PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The Role of Phosphorylation in the Regulation of Fatty Acid Synthesis by Insulin and other Hormones [and Discussion]

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The role of phosphorylation in the regulation of fatty acid synthesis by insulin and other hormones

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Insulin stimulates fatty acid synthesis in white and brown fat cells as well as in liver and mammary tissue. Hormones that increase cellular cyclic AMP concentrations inhibit fatty acid synthesis, at least in white adipose tissue and liver. These changes in fatty acid synthesis occur within minutes. In white fat cells, they are brought about not only by changes in glucose transport but also changes in the activities of pyruvate kinase, pyruvate dehydrogenase and acetyl-CoA carboxylase.

The basis of the alterations in pyruvate kinase activity in fat cells is not understood. Unlike the liver isoenzyme, the isoenzyme present in fat cells does not appear to be phosphorylated either in the absence or presence of hormones. The changes in pyruvate dehydrogenase activity in fat cells are undoubtedly due to changes in phosphorylation of the α subunits. Insulin appears to act by causing the parallel dephosphorylation of all three sites. The persistence of the effect of insulin during the preparation and subsequent incubation of mitochondria has allowed the demonstration that insulin acts mainly by stimulating pyruvate dehydrogenase phosphatase rather than inhibiting the kinase.

Acetyl-CoA carboxylase within fat cells is phosphorylated on a number of different sites. The exposure of cells to insulin leads to activation of the enzyme and this is associated with increased phosphorylation of a specific site on the enzyme. Exposure to adrenalin, which results in a marked diminution in activity, also causes a small increase in the overall level of phosphorylation, but this increase is due to an enhanced phosphorylation of different sites; probably those phosphorylated by cyclic-AMP-dependent protein kinase.

Acetyl-CoA carboxylase is one of a number of proteins in fat cells that exhibit increased phosphorylation with insulin. Others include ATP-citrate lyase, the ribosomal protein S_6, the β subunit of the insulin receptor and a heat and acid stable protein of M_r 22000. Changes in phosphorylation of ATP-citrate lyase do not appear to result in any appreciable changes in catalytic activity. A central aspect of insulin action may be the activation and perhaps release of a membrane-associated protein kinase. Plasma membranes from fat cells have been shown to contain a cyclic-nucleotide-independent kinase able to phosphorylate and activate acetyl-CoA carboxylase. Furthermore, high-speed supernatant fractions from cells previously exposed to insulin contain elevated levels of the same or similar kinase activity capable of phosphorylating both ATP-citrate lyase and acetyl-CoA carboxylase.

Introduction

In mammals the principal sites of fatty acid synthesis are white and brown fat, liver and the lactating mammary gland. Rates of fatty acid synthesis in these tissues alter greatly according to the nutritional and hormonal status of the animal. In part these changes are brought about by long-term changes in the concentrations of enzymes in the pathway (Block & Vance 1977) but various hormones can also alter rates of fatty acid synthesis in the short term through changes in the catalytic properties of a number of enzymes catalysing key steps in the pathway (table 1).

Insulin stimulates fatty acid synthesis in all four tissues but the greatest proportional effects are in adipose tissue (Volpe & Vagelos 1976; Hardie 1980; McCormack & Denton 1977; Munday & Williamson 1981). In white adipose tissue and liver it is also evident that hormones that increase the cell content of cyclic AMP cause inhibition of fatty acid synthesis (see Volpe & Vagelos (1976) and Hardie (1980)). This has not been clearly shown for brown fat and mammary tissue, probably because of the lack of adequate *in vitro* preparations of these tissues. Rates of fatty acid synthesis in liver cells can also be altered by hormones that are thought to act primarily through increases in cytoplasmic Ca^{2+} concentration, namely vasopressin, angiotensin and α-agonists. However, the situation is far from straightforward because fatty acid synthesis in rat liver cells incubated with glucose is stimulated by vasopressin and angiotensin but may be inhibited by α-agonists (Assimacopoulos-Jeannet *et al.* 1981; Ly & Kim 1982).

