Reversibility of the insulin-stimulated phosphorylation of ATP citrate lyase and a cytoplasmic protein of subunit M_r 22 000 in adipose tissue

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Exposure of rat epididymal adipose tissue to insulin leads to a 2-fold increase in incorporation of [^{32}P]phosphate into ATP citrate lyase and a cytoplasmic protein of M_r 22 000. A smaller increase and decrease in incorporation into cytoplasmic proteins of M_r 63 000 and 20 000 respectively were also observed. Subsequent addition of anti-insulin serum largely reversed these changes within 15 min, indicating that the alterations in ^{32}P incorporation represented changes in net phosphate content of the proteins.

The effect of insulin on ^{32}P incorporation into rat fat-cell phosphoproteins has been studied in several laboratories (see Denton *et al.*, 1981, for review). It has been shown that the ^{32}P labelling of a protein of subunit M_r 130 000 as determined by SDS/polyacrylamide-gel electrophoresis is increased after exposure to insulin of fat-cells incubated with $^{32}\text{P}_i$ (Benjamin & Singer, 1975; Forn & Greengard, 1976; Avruch *et al.*, 1976a,b; Hughes *et al.*, 1977, 1980). This phosphoprotein has now been identified as ATP citrate lyase (EC 4.1.3.8) (Janski *et al.*, 1979; Alexander *et al.*, 1979). Studies from our laboratory have identified large increases in the ^{32}P labelling of fat-cell proteins with subunit M_r 35 000 (Hughes *et al.*, 1977, 1980) and 22 000 (Belsham & Denton, 1980; Belsham *et al.*, 1980a). The former protein may be the ribosomal protein S6, whose phosphorylation has been shown to be increased after exposure to insulin in both 3T3 L1 pre-adipocytes (Smith *et al.*, 1979) and HeLa cells (Lastick & McConkey, 1981), and Belsham & Denton (1980) have suggested that the latter protein may be related to the general protein phosphatase inhibitor protein termed inhibitor-1 (Huang & Glinemann, 1976; Foulkes & Cohen, 1979). Smaller increases in the ^{32}P content of a plasma-membrane-associated protein (subunit M_r 61 000; Belsham *et al.*, 1980b) and of a cytoplasmic protein (subunit M_r 62 000; Benjamin & Clayton, 1978) have also been reported. In addition, insulin increases the phosphorylation of a single site on acetyl-CoA carboxylase (EC 6.4.1.2) (subunit M_r

230 000), although only a 10–20% increase in total ^{32}P labelling of this protein is observed under these conditions (Brownsey & Denton, 1982).

It is clearly important to be sure that the elevated ^{32}P labelling of this series of phosphopeptides observed after exposure of fat-cells to insulin does represent a true increase in phosphate content of the proteins, especially as it has been widely argued that an important common element in the mechanism of insulin action may be the dephosphorylation of proteins (see Cohen, 1980). If insulin activated a protein phosphatase which removed phosphate groups not in radioactive equilibrium with the cytosolic ATP pool, it is conceivable that re-phosphorylation could lead to elevated ^{32}P labelling, although this might be accompanied by an actual decrease in phosphate content of the proteins. Such a phenomenon has been encountered with the stimulation of ^{32}P incorporation into phosphatidylinositol in the plasma membrane after hormonal stimulation of a wide range of cell types [see Lapetina & Michell (1973) and Michell (1975) for reviews]. The increased ^{32}P labelling of this lipid reflects elevated turnover of the phosphoinositol head groups while the actual concentration of phosphatidylinositol is decreased. Similarly, if the terminal phosphate group of ATP, or an alternative phosphate donor molecule, were not in radioactive equilibrium with the phosphate in the medium, in the absence of insulin, then an increase in ^{32}P labelling of certain proteins could occur, at least theoretically, after exposure to insulin without there being a net increase in phosphate content.

Previous studies from our and other laboratories concerned with insulin-stimulated ^{32}P labelling of proteins in adipocytes have attempted to ensure that

Abbreviation used: SDS, sodium dodecyl sulphate.

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the above phenomena were unlikely explanations for the observed effects by taking care that steady-state ^{32}P labelling had been achieved before, and after, the addition of insulin. However, a more rigorous method of proving that increased incorporation of ^{32}P in the presence of insulin does represent a net increase in phosphorylation is to show that the subsequent removal of insulin results in the specific loss of ^{32}P from the appropriate proteins. In this paper, we show that the insulin-stimulated ^{32}P labelling of specific phosphoproteins in epididymal-fat-pad pieces can be rapidly reversed by the removal of insulin with anti-insulin serum.

