity while the addition of arg-pro-val-lys, to produce CLIP, abolishes the lipogenic
activity.

Because this active val-tyr-pro sequence contains the only tyrosine residue in the BCT molecule, this has provided a problem for future investigation of BCT-receptors. Hence, some way, other than iodination, will have to be found to allow isotopic labelling of the peptide.

IVd Consideration of general aspects of Bcell tropin physiology

BCT has an unusual and possibly unique, physiological role in that its action stimulates the release of a hormone, insulin (see Ia), while it itself has an insulin-like action on adipocytes. The uniqueness of the reaction is that a hormone (BCT) stimulates the release of a second hormone (insulin) which has some identical actions to that of the first hormone. Why this happens is not known; since insulin stimulates adipose tissue lipogenesis it would seem that an additional lipogenic role for BCT would not be required.

A possible role for such a system may be that under certain physiological conditions a 'reinforcing' of the signal to lipogenesis is needed. Another possibility is that the BCT is involved in 'fine tu-ning' of lipogenesis. It may be that BCT causes the emphasis of the insulin-like stimulation of lipogenesis to be placed on the adipose tissue; this may occur since BCT did not stimulate liver lipogenesis (Beloff-Chain et al 1982). It is also possible that BCT has a role in promoting lipogenesis under conditions of insulin resistance (as discussed below).

The physiological action of BCT upon the adipose tissue may require the presence of insulin which promotes glucose
transport. BCT has only a weak glucose transport action (figure 28), and if it was acting alone on the adipose tissue and stimulating the conversion of glucose into lipid this could deplete the intracellular glucose. However in vivo, by stimulating insulin release it probably acts in the presence of insulin which would concurrently promote transport into the tissue.

Another reason why insulin may also be required is to prevent futile cycling. Since BCT has no antilipolytic action (tables 10 and 11), by stimulating lipogenesis and not effecting the rate of lipolysis, BCT alone would cause futile cycling. However, with insulin present lipolysis will be inhibited.

Figure 27 shows that BCT and insulin, at maximal concentration, do not have an additive effect. Hence the effect that BCT has upon the adipose tissue may depend on the localised insulin concentration. If insulin concentrations are such that maximal stimulation of the adipose tissue occurs then presumably the action of BCT would be irrelevant.

Whatever the function of BCT in vivo, the control of its release appears to be linked with the dietary state of the animal. Fed animals have been reported as releasing more pituitary insulin secretagogue (BCT) than fasted animals. (Beloff-Chain et al 1977b, 1978). Furthermore, administration of a glucose load or a high food intake, both increase the release of insulin secretagogue activity from the pituitary (Beloff-Chain et al 1978). Hence it is probable that the control of BCT is linked with feeding.

The hypothalamus, the main region of the brain responsible
for the control of food intake (see Bray and York 1979; Morley J.E. 1980), is presumably partially responsible for the control of BCT release. It is linked to the pars intermedia by a variety of neural fibres, via which the hypothalamus exerts its control (Howe 1973).

Additional control mechanisms appear to act upon the NIL. BCT appears to have an inhibitory feedback control on BCT secretion from the NIL (see IVa). Furthermore insulin may also inhibit BCT release; a preliminary observation showed that if ob/ob NIL were incubated in media containing insulin then the BCT released is reduced compared to an incubation without insulin (J. Morton, unpublished data). A feedback inhibitory action of insulin on BCT secretion from the pars intermedia would prevent excessive release of BCT; such a mechanism may have become desensitised or defective in the ob/ob mouse.

The half life of BCT in the bloodstream is of crucial importance to its action. Experiments in which BCT was incubated with adipose tissue pieces (tables 8 and 9) suggested that it might be broken down by proteolytic action, in this case BCT would not be expected to have a significant effect upon the adipose tissue. However, the injection of BCT into the carotid artery of a rat stimulated insulin release and also lipogenesis in subcutaneous adipose tissue (Beloff-Chain et al 1982a). Thus, in this experiment at least, BCT maintained its integrity sufficient time to manifest biological activity. Furthermore, in vivo, BCT will be constantly released by the pituitary and the levels of BCT in the periphery will reach an equilibrium concentration depending on the rate of release and rate of degradation. Since BCT is a potent peptide, the
concentration attained in the plasma could be sufficient to act upon the adipose tissue.

Because the action of BCT upon two different tissues elicits two dissimilar responses, one a secretagogue action the other an anabolic action it would be of interest to discover, how this occurs at a molecular level.