TABLE 1. ACUTE EFFECTS OF HORMONES ON SYNTHESIS OF FATTY ACIDS

tissue	increases	decreases
white adipose tissue	insulin	β-agonists, ACTH
brown adipose tissue	insulin	?
mammary tissue	insulin	?
liver	insulin	glucagon
	vasopressin, angiotensin	α-agonists

In this article we shall summarize evidence that indicates that the opposing effects of insulin and hormones that increase cyclic AMP levels are brought about by changes in the phosphorylation of pyruvate dehydrogenase and acetyl-CoA carboxylase. Some emphasis will be given to studies on rat epididymal fat cells. We have concentrated our own studies on this system because large and reproducible effects of hormones can be obtained with *in vitro* preparations. This tissue has a second major advantage: the low levels of protease activity in cell extracts, especially in comparison with liver. Both pyruvate dehydrogenase and acetyl-CoA carboxylase are extremely susceptible to limited proteolysis, which can alter their catalytic properties (Wieland 1975; Hardie 1980; Song & Kim 1981).

GENERAL ASPECTS OF THE REGULATION OF FATTY ACID SYNTHESIS

Both insulin and adrenalin increase glucose uptake into rat epididymal fat cells, but whereas suinlin stimulates the conversion of glucose to fatty acids, adrenalin inhibits the process. The hormones must therefore bring about opposing changes in activities of key enzymes that determine the rate of fatty acid synthesis within fat cells. Alterations in activities of enzymes in extracts prepared from cells previously exposed to hormones were sought and persistent changes in activities of three enzymes found, roughly paralleling the changes in rates of fatty acid synthesis (figure 1). No changes were found in the activities of other enzymes in the pathway, including ATP-citrate lyase (Coore *et al.* 1971; T. J. Hopkirk & R. M. Denton, unpublished). Similar changes have been found in studies *in vivo* in which the circulating insulin level was varied within the physiological range by injections of glucose or anti-insulin serum (Stansbie *et al.* 1976; Denton *et al.* 1979).

Similar techniques have been applied to other tissues. In the liver, inhibition of fatty acid synthesis with glucagon or α-agonists has been reported to be associated with decreases in

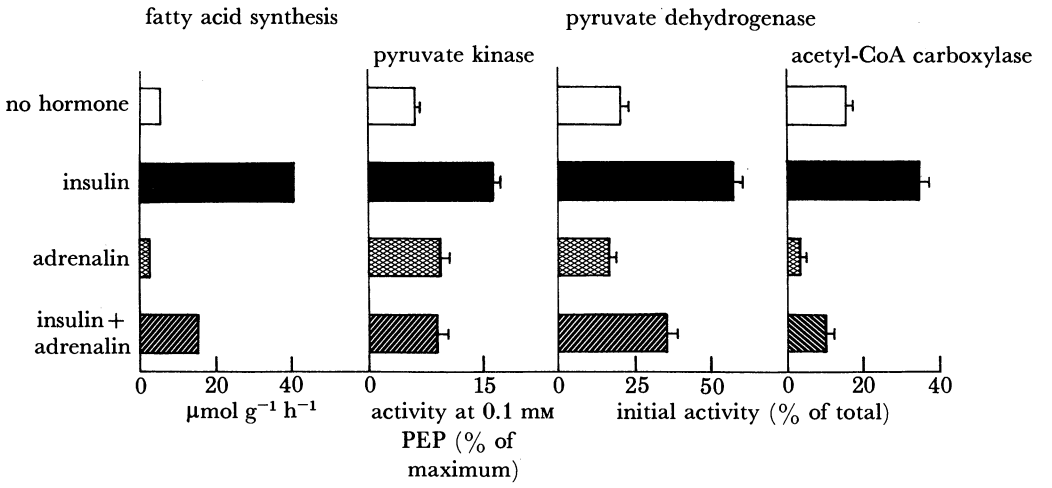

FIGURE 1. Effects of insulin and adrenalin on fatty acid synthesis in rat epididymal fat cells involves parallel changes in the proportion of pyruvate kinase, pyruvate dehydrogenase and acetyl-CoA carboxylase in their respective active forms. Data taken from Denton & Halperin (1968), Martin *et al.* (1972), Denton *et al.* (1979), Brownsey *et al.* (1979) and unpublished observations. Maximum activities were taken for pyruvate dehydrogenase as the activity measured after treatment of extracts with pyruvate dehydrogenase phosphate phosphatase in the presence of $MgCl_2$ and $CaCl_2$, for acetyl-CoA carboxylase as the activity after treatment of extracts with citrate, and for pyruvate kinase as the activity assayed at a saturating concentration of phosphoenolpyruvate (PEP). Neither of the hormones altered maximum activity except for a 20–30% diminution in the maximum activity of acetyl-CoA carboxylase in fat cells incubated with adrenalin (in the presence or absence of insulin).