Materials and methods

Materials

Collagenase was obtained from International Enzymes, Windsor, Berks., U.K. (lot no. 504-22). $^{32}\text{P}_i$ was from New England Nuclear (Dreieich, W. Germany). Insulin was from Sigma (London) Chemical Co., Poole, Dorset BH17 7NH, U.K., and was dissolved in 3 mM-HCl (0.5 mg/ml) and stored at -20°C until use. Other reagents were from BDH Chemicals (Poole, Dorset BH12 4NN, U.K.). Anti-insulin serum, which binds $5\mu\text{g}$ of ^{125}I -labelled insulin/ml at 4°C , was a kind gift from Dr. A. S. Cole of this Department as used previously (Stansbie *et al.*, 1976; McCormack & Denton, 1977). Rabbit skeletal-muscle ^{32}P -labelled inhibitor 1 was a kind gift from Dr. A. Aitken and Professor P. Cohen (Department of Biochemistry, University of Dundee).

Incubation and extraction of fat-pad pieces

Epididymal fat-pads were obtained from male albino Wistar rats (160–200 g) with free access to water and stock laboratory diet (breeding diet; Oxoid, Basingstoke, Hants., U.K.). After decapitation of the animals the fat-pads were removed and cut into three or four pieces per pad and pre-incubated in bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing 1.25 mM- CaCl_2 , 5 mM-glucose and 1 mM- P_i . The tissue (six pieces, i.e. approx. 500 mg fresh wt. in total) was then incubated for 2 h in 3 ml of the bicarbonate-buffered medium as above but containing 0.2 mM- $^{32}\text{P}_i$ (approx. 500 d.p.m./pmol). At the end of the incubation period, additions were made as described in Figure and Table legends, and incubations continued for a further 15 or 30 min. Dinonyl phthalate (2 ml) was added and brief centrifugation (1000 g for 30 s) used to separate the tissue from the medium. The fat-pad pieces were extracted in 2 ml of medium containing 0.25 M-sucrose, 10 mM-Tris/HCl, 2 mM-EGTA, 20 mM-KF, 5 mM-EDTA and 7.5 mM-

reduced glutathione, pH 7.4, at 0°C for 15 s at speed 5 with a Polytron PT-10 homogenizer. The extracts were centrifuged in conical glass tubes at 1000 g for 30 s, and the infranats removed by syringe from between the fat-plug and cell debris and then centrifuged at 10000 g for 1 min in an Eppendorf centrifuge. The supernatants were treated with trichloroacetic acid (2 or 15%, w/v) and the protein was sedimented by centrifugation (10000 g for 1 min). Protein soluble in 2% trichloroacetic acid was treated with further trichloroacetic acid (15% final concn.) and kept at 0°C for 2 h, and the protein was sedimented by centrifugation at 10000 g for 2 min.

Incubation of isolated fat-cells

Fat-cells were prepared by collagenase digestion, incubated with $^{32}\text{P}_i$ and extracted as described previously (Belsham *et al.*, 1980a).

Separation of [^{32}P]phosphopeptides by SDS/polyacrylamide-gel electrophoresis

Separation was performed in 1 cm tracks on 7–10% (w/v) polyacrylamide slab gels (14 cm $\times$ 14 cm) by using solutions described by Laemmli (1970). Protein pellets were washed in diethyl ether and redissolved in 'SDS gel buffer', containing 62.5 mM-Tris/HCl, pH 7.4, SDS (40 mg/ml), sucrose (200 mg/ml), Bromophenol Blue (0.2 mg/ml) and 100 mM-2-mercaptoethanol by heating at 100°C for 5 min. Electrophoresis was performed at 20°C for 2.5–3 h at 30–35 mA/gel slab. The gels were washed with 20% trichloroacetic acid in 50% (w/v) methanol, and then stained with Coomassie Brilliant Blue. After destaining, the gels were dried under vacuum on to cellophane and then exposed to Kodak No-Screen film for 1–4 days. Incorporation of ^{32}P into protein was assessed by densitometric scanning of the radioautograph at 625 nm as described previously (Brownsey *et al.*, 1979; Belsham *et al.*, 1980b).