A possible mechanism of the action of BCT as an insulin secretagogue could be by the stimulation of adenylate cyclase, to produce cAMP, and/or the elevation of intracellular Ca\(^{2+}\) (see Sharp 1979; Hedeshkov 1980). It has been shown in adipose tissue that there is no effect of BCT on adenylate cyclase (tables 12 and 13). In addition, a second messenger role for Ca\(^{2+}\) appears to be improbable, in adipose tissue (see IVb). It is possible that BCT has different mechanisms of action in the different tissues. This, presumably, means that there are two different BCT-receptor types. The presence of two types of BCT-receptor could explain why collagenase treatment of islets reduced the responsiveness to BCT (Beloff-Chain et al 1980b) whereas the adipocytes produced by collagenase treatment are at least partially responsive to BCT.

If there are two types of BCT-receptor, it would seem that both would require the N-terminal sequence of the peptide. Evidence presented in this thesis (figure 31) has demonstrated that the lipogenic activity of BCT requires the three N-terminal residues val-tyr-pro. This has also been shown to be the case with the insulin secretagogue activity of BCT; N-Ac-BCT and des-val-tyr-pro-BCT had reduced or no insulin releasing activity (Beloff-Chain et al 1981, 1982b).
The possible role of β-cell tropin in the obese-hyperglycaemic (ob/ob) mouse

Since very little work has been carried out on the action of BCT in the ob/ob mouse, it is only possible to speculate as to a role for this peptide in the aetiology of the ob/ob syndrome.

Despite the fact that the release of BCT from ob/ob and +/+ NIL was not significantly different in results presented in this thesis, which was probably due to the invitro incubation method used, previous work has shown that the ob/ob mouse secreted an elevated level of BCT compared to its lean littermates. This has been determined by the insulin secretagogue activity of ob/ob and +/+ NIL (Beloff-Chain et al 1975a, 1978, 1979; Dunmore and Beloff-Chain 1982), and the fact that BCT was only detected in ob/ob mouse plasma (Billingham et al 1982). Hence, in addition to the various other endocrinological disorders (see Iaiii), the ob/ob mouse has elevated plasma BCT levels. This physiologically abnormal BCT release probably contributes to the hyperinsulinaemia of the ob/ob mouse.

Recent evidence has shown that the onset of lipid accumulation in the preobese ob/ob mouse arises concurrently with the hyperinsulinaemic syndrome (Dubuc 1976; Godbole et al 1980). Hence the excessive insulin secretion probably is an important contributory factor to increased lipid accumulation.

The problem in relating BCT levels to the hyperinsulinaemia of the preobese ob/ob mouse is that no longitudinal hormone
profile of BCT release in the ob/ob mouse has been ions. However, Beloff-Chain et al (1978) have demonstrated that the insulin secretagogue activity released from the pituitaries of 3 week old and 3 month old ob/ob mice is similar. From this observation it can be deduced that at 3 weeks of age the ob/ob mouse has an elevated plasma BCT level. Furthermore, islets of the ob/ob mouse are resistant to BCT at 3 months of age but are hypersensitive at 3 weeks (Beloff-Chain and Hawthorn 1976; Beloff-Chain et al 1977b, 1978). Hence it is highly probable that BCT plays a role in the early aetiology of the ob/ob syndrome.

In the lean (+/+) mouse the liver is the predominant lipid synthesising organ whereas in the ob/ob mouse it is the adipose tissue which is mainly responsible for excessive lipid synthesis, although the liver lipogenic rate is also increased (Hems et al 1975). Furthermore, Godbole et al (1980) demonstrated an increased carcass (adipose) lipogenic rate at 15 days of age in the preobese ob/ob mouse whereas the liver lipogenic rate was elevated at 35 days of age. Hence, it would seem that the adipose tissue is mainly responsible for the excessive stores of lipid in the ob/ob mouse (Hems et al 1975).

Due to the inability of BCT to stimulate liver lipogenesis in vivo (Beloff-Chain et al 1982a), it is possible that the elevated BCT levels, in the ob/ob mouse, by preferentially stimulating lipogenesis in the adipose tissue may contribute to the increased lipogenic rate, especially in the early aetiology of the obese syndrome. Depending at what age in the ob/ob mouse the BCT levels become elevated, BCT in association with the hyperinsulinaemia could be responsible for the
adipose tissue lipogenic rate becoming elevated before the hepatic lipogenic rate.

At about 4 weeks of age the ob/ob mouse develops insulin resistance (Stauffacher and Renold 1969; Dubuc 1976), probably as a means of protecting against hyperinsulinaemia. Hence, it is also possible that the islets of Langerhaus and the adipose tissue will react to the high plasma BCT levels of the ob/ob mouse (Billingham et al 1982) by developing a resistance to BCT. As mentioned above, this resistance has already been demonstrated with pancreatic islets from 3 month old ob/ob mice.