acetyl-CoA carboxylase activity while stimulated rates of synthesis with vasopressin and insulin are accompanied by increases in activity (Witters *et al.* 1979; Geelen *et al.* 1978; Assimacopoulos-Jeannet *et al.* 1981). All four hormones may activate pyruvate dehydrogenase (see Denton *et al.* (1981) and Assimacopoulos-Jeannet *et al.* (1982)). Hepatic pyruvate kinase is inhibited by glucagon and α-agonists and these effects are opposed by insulin (see Engstrom (1980) and Claus & Pilkis (1981) for reviews). Evidence for the activation of both pyruvate dehydrogenase and acetyl-CoA carboxylase in brown adipose tissue and mammary tissue has been obtained *in vivo* (McCormack & Denton 1977; McCormack 1982; Baxter *et al.* 1979; McNeillie & Zammit 1982).

ROLE OF PHOSPHORYLATION IN THE HORMONAL CONTROL OF PYRUVATE KINASE AND PYRUVATE DEHYDROGENASE ACTIVITY IN RAT EPIDIDYMAL FAT CELLS

Pyruvate kinase

It is well established that the hormonal control of pyruvate kinase in the liver is brought about by changes in phosphorylation (see Engstrom 1980). Although the enzyme in fat cells is a different isoenzyme from that present in liver it seemed reasonable to suppose that alterations in phosphorylation may also be involved in its hormonal regulation. Not only were the changes observed due to alterations in the K_m for phosphoenolpyruvate rather than in V_{max} but the effects persisted during incubation of extracts (Denton *et al.* 1979). However, more recent studies carried out in this laboratory by Elizabeth Levett and Timothy Hopkirk indicate that the fat-cell enzyme is not phosphorylated to any appreciable extent either in the presence or in

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

the absence of hormones. No satisfactory explanation for the persistent changes in activity in extracts has been found. Possibilities include different amounts of a tightly bound but hitherto unrecognized effector or a covalent modification other than phosphorylation, e.g. limited proteolysis. Changes in K_m for phosphoenolpyruvate after limited proteolysis of liver, pyruvate kinase and other isoenzymes, are well documented (Engstrom 1980; Ibsen *et al.* 1981).

Pyruvate dehydrogenase

Studies on the role of phosphorylation in the hormonal regulation of fat-cell pyruvate dehydrogenase have been extensively reviewed elsewhere (Denton *et al.* 1978; Denton & Halestrap 1979; Denton *et al.* 1981) and only a brief summary will be given here.

Early studies suggested that the effects of insulin and adrenalin were the result of alterations in the fraction of the complex in its active, non-phosphorylated form because incubation of extracts with pyruvate dehydrogenase phosphatase in the presence of Mg^{2+} and Ca^{2+} led to activation and the loss of the hormone effects (figure 1). Direct demonstration that the effect of insulin was due to dephosphorylation has only been achieved recently by the specific immunoisolation of pyruvate dehydrogenase from fat cells previously incubated with medium containing $^{32}P_i$. Studies of others have shown that purified preparations of pyruvate dehydrogenase are phosphorylated on three different sites on the α subunits (Yeaman *et al.* 1978; Sugden *et al.* 1979; Sale & Randle 1982). Peptide analysis of the α subunits in pyruvate dehydrogenase isolated from fat cells indicated for the first time that all three sites were phosphorylated in the intact cell (Hughes *et al.* 1980). No evidence was found that insulin caused any appreciable change in the relative occupancy of the three sites, rather it appeared that there is a parallel dephosphorylation of all three sites (Hughes *et al.* 1980).