Exposure of the film was such that peak heights did not exceed an absorbance value of 1.0. Incorporation was calculated on the basis of peak height measured above the baseline (taken as the lowest absorbance on the radioautograph) and expressed as percentage of the relevant no-treatment control value in the same experiment. In all cases, control and experimental values were determined from parallel tracks on the same autoradiograph. Phosphoprotein band numbers are as used previously by this laboratory (Belsham *et al.*, 1980a,b). The loading of protein was assessed by scanning the Coomassie Blue-stained protein bands in the dried gels. However, differences in loading within each experiment were less than 5%, and no corrections were therefore necessary.

Results and discussion

We are unaware of any previous studies concerning the reversal of insulin-stimulated protein phosphorylation. However, Forn & Greengard (1976) have shown that the adrenaline-stimulated ^{32}P labelling of phosphoproteins in isolated fat-cells could be rapidly reversed by adding the β -blocker propranolol. In preliminary experiments, we tried to reverse the insulin-stimulated protein phosphorylation in isolated fat-cells with guinea-pig anti-(bovine insulin) serum, but found that this serum exhibited appreciable insulin-like activity. The insulin-like activity of anti-insulin serum on fat-cells has been encountered previously (Kahn *et al.*, 1978), but not by all workers (e.g. Karnieli *et al.*, 1981). We were aware that this serum did not exhibit insulin-like activity *in vivo* (Stansbie *et al.*, 1976) or in intact fat-pads (Belsham, 1981), and thus we turned to the study of protein phosphorylation within intact fat-pads, since previous studies by others had indicated that it was possible to observe the incorporation of ^{32}P from P_i in the incubation medium into intracellular phosphoproteins with fat-pad pieces (Benjamin & Singer, 1974; Lee & Kim, 1979).

In the present studies it was found that after a preincubation period of 2 h in the absence of hormones the incorporation of ^{32}P into the major phosphoproteins in small fat-pad pieces had reached steady-state values. Incubation for a further 30 min

period did not result in any further incorporation (Table 1). This period of preincubation is twice that previously used in this laboratory for isolated fat-cells. As is evident from Fig. 1, the pattern of labelled phosphoproteins in whole-cell extracts of the fat-pad pieces was strikingly similar to that found in the isolated fat-cells. Moreover, the effects of exposure to either insulin or adrenaline for 15 min after the incubation period with $^{32}\text{P}_i$ resulted in rather similar changes in phosphorylation (Fig. 1, Table 1). The major effects of insulin at a saturating concentration in both preparations were increases in the incorporation of ^{32}P into phosphoproteins of M_r 130 000 and 35 000 (Table 1, Fig. 1). Adrenaline also increased the phosphorylation of these phosphoproteins, and in addition, a particularly marked increase in the incorporation into a protein of M_r 65 000 was evident. The increase in the ^{32}P labelling of the M_r -35 000 phosphoprotein band with insulin appeared to be less in fat-pad pieces than in the isolated cells (see also below).

Exposure of fat-cells to either insulin or adrenaline results in the steady-state incorporation of $^{32}\text{P}_i$ into acetyl-CoA carboxylase (M_r 230 000, designated band 1; Brownsey *et al.*, 1979; Brownsey & Denton, 1982). There was an indication that similar increases also occur in fat-pad pieces, but the increases were not statistically significant (Fig. 1, Table 1). Studies with isolated fat-cells have also led to the finding that the phosphorylation of a rather minor cytoplasmic phosphoprotein of subunit M_r

Table 1. Effects of insulin and adrenaline on the incorporation of $^{32}\text{P}_i$ into adipose-tissue cytoplasmic phosphoproteins within fat-pad pieces previously preincubated with $^{32}\text{P}_i$ for 2 h

Fat-pad pieces were preincubated for 2 h in hormone-free medium containing $^{32}\text{P}_i$ as described in the Materials and methods section. Incubations were then continued in the same medium either with no hormone (for 30 min) or with insulin (0.5 $\mu\text{g}/\text{ml}$) or adrenaline (0.2 $\mu\text{g}/\text{ml}$) for 15 min. Fraction-A samples were 10 000 $\text{g} \times 1$ min supernatants precipitated with 15% (w/v) trichloroacetic acid and separated on a 7% (w/v) polyacrylamide gel; fraction-B samples were the same as fraction A, but separated on 10% (w/v) polyacrylamide gels to improve resolution of lower molecular-weight proteins; fraction-C samples were proteins soluble in 2% (w/v) trichloroacetic acid, then precipitated with 15% trichloroacetic acid and separated on 10% (w/v) polyacrylamide gels. Incorporation of ^{32}P is given as a percentage of the no-treatment control value taken as the incorporation into the appropriate phosphoprotein band after 2 h preincubation period and expressed as the mean $\pm$ S.E.M. for 4–12 observations on separate groups of pad pieces. * $P < 0.01$ compared with no-treatment control. Phosphoprotein band numbers are as indicated on Figs. 1–3 and used previously (Belsham *et al.*, 1980a,b).

