Actions of Insulin, Epinephrine, and Dibutyryl Cyclic Adenosine 5′-Monophosphate on Fat Cell Protein Phosphorylations. Cyclic Adenosine 5′-Monophosphate Dependent and Independent Mechanisms†

William B. Benjamin*,‡ and Irwin Singer

With the Assistance of Susan T. Fish

ABSTRACT: Endogenous and hormone-induced protein (polypeptide) phosphorylations were studied in isolated rat fat cells, in fat pads, and in subcellular fractions obtained from fat tissue under different physiological conditions. Insulin (25–100 μU/ml) increased the incorporation of ^{32}P into two proteins: insulin-phosphorylated proteins (IPP 140 and IPP 50; ~140,000 and 50,000 daltons, respectively). Epinephrine (10^{-7}–10^{-6} M) increased the incorporation of ^{32}P into another protein: epinephrine-phosphorylated protein (EPP 60–65; ~60,000–65,000 daltons). Endogenous IPP 140 phosphorylation in fat cells obtained from fasted and refed rats was similar to that of insulin in normal cells. Studies of insulin and epinephrine interactions showed that insulin increased IPP 140 phosphorylation even in the presence of epinephrine or lithium (25 mM × 10^{-3} M). Dibutyryl cyclic AMP (5 × 10^{-4} M) markedly stimulated EPP 60–65 phosphorylation, but neither epinephrine (10^{-7}–10^{-6} M) nor dibutyryl cyclic AMP reproduced insulin's phosphorylation of IPP 140. Lithium inhibited both endogenous and epinephrine-stimulated EPP 60–65 phosphorylation, but did not inhibit that induced by dibutyryl cyclic AMP. These findings suggest that insulin stimulated a specific, cyclic AMP independent protein kinase for IPP 140 phosphorylation. Cell-free extracts from insulin-treated fat tissue catalyzed the specific transfer of ^{32}P from ATP to IPP 140 more rapidly than control extracts. No differences in the total receptor protein or total protein kinase activity using [γ-^{32}P]ATP were noted between insulin-treated and control preparations. IPP 140 may be either (a) an insulin-sensitive protein kinase (phosphotransferase) or (b) a protein whose function is regulated by an insulin-sensitive protein kinase or phosphatase.

For insulin–cell membrane interactions (Krahl, 1961; Cuatrecasas, 1969, 1971) to produce diverse effects on intracellular metabolism (Gellhorn and Benjamin, 1965, 1966; Benjamin and Gellhorn, 1966; Fritz, 1972) both the qualitative and quantitative nature of the membrane interaction must be conveyed to an appropriate effector system. Protein kinases are particularly important in these information transfer systems (Greengard and Kuo, 1970; Walsh and Ashby, 1973; Langan, 1973), and cyclic AMP[1] specifically activates several of these enzymes (Walsh et al., 1968). However, only some of insulin's many metabolic effects can be explained by insulin's modulation of the levels or effects of cyclic AMP. Although insulin may affect both adenylate cyclase (Illiano and Cuatrecasas, 1972) and phosphodiesterase activities (Loten and Sneyd, 1970; Manganiello and Vaughan, 1973; Zinman and Hollenberg, 1974), there is a dissociation between at least some of insulin's actions and the intracellular levels or effects of cyclic AMP (Rodbell et al., 1968; Glinsmann and Mortimore, 1968; Craig et al., 1969; Robison et al., 1971).

These and more recent observations (Bishop et al., 1971; Khoo et al., 1973; Taylor et al., 1973; Kono and Barham, 1973; Tolbert et al., 1973) suggest that an insulin-dependent, cyclic AMP independent protein kinase system may link the insulin–membrane receptor complex to some biochemical responses to insulin. Our initial studies (Benjamin and Singer, 1974a–c) imply such a mechanism of insulin action on fat cell protein phosphorylation. In either the absence or the presence of adenylate cyclase stimulation, physiological concentrations of insulin rapidly and specifically increased $^{32}P_i$ incorporation into a high molecular weight protein–polypeptide of approximately 140,000 daltons (IPP 140).[2] The present study extends our investiga-

† From the Departments of Medicine, University of Pennsylvania School of Medicine, and Philadelphia Veterans Administration Hospital, Philadelphia, Pennsylvania 19174. *Received December 6, 1974.* During the tenure of this project, Dr. William B. Benjamin was the recipient of a Research Career Development Award from the U.S. Public Health Service (5-KD-4CA-2461) and was supported by grants from the Alma Toorock Memorial and Young People's Cancer Fund of the University of Pennsylvania; Dr. Irwin Singer was an Established Investigator of the American Heart Association (69–106) and was supported by grants from the U.S. Public Health Service (5-RO1-HL-14012-03 and 9-RO1-AMHL-17344-04) and by the Philadelphia Veterans Administration Hospital (103.14M).

‡ Present address: Department of Physiology and Biophysics, State University of New York—Stony Brook, Stony Brook, New York 11794.

[1] Abbreviations used are: cyclic AMP, cyclic adenosine 3′,5′-monophosphate, sodium; dibutyryl cyclic AMP, cyclic N^6,O^2-dibutyryladenosine 3′,5′-monophosphate; dibutyryl cyclic GMP, cyclic N^2,O^2-dibutyrylguanosine 3′,5′-monophosphate; EDTA, disodium ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; Hepes, hydroxyethylpiperazine-N-ethanesulfonic acid; MEM