In theory the effects of insulin could be brought about by activation of pyruvate dehydrogenase phosphatase or inhibition of the kinase. It has proved possible to show that the first possibility is correct by making use of the persistence of the effects of insulin during preparation and subsequent incubation of intact mitochondria from both white and brown adipose tissue (Hughes & Denton 1976; Denton *et al.* 1978; McCormack 1982). The mechanism involved in the short-term effects of insulin is quite distinct from that operating during the long-term exposure to low concentrations of insulin as in diabetes or starvation, where the studies of Randle's group indicate that alterations in kinase activity are involved (Kerbey & Randle 1982). Because no change in phosphatase activity is found in mitochondrial extracts, it seemed possible that insulin might increase phosphatase activity by increasing the intramitochondrial concentration of Ca^{2+}, which is an activator of the enzyme (Denton *et al.* 1972). However, recent studies with two other intramitochondrial enzymes (NAD-isocitrate dehydrogenase and oxoglutarate dehydrogenase), which are also activated by Ca^{2+} (Denton & McCormack 1980) in the same concentration range as pyruvate dehydrogenase phosphatase, as a means of assessing intramitochondrial Ca^{2+}, suggest that this hypothesis may be incorrect (J. G. McCormack, S. E. Marshall & R. M. Denton, unpublished). We have been unable to obtain any effect of insulin on pyruvate dehydrogenase activity in cell-free systems similar to those of Jarett *et al.* (1981). Some of the problems in this field are discussed in greater detail in a recent review (Denton *et al.* 1981).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Role of phosphorylation in the hormonal regulation of acetyl-CoA carboxylase activity

Initial indications that this enzyme might be regulated by phosphorylation were that the enzyme purified from rat liver contained convalently bound and alkali-labile phosphate (Inoue & Lowenstein 1972) and that incubation of a partly purified preparation of the liver enzyme with Mg-ATP resulted in a loss of activity (Carlson & Kim 1973). The first demonstration of the rapid incorporation of ^{32}P from medium phosphate into acetyl-CoA carboxylase within fat cells was achieved by using rat epididymal fat cells (Brownsey *et al.* 1977). Acetyl-CoA carboxylase was isolated by rapid immunoprecipitation and shown to bind to Sepharose–avidin. Subsequent studies with similar techniques have demonstrated phosphorylation within chicken and rat hepatocytes (Pekala *et al.* 1978; Witters *et al.* 1979).

Investigations of the effects of insulin and adrenalin upon the level of phosphorylation in fat cells have revealed that both hormones cause modest increases in overall phosphorylation. With

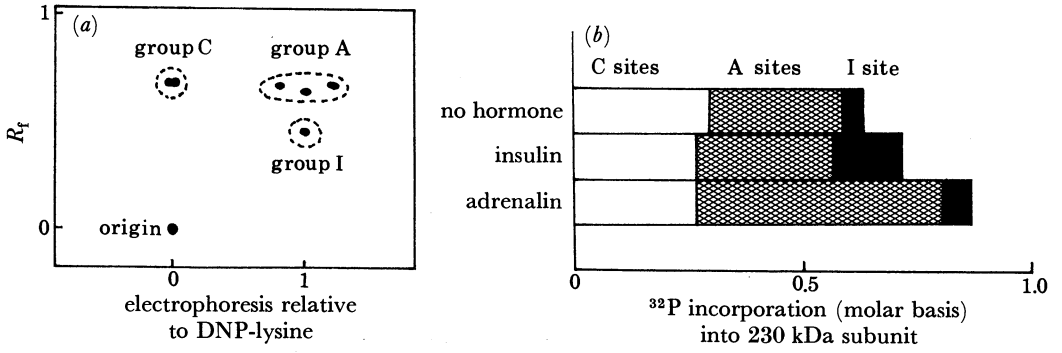

Figure 2. Effects of insulin and adrenalin on the phosphorylation of specific sites on acetyl-CoA carboxylase in rat epididymal fat cells. Data taken from Brownsey & Denton (1982). Cells were incubated with [^{32}P]phosphate and appropriate hormones; [^{32}P]acetyl-CoA carboxylase was isolated by immunoprecipitation and overall ^{32}P incorporation determined. Peptides released by tryptic digestion were then separated by two-dimensional thin-layer chromatography and ^{32}P-labelled peptides located by radioautography and assigned to groups C, A and I according to mobility, as indicated in (*a*). (*b*) The incorporation into these three groups, with the assumption that these sites represent all the phosphorylation sites on acetyl-CoA carboxylase (see Brownsey & Denton 1982).

adrenalin, a halving of the initial activity of acetyl-CoA carboxylase is associated with a 40% increase in phosphorylation whereas with insulin a doubling of activity is associated with a 15% increase in phosphorylation (Brownsey *et al.* 1979; Brownsey & Denton 1982). Qualitatively similar results have been obtained with liver cells incubated with either glucagon or insulin (Witters *et al.* 1979; Witters 1981).