Fraction	Phosphoprotein band no.	$10^{-3} \times$ subunit M_r	Incorporation of ^{32}P (as % of no-treatment control) in pieces incubated with:		
			No hormone after further 30 min	Insulin after 15 min	Adrenaline after 15 min
A	1	230	89 $\pm$ 23	137 $\pm$ 22	146 $\pm$ 20
	2	130	120 $\pm$ 15	207 $\pm$ 22*	186 $\pm$ 24*
	3	85	96 $\pm$ 13	96 $\pm$ 6	95 $\pm$ 9
	4A	65	—	117 $\pm$ 7	198 $\pm$ 17
B	7	35	—	150 $\pm$ 11*	149 $\pm$ 12
	8	26	—	114 $\pm$ 7	151 $\pm$ 6
C	—	45	146 $\pm$ 10	110 $\pm$ 7	122 $\pm$ 8
	9	22	92 $\pm$ 10	286 $\pm$ 18*	174 $\pm$ 10*
	—	15	87 $\pm$ 12	138 $\pm$ 10	137 $\pm$ 10

Fig. 1. Radioautographs locating ^{32}P -labelled phosphoproteins in whole-cell extracts of (a) isolated fat-cells and (b) fat-pad pieces separated by SDS/polyacrylamide-gel electrophoresis

Fat-cells were incubated with medium containing $^{32}\text{P}_i$ for 60 min and then for a further 15 min in the same medium with either (i) no added hormone, (ii) insulin ($0.5\text{ }\mu\text{g/ml}$), or (iii) adrenaline ($0.2\text{ }\mu\text{g/ml}$). Fat-pad pieces were incubated with medium containing $^{32}\text{P}_i$ for 120 min before the same additions were made and incubations continued for a further 15 min. Further details are given in the Materials and methods section. After centrifugation of extracts for 30 s at $1000g$, proteins in the infranant were precipitated with 10% trichloroacetic acid and separated on parallel tracks of the same gel containing 7.5% polyacrylamide.

22 000 is greatly increased by insulin and to a lesser extent by adrenaline (Belsham *et al.*, 1980a; Belsham & Denton, 1980). Similar changes were also evident in fat-pad pieces. As found previously with fat-cells, this phosphoprotein is not precipitated by either 2% (w/v) trichloroacetic acid or by heat treatment at 100°C for 10 min, but is precipitated by 15% (w/v) trichloroacetic acid. There were only three major ^{32}P -labelled phosphoproteins in the 2–15% (w/v)-trichloroacetic acid fraction (Fig. 2a). This fraction is designated fraction C in Table 1. In addition to the M_r -22 000 [^{32}P]phosphoprotein, there were also labelled bands corresponding to M_r 45 000 and 15 000. Again this pattern is similar to that found in the corresponding extract of isolated fat-cells (Fig. 2b; Belsham & Denton, 1980). Exposure to insulin and adrenaline increases the incorporation of $^{32}\text{P}_i$ into the M_r -22 000 phosphoprotein in both cells and pads (Belsham & Denton, 1980; Table 1). However, it was noticed during the present studies that in pad pieces exposed to insulin there was a decrease in the incorporation of $^{32}\text{P}_i$ into a phosphoprotein of M_r 20 000, which

Fig. 2. Densitometric scans showing the ^{32}P -labelled phosphoproteins of the 2–15%-trichloroacetic acid fraction of (a) fat-pad pieces and (b) isolated fat-cells

The cells and pad pieces were incubated as in Fig. 1 with insulin ($0.5\text{ }\mu\text{g/ml}$) present after the final 15 min period. Extracts were centrifuged at $10000g$ for 1 min and then the supernatants were treated with first 2 and then 15% trichloroacetic acid. The proteins precipitated between 2 and 15% trichloroacetic acid were separated on parallel tracks on a 10% (w/v) polyacrylamide gel. Also shown (c) is the migration of ^{32}P -labelled inhibitor-1 purified from rabbit skeletal muscle and that of: (i) α -chain of pig heart pyruvate dehydrogenase (M_r 42 000); (ii) chymotrypsinogen A (M_r 25 600) and (iii) cytochrome c (M_r 12 000). 45K and 22K represent bands of M_r 45 000 and 22 000 (also referred to as band 9).

was rather poorly separated from the M_r -22 000 band (see below and Fig. 5).