As has previously been described (see Ib), resistance can be achieved either by 'down-regulating' receptors or by developing a post-receptor defect, or by a combination of both.

It is not known if ob/ob adipose tissue develops BCT resistance, although it is probable that in response to the high BCT levels the adipose tissue at least down-regulates its BCT-receptors. In the case of the pancreatic islets from ob/ob mice, the resistance to BCT was abolished after a 48h fast, indicating that the resistance was probably largely due to the down-regulation of the BCT-receptors (Beloff-Chain et al 1977b).

Down-regulation has been demonstrated with the insulin-receptors in adipocyte plasma membranes of the ob/ob mouse (Freychet et al 1972), and furthermore, it has been found in ob/ob liver plasma membranes and in intact ob/ob thymocytes (Kahn et al 1972a, b; Soll et al 1975a, b).

Le Marchand et al (1977) demonstrated that the down-regulation of insulin-receptors was ameliorated in ob/ob
liver and adipose tissue plasma membranes after the reduction of the plasma insulin concentration by fasting and streptozotocin treatment. However, this increase in insulin-receptors was not accompanied by an improvement of insulin responsiveness to lipogenesis, in either tissue. This demonstrates the existence of a post-receptor defect in ob/ob adipose tissue and liver. In the ob/ob adipose tissue, the nature of the post-receptor defect may be important to the role of BCT in the ob/ob mouse; the defect may reside in part of the insulin-response mechanism at which BCT may also act, in which event the adipose tissue will also be unresponsive to BCT. However, as it is possible the insulin resistance post-receptor defect is not associated with the BCT mechanism of action; insulin-resistance may occur while the ob/ob adipose tissue is still responsive to BCT. As it has been demonstrated that the action of insulin and BCT on lipogenesis is not additive, it would appear more likely that if BCT is biologically significant as a lipogenic agent in the ob/ob mouse, it would be active under conditions in which the tissue is resistant to insulin.

Despite the existence of insulin resistance in the tissues of the ob/ob mouse (see Ib), there may still be a residual sensitivity to insulin. It has been demonstrated that the ob/ob adipose tissue lipogenic rate can be lowered by removing insulin 'trapped' in the tissue in vitro, or by lowering ob/ob plasma insulin levels in vivo (Loten et al 1974, 1976). This evidence implies that the ob/ob adipose tissue retains some capacity to respond to insulin. The same could possibly also apply to BCT, if a BCT post-receptor defect developed, and thus
BCT could still contribute to the 'increased' lipogenic rate of the ob/ob adipose tissue.

The physiological role of BCT in the ob/ob mouse and also in human obesity has yet to be determined. The significance of this peptide hormone which not only stimulates insulin release but also has an insulin-like action on the adipose tissue could be considerable in the physiologically abnormal obese state.
V Possible future work

In view of the fact that the work involved with the biosynthesis of BCT only started to produce positive results in the last few days of the project, a major priority would be the completion of these studies. The aim being to identify the prohormone, whether or not it is POMC, and to discover the manner in which the prohormone is processed. After identification of the prohormone, another aim would be the isolation, and characterization of the enzyme responsible for BCT synthesis. It would also be of interest to discover when in relation to secretion, BCT is synthesised.

This thesis has produced only preliminary results on the effect of BCT on adipocytes, and this leaves a wide range of possible experiments which could be carried out. One possible way of progressing with this work is to further characterize the action of BCT on the adipocyte, possibly by investigating its effect on cholesterol and glycogen synthesis. Another interesting field of research would be the mechanism of action of BCT in the adipocyte. Among experiments which could be carried out would be the effect of BCT on the activation and deactivation of specific enzymes, especially PDH and ACC. It would also be interesting to investigate the effect of BCT on certain protein phosphorylations, in the adipocyte, to determine if it has the same effect as insulin.

Yet another line of research possible is the identification of the BCT-receptors. These experiments may be complicated by the inability to demonstrate specific $^{125}$I-BCT binding. The iodine may prevent the peptide binding to its receptor
(see IV c), in which case an alternative method of isotopically labelling BCT would have to be found.

As BCT has only recently been identified, the biological implications of its action have only just started to be explored. It would, therefore, seem that the possibilities for future work involving BCT are virtually boundless. Although some immediate experiments can be suggested, the course of future work will depend on how the BCT 'story' unfolds.
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