Because studies with purified acetyl-CoA carboxylase from mammary gland suggested that phosphorylation could occur at multiple sites in the enzyme (Hardie & Cohen 1978), the extent of multisite phosphorylation was explored in fat cells (Brownsey & Hardie 1980; Brownsey & Denton 1982). Fat cells were incubated with $^{32}P_i$ in the presence or absence of hormones, [^{32}P]acetyl-CoA carboxylase was isolated by immunoisolation and the peptides released by trypsin digestion were separated by a two-dimensional system (Brownsey & Denton 1982); (figure 2). Three groups of ^{32}P-labelled peptides were separated and designated the C, A and I groups. Adrenalin but not insulin increased incorporation into the A group about twofold whereas insulin increased incorporation into the I peptide some fivefold. Incorporation

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

into this site was also increased by adrenalin but to a smaller extent. Neither hormone affected the phosphorylation of the C group of peptides. These results indicated that treatment of fat cells with insulin and adrenalin led essentially to the phosphorylation of different sites on acetyl-CoA carboxylase.

The sites phosphorylated in cells exposed to adrenalin appear to be the same as those phosphorylated by cyclic-AMP-dependent protein kinase (Brownsey & Hardie 1980; Brownsey, unpublished). Moreover, the changes in kinetic properties are very similar, including a diminution in the maximum activity even after treatment with high concentrations of citrate and an increase in the concentration of citrate capable of causing half-maximal activation (Brownsey *et al.* 1979, 1981; Hardie & Guy 1980; Brownsey & Denton 1982; Tipper & Witters 1982). It seems reasonable to conclude that the inhibition of fatty acid synthesis found in fat cells incubated with adrenalin is brought about, at least in part, by the phosphorylation of acetyl-CoA carboxylase by cyclic-AMP-dependent protein kinase, and that a similar mechanism is the basis of the inhibition of fatty acid synthesis by glucagon in liver cells.

In contrast, phosphorylation of the I site by a cyclic-AMP-independent kinase appears to explain the activation of acetyl-CoA carboxylase in cells exposed to insulin. In a later section, evidence will be presented that fat cells not only contain a protein kinase able to phosphorylate acetyl-CoA carboxylase on this site but also that this phosphorylation is associated with increases in enzyme activity.

There are two further observations with a bearing on the hormonal control of acetyl-CoA carboxylase activity. First, the increases in acetyl-CoA carboxylase activity persist through a 100-fold purification of the enzyme, which is clearly consistent with the insulin's bringing about its effect by covalent modification rather than by effector binding. Second, changes in activity brought about by insulin are not strictly the inverse of those seen with adrenalin. Whereas the effects of adrenalin are still apparent after incubation of tissue extracts with citrate, the effects of insulin are abolished. Conversely, the effects of adrenalin disappear if tissue extracts are incubated with Mg^{2+} and Ca^{2+} but those of insulin remain (Brownsey *et al.* 1979; Brownsey & Denton 1982). Under these latter conditions partial but not complete dephosphorylation of acetyl-CoA carboxylase occurs. These observations support the view that adrenalin and insulin are acting primarily through different modes of regulation, e.g. by altering the occupancy of different phosphorylation sites on the enzyme.

Hormonal control of the phosphorylation of ATP-citrate lyase

ATP-citrate lyase is phosphorylated in fat and liver cells, and the extent of its phosphorylation is, like that of acetyl-CoA carboxylase, increased both by insulin and by hormones that increase cyclic AMP concentrations (Linn & Srere 1979; Ramakrishna & Benjamin 1979; Alexander *et al.* 1979). Moreover, at least in liver cells, additive effects of insulin and glucagon are observed when both hormones are present at maximal doses (Alexander *et al.* 1979). However, careful and extensive studies by Avruch's group have shown that the same site is phosphorylated after an exposure of liver cells to glucagon and to insulin and that this is the same site phosphorylated, albeit rather slowly, by cyclic-AMP-dependent kinase (Pierce *et al.* 1981, 1982). The situation in 3T3-L1 preadipocytes and rat fat cells appears similar except that no clearcut additive effects of insulin and adrenalin are obtained (Swergold *et al.* 1982, T. J. Hopkirk, R. W. Brownsey & R. M. Denton, unpublished).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