Previously we have pointed out that the M_r -22 000 phosphoprotein had rather similar physical properties to inhibitor-1 of the general phosphoprotein phosphatase. However, as shown in Fig. 2, it is evident that this phosphoprotein from both isolated fat-cells and pad pieces has an apparent molecular weight on SDS/polyacrylamide-gel electrophoresis which is appreciably less than that from rabbit skeletal muscle.

Since the cytoplasm contains the phosphoproteins whose ^{32}P labelling is most markedly stimulated by insulin, the ability of anti-insulin serum to reverse the effects of insulin on protein phosphorylation has been studied in cell extracts after brief centrifugation to remove mitochondria and much of the plasma membranes. To maximize the possibility of reversal, a non-saturating concentration of insulin (5 ng/ml) has been employed. This is within the physiological range and results in approx. 2-fold increases in the phosphorylation of

the phosphoproteins with subunit M_r of 130 000 (ATP citrate lyase) and 22 000.

The combined results from a series of experiments investigating the ability of anti-insulin serum to reverse the effects of insulin on ^{32}P labelling of fat-cell proteins are shown in Table 2. An excess of anti-insulin serum, sufficient to neutralize approx. 100 ng of insulin/ml, was used. This serum alone had no significant effects on phosphoprotein bands 1 (M_r 230 000), 3 (M_r 85 000) and 8 (M_r 26 000), which insulin also did not affect. However, the serum did appear to cause small insulin-like increases in the phosphorylation of ATP citrate lyase (M_r 130 000) and the acid-soluble 22 000- M_r protein. This may represent a limited activity related to that seen in isolated fat-cells discussed above. Nevertheless, if anti-insulin serum was added to fat-pad pieces for 15 min after the exposure of the tissue to insulin for 15 min, the effect of insulin on ATP citrate lyase phosphorylation was diminished by $67 \pm 21\%$ ($P < 0.02$; 7 separate observations) and on the 22 000- M_r protein by $74 \pm 9\%$ ($P < 0.01$; 11 separate observations). Continued exposure to insulin alone during this time led, if anything, to further elevated ^{32}P labelling of these two phosphoproteins (Table 2). It should be noted that there are no appreciable changes in the incorporation of ^{32}P into these phosphoproteins over the 30 min treatment period in cells incubated in the absence of hormones (Table 1).

Densitometric scans of the radioautograph from one representative experiment showing the ^{32}P incorporation into cytoplasmic proteins separated in the presence of SDS on a 7% (w/v) polyacrylamide gel are shown in Fig. 3. In addition to the changes in ATP citrate lyase phosphorylation, it can be seen that the ^{32}P content of a relatively minor phosphoprotein (marked by †, subunit M_r 63 000) was increased by insulin, and this change was also reversed by anti-insulin serum. It seems probable

Fig. 3. Densitometric scans showing the reversal of insulin-stimulated ^{32}P -labelling of adipose-tissue cytoplasmic proteins

Fat-pad pieces were incubated with $^{32}\text{P}_i$ for 2 h. Additions (for the period indicated below) were made and incubations terminated by extraction of the tissue as described in the Materials and methods section. The proteins in the supernatant fraction were precipitated with trichloroacetic acid (15%, w/v) and separated on parallel tracks of 7% (w/v) polyacrylamide gel in the presence of SDS (as Fraction A of Tables 1 and 2). The Figure shows densitometric traces from a radioautograph of a representative experiment; (a) no-treatment control; (b) insulin (5 ng/ml) for 15 min; (c) insulin (5 ng/ml) for 30 min; (d) insulin (5 ng/ml) for 15 min, then plus anti-insulin serum (20 µl/ml) for a further 15 min. R_F values were calculated relative to the mobility of Bromophenol Blue, which was allowed to migrate 10 cm. Samples were derived from approx. 100 µl of tissue extract. Phosphoprotein band numbers (see Brownsey *et al.*, 1979; Belsham *et al.*, 1980b) are indicated on trace (c); † band of M_r 63 000 (see the text).