A perplexing aspect of the phosphorylation of ATP-citrate lyase is that no appreciable changes in catalytic activity have been observed associated with changes in phosphorylation either within intact cells or with the purified enzyme. It has been suggested that phosphorylation might affect the location of the enzyme within cells (Janski & Cornell 1982) or its rate of breakdown (Guy *et al.* 1981). However, it is strange that hormones that have essentially opposing effects on fatty acid synthesis should bring about such apparently similar changes in phosphorylation. ATP-citrate lyase may account for up to 5% of the soluble protein in liver and fat cells, and this high concentration has led to the proposal that phosphorylation is just adventitious (Pierce *et al.* 1982). However, phosphorylation in cells appears to be in the range 0.1–0.4 mol per mole of subunit, corresponding to 0.4–1.6 mol per tetramer, which is by no means a trivial level. Moreover, because the activation of two different kinases is almost certainly involved in the effects of insulin and those hormones that act through cyclic AMP, it seems unlikely that two different protein kinases would both phosphorylate the same protein adventitiously.

Studies on protein kinase activity involved in insulin action on fat cells

After the recognition that insulin increased the phosphorylation of a specific site on acetyl-CoA carboxylase, we searched for a protein kinase activity in fat cells that could phosphorylate the appropriate site with concomitant activation of the enzyme. Purified fat-cell membranes were found to contain substantial activities of such a kinase (figure 3). The kinase employed Mg-ATP, is insensitive to cyclic nucleotides and the specific (Walsh) inhibitor protein, and is active in the absence of Ca^{2+}. Phosphorylation of acetyl-CoA carboxylase occurs in the I site as defined in figure 2, but there is also appreciable phosphorylation of C sites. The changes in catalytic activity are similar to those that follow exposure of fat cells to insulin. Little or no evidence of phosphatase activity was apparent under these conditions (Brownsey *et al.* 1981; Brownsey 1983). Fat-cell plasma membranes also contain a cyclic-nucleotide-independent

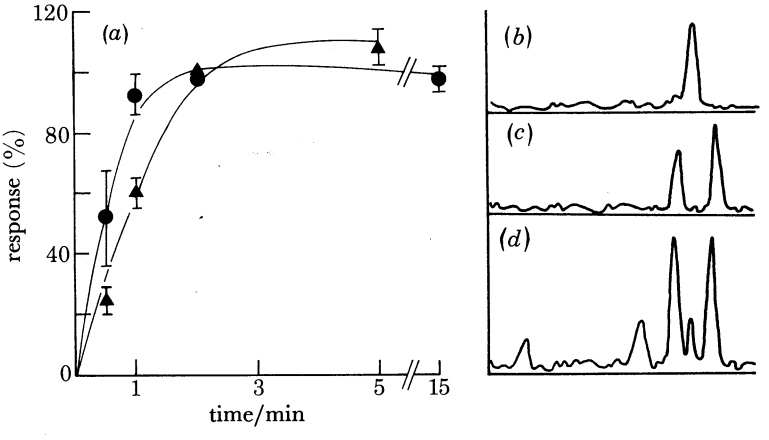

Figure 3. Parallel phosphorylation and activation of acetyl-CoA carboxylase upon incubation of a partly purified preparation of the fat-cell enzyme with fat-cell plasma membranes and [γ-^{32}P]ATP. Data taken from Brownsey *et al.* (1981). (*a*) Acetyl-CoA carboxylase activity (▲) was assayed in the absence of citrate, and ^{32}P incorporation (●) was measured after separation of acetyl-CoA carboxylase by sodium dodecyl sulphate polyacrylamide gel electrophoresis. There were no changes in activity in the absence of ATP or plasma membranes. (*b–d*) Densitometric traces of radioautographs of the separated ^{32}P-labelled proteins after incubations of (*b*) acetyl-CoA carboxylase alone, (*c*) plasma membranes alone, (*d*) acetyl-CoA carboxylase and plasma membranes with [γ-^{32}P]ATP.

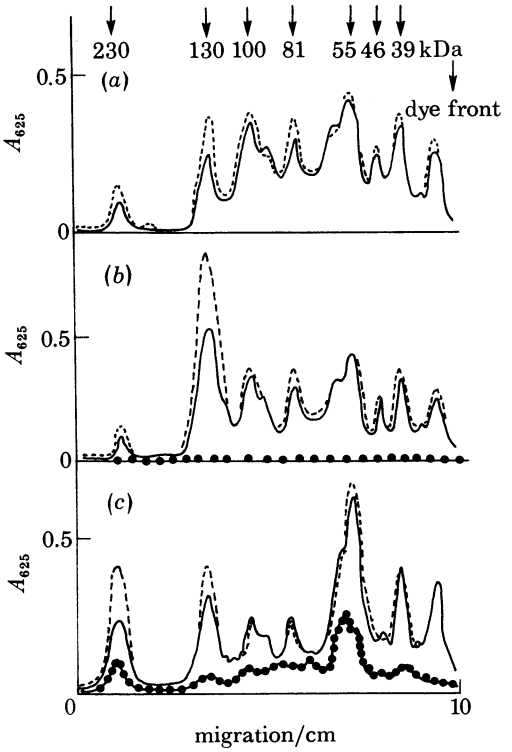

FIGURE 4. Increased protein kinase activity in fresh high-speed supernatant fractions of rat epididymal adipose tissue previously exposed to insulin. Fractions from control (solid line) or insulin-treated tissue (broken line) were prepared by centrifugation of tissue extracts for 10 min at 100000*g* and then incubated for 2 min at 30 °C in the presence of added $MgCl_2$ (5 mM) and [γ-^{32}P]ATP (10 μM and 33 Bq pmol^{-1}) plus (*a*) no further additions, (*b*) purified fat-cell ATP-citrate lyase (100 μg ml^{-1}) or (*c*) purified fat-cell acetyl-CoA carboxylase (100 μg ml^{-1}), ^{32}P-labelled phosphoproteins were separated by slab-gel sodium dodecyl sulphate polyacrylamide electrophoresis. After radioautography the densitometric scans shown in the figure were obtained. The figure also indicates by dotted lines the pattern of protein labelling obtained when the purified proteins were incubated with [γ-^{32}P]ATP but without any added tissue fraction.

TABLE 2. EFFECTS OF PRIOR EXPOSURE OF RAT EPIDIDYMAL ADIPOSE TISSUE TO INSULIN ON THE RATE OF PHOSPHORYLATION OF ACETYL-CoA CARBOXYLASE AND ATP-CITRATE LYASE BY HIGH-SPEED SUPERNATANT FRACTIONS

(Details are as given in the legend to figure 4. Results are rates of phosphorylation by cytoplasmic fraction from insulin-treated tissue, as percentages of controls, from 12 separate experiments; means ± s.e.m.; *, $P < 0.01$.)

protein	endogenous proteins	added proteins
acetyl-CoA carboxylase	131 ± 8*	202 ± 22*
ATP-citrate lyase	180 ± 20*	202 ± 36*
100 kDa	109 ± 8	—
55 kDa	106 ± 5	—
39 kDa	113 ± 8	—

protein kinase able to phosphorylate ATP-citrate lyase (R. W. Brownsey & T. J. Hopkirk, unpublished). However, it has not so far proved possible to demonstrate consistent changes in this cyclic-nucleotide-independent protein kinase activity after direct addition of insulin to fat-cell membrane preparations.

More recently we have been able to demonstrate a similar cyclic-nucleotide-independent protein kinase activity in the high-speed supernatant fraction of fat cells (figure 4; table 2). In

these experiments the fraction was prepared as rapidly as possible from rat epididymal adipose tissue previously incubated in the presence or absence of insulin. As indicated in figure 4 and table 2, the phosphorylation of both endogenous and added purified acetyl-CoA carboxylase and ATP-citrate lyase was significantly greater after insulin treatment. Analysis of time-courses indicates that although the rates of phosphorylation are modest compared with the rate associated with the plasma membrane preparation they still greatly exceed rates of dephosphorylation. Thus there seems little doubt that incubation of rat epididymal adipose tissue with insulin leads to a substantial increase in protein kinase activity able to phosphorylate at least two of the proteins exhibiting enhanced phosphorylation in cells exposed to insulin. Some evidence for persistent increases in the ability of cell extracts to phosphorylate proteins after exposure of fat cells or 3T3-L1 preadipocytes to insulin have also been reported by others (Benjamin & Singer1975; Smith *et al.* 1980).

Discussion

Our knowledge of the role of protein phosphorylation in the regulation of fatty acid synthesis is still very incomplete. For acetyl-CoA carboxylase many questions concerning the relation between phosphorylation at the various sites, the state of polymerization of the enzyme and catalytic activity remain to be answered. Presumably in the cell the metabolic flux through acetyl-CoA carboxylase is governed by a combination of changes in substrate concentration, alterations in the levels of possible effectors (including citrate, fatty acyl-CoA, CoA, purine nucleotides and malonyl-CoA) as well as the modifications in phosphorylation emphasized so far in this article. Determining the relative importance of these various means of control is a challenge that remains for future studies.