Table 2. Effects of insulin and anti-insulin serum on the incorporation of $^{32}\text{P}_i$ into cytoplasmic phosphoproteins within intact fat-pad pieces

The pieces were preincubated for 2 h with $^{32}\text{P}_i$ and the incubations continued with additions of insulin (5 ng/ml) and/or anti-insulin serum (20 µl/ml) (AIS) as indicated. Other details, including preparation of fractions and expression of results, are given in Table 1. * $P < 0.05$ compared with no-treatment control; ** $P < 0.01$ compared with no-treatment control; † $P < 0.01$ compared with incubations with insulin after both 15 and 30 min on paired basis. All results are given as means $\pm$ S.E.M. for 6–12 observations.

Fraction	Phosphoprotein band no.	$10^{-3} \times$ Approx. M_r	Incorporation of ^{32}P (% of no-treatment control)			
			Effect of insulin (15 min)	Effect of insulin (30 min)	Effect of AIS (15 min)	Effect of insulin (15 min) followed by AIS (15 min)
A	1	230	108 $\pm$ 5	116 $\pm$ 14	99 $\pm$ 14	120 $\pm$ 20
	2	130	209 $\pm$ 22**	239 $\pm$ 10**	135 $\pm$ 17	157 $\pm$ 25**†
	3	85	94 $\pm$ 11	95 $\pm$ 8	109 $\pm$ 14	118 $\pm$ 11
B	7	35	120 $\pm$ 12	138 $\pm$ 10**	125 $\pm$ 12	139 $\pm$ 10**
	8	26	97 $\pm$ 9	103 $\pm$ 10	107 $\pm$ 10	131 $\pm$ 12
C	9	22	219 $\pm$ 19**	247 $\pm$ 15**	143 $\pm$ 14*	145 $\pm$ 10**†

that this band is the same as that (subunit M_r 62000) previously reported by Benjamin & Clayton (1978); it was not always well resolved from phosphoproteins of similar subunit M_r in the systems used in the present study.

Fig. 4 shows densitometric scans of extracts prepared by precipitation of protein soluble in 2% (w/v) trichloroacetic acid and in 15% (w/v) trichloroacetic acid. In addition to the large increase in the phosphorylation of the M_r -22000 band with insulin, there was also apparent a small decrease in phosphorylation of a protein of subunit M_r 20000. This effect is masked by the ^{32}P incorporation into the M_r -22000 band in the densitometric scans shown in Fig. 4, but can be seen in the expanded

Fig. 4. Effects of insulin and anti-insulin serum on the phosphorylation of proteins soluble in 2% (w/v) trichloroacetic acid

Fat-pad pieces were incubated with $^{32}\text{P}_i$ for 2 h. Additions (as indicated below) were made and incubations continued for the appropriate time. Supernatant fractions were prepared and proteins soluble in 2% (w/v) trichloroacetic acid but precipitated by 15% (w/v) trichloroacetic acid were separated on a 10% (w/v) polyacrylamide gel in the presence of SDS (as Fraction C of Tables 1 and 2). The Figure shows densitometric scans of the radioautograph from a representative experiment: (a) no-treatment control; (b) insulin (5 ng/ml) for 30 min; (c) anti-insulin serum (20 µl/ml) for 15 min; (d) insulin (5 ng/ml) for 15 min alone, then for a further 15 min with anti-insulin serum (20 µl/ml). R_F values were calculated from the mobility of Bromophenol Blue, which was allowed to migrate 10 cm. Samples were derived from 1 ml of tissue extract.

segment of these same scans shown in Fig. 5. This decrease in phosphorylation of the 20000- M_r protein was also reversed by treatment with anti-insulin serum. The difference scans clearly reveal these changes and are typical of ten separate experiments (Fig. 5).

The effect of insulin (5 ng/ml) on the phosphorylation of band 7 (M_r 35000) was only significant ($138 \pm 10\%$ of control; $P < 0.05$; 12 observations) after 30 min exposure to the hormone, and no reversal was observed (Table 2). With saturating concentrations of insulin (500 ng/ml) the phosphorylation of band 7 was $147 \pm 11\%$ of the control ($P < 0.01$ for 12 observations) after 15 min exposure of fat-pad pieces to the hormone (Table 1). In this and previous studies with isolated fat-cells with saturating concentrations of insulin, larger increases (about 4-fold) in the phosphorylation of this band were apparent (Hughes *et al.*, 1977, 1980; Fig. 1). We do not understand the reason for the apparent discrepancy between the two incubation systems.

Fig. 5. Expanded segment of densitometric scans shown in Fig. 4

The radioautograph used for the preparation of Fig. 4 was also scanned at a slower rate and the scale expanded to improve the resolution of the 22000- and 20000- M_r phosphoproteins. The Figure shows segments of scans 4(a), 4(b) and 4(d) and difference scans as indicated.

Significance of the reversal of insulin-stimulated phosphorylation by anti-insulin serum

Studies of fat-cell protein phosphorylation by Avruch and co-workers have involved extensive washing of cells in hormone- and $^{32}\text{P}_i$ -free medium at the end of the incubation period and before cell breakage (Avruch *et al.*, 1976a,b; Crapo *et al.*, 1980). In view of the quite rapid reversal of insulin-stimulated phosphorylation after removal of insulin, it seems probable that some loss of insulin-stimulated ^{32}P labelling could occur during such procedures. This may explain the small hormone effects seen by those workers; for example, only 20–30% increases in the phosphorylation of the band corresponding to ATP citrate lyase were reported after exposure of fat-cells to insulin (Avruch *et al.*, 1976a,b; Crapo *et al.*, 1980). The technique of spinning the cells up through dinonyl phthalate as used in studies from our laboratory is rapid and involves no dilution of the incubation medium.

The most important conclusion that can be drawn from the results of the present study is that the substantial increases in ^{32}P incorporation into ATP citrate lyase and the cytoplasmic protein of subunit M_r 22 000 observed in fat-cells exposed to insulin do represent genuine net increases in the phosphorylation of these proteins. The increase in phosphorylation could be the result of an increase in protein kinase activity or a decrease in phosphoprotein phosphatase activity, or both. On removal of insulin these changes are reversed and the ^{32}P content of the two phosphoproteins is decreased, reflecting dephosphorylation. If the increase in ^{32}P incorporation with insulin had been brought about by some turnover phenomenon (perhaps because phosphatase activity was elevated by insulin from an essentially inactive state) or an increase in ATP specific radioactivity, then on removal of the insulin stimulus the elevated ^{32}P labelling would have persisted or even increased.

The phosphorylation of these two proteins is also increased (but to a lesser extent) in cells exposed to adrenaline (Benjamin & Singer, 1975; Forn & Greengard, 1976; Avruch *et al.*, 1976a,b; Brownsey *et al.*, 1979; Belsham & Denton, 1980; Fig. 1). It seems likely that adrenaline and insulin may cause increased phosphorylation of different sites, perhaps through activation of cyclic AMP-dependent and -independent protein kinases respectively (Brownsey *et al.*, 1981; Denton *et al.*, 1981). Ramakrishna & Benjamin (1981) have reported that a cyclic AMP-independent kinase from liver phosphorylates fat-cell ATP citrate lyase on a site distinct from that phosphorylated by cyclic AMP-dependent protein kinase. With fat-cell acetyl-CoA carboxylase, insulin and adrenaline appear to bring about activation and inhibition respectively by initiating the

increased phosphorylation of separate sites on the enzyme (Brownsey *et al.*, 1981; Brownsey & Denton, 1982).

The identity of the M_r 22 000 phosphoprotein remains to be established. Although it shares some physical properties with inhibitor-1, it clearly has a lower molecular weight on SDS/polyacrylamide-gel electrophoresis than inhibitor-1 from rabbit skeletal muscle. However, its physical properties, and in particular its solubility in 2% (w/v) trichloroacetic acid and after boiling, are also shared by other phosphorylated proteins of approximately similar mobility. These include 'nucleoplasmin' (Laskey, 1981) and 'G-substrate' (Aswad & Greengard, 1981). We have speculated previously that the phosphorylated form of the M_r 22 000 phosphoprotein in fat-cells exposed to insulin may stimulate phosphoprotein phosphatase activity (Denton *et al.*, 1981).

Thus insulin is able to initiate the increased phosphorylation of certain proteins, e.g. ATP citrate lyase, a 22 000- M_r protein and acetyl-CoA carboxylase, as well as the decreased phosphorylation of others, including pyruvate dehydrogenase and presumably glycogen synthase. Any hypothesis concerning the mechanism of insulin action must be able to account for both these phenomena (see Denton *et al.*, 1981).

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