Nevertheless the study of the regulation of fatty acid synthesis has yielded some new insights into insulin action. In particular the recognition that the pathway contains two enzymes exhibiting increased phosphorylation after exposure to insulin highlights the possibility that increased phosphorylation of cell proteins may be a central and rather important feature of the mechanism of action of insulin (Denton *et al.* 1981). The levels of phosphorylation of other proteins are also increased in fat cells and liver cells exposed to insulin; these include the ribosomal protein S_6 (Smith *et al.* 1980), a thermostable protein of M_r 22000 (Belsham *et al.* 1980, 1982) and, at least in liver cells, a cytoplasmic protein of M_r 46000 (Avruch *et al.* 1978). The most recent example and perhaps the most exciting is the β subunit of the insulin receptor itself (Kasuga *et al.* 1982*a*). In this case it has been demonstrated that increased phosphorylation occurs with solubilized and even extensively purified preparations of the receptor after the binding of insulin (Kasuga *et al.* 1982*b*; Van Obberghen & Kowalski 1982; Machicao *et al.* 1982). Because most of the phosphorylation in these systems appears to occur on tyrosine residues it seems that the insulin receptor itself (or a protein closely associated with it) has tyrosine kinase activity. Increased phosphorylation of the β subunit has also been shown in intact cells, but in this situation phosphorylation appears to occur principally on serine residues with only a modest amount of tyrosine phosphorylation (Kasuga *et al.* 1982*c*). These important findings, together with the work on the other intracellular proteins which are phosphorylated to a greater extent, mainly on serine residues, in insulin-treated cells, suggest that separate tyrosine- and serine-protein kinases may be activated by insulin. However, the relation between the kinases remains to be established.

Our own work on the cyclic-nucleotide-independent protein kinase activity capable of

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

phosphorylating ATP-citrate lyase and acetyl-CoA carboxylase in fat cells suggests that much of the activity is associated with the plasma membrane, but increased activity is apparent in the supernatant fraction after exposure of the cells to insulin. Our present working hypothesis (Denton *et al.* 1981) is that the binding of insulin to its plasma-membrane receptors leads to the dissociation of the kinase activity from specific sites on the inner face of the plasma membrane. The form of activation may be viewed as being analogous to the dissociation of the regulatory and cataiytic subunits of cyclic-AMP-dependent protein kinase. An attractive possibility is that dissociation and activation may be initiated by phosphorylation of tyrosine residues catalysed by the tyrosine protein kinase associated with the insulin receptor. It should also be emphasized in this context that it is becoming increasingly evident that a characteristic of insulin action on cells may be translocation of protein between the plasma membrane and intracellular locations. Strong evidence has been put forward recently that the activation by insulin of glucose transfer across the plasma membranes of fat and other cells is due to the recruitment of glucose transporters from intracellular sites (Karnieli *et al.* 1981; Kono *et al.* 1982). Rapid internalization of insulin–receptor complexes also appears to follow the binding of insulin to plasma membrane receptors (Olefsky & Kao 1982; Desbuquois *et al.* 1982).

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Discussion

S. J. Yeaman (*Department of Biochemistry, University of Newcastle upon Tyne, U.K.*). It is noticeable that the peptides from acetyl-CoA carboxylase that are phosphorylated in response to insulin are also phosphorylated after adrenalin treatment. Are any results available about whether the same residue in the same peptide is phosphorylated in both cases?

R. M. Denton. I think it is important to emphasize that the major effect of adrenalin is to increase the incorporation of ^{32}P into phosphopeptides (denoted the A-phosphopeptides) of acetyl-CoA carboxylase that probably correspond to sites phosphorylated by cyclic-AMP-dependent protein kinase. Insulin does not alter the incorporation of ^{32}P into these sites by any appreciable amount. On the other hand, adrenalin does seem to cause some increased incorporation into a phosphopeptide that migrates in the two-dimensional system we use identically with the phosphopeptide (the I phosphopeptide) that exhibits very marked increased phosphorylation after exposure of cells to insulin. However, the incorporation after adrenalin treatment is very variable and considerably smaller than that seen with insulin. We have no data at present on whether or not incorporation is in the same or different residues. Present studies are concerned with separating the phosphopeptides by high-performance liquid chromatography so that sufficient amounts of the purified peptide can be obtained for us to tackle this important question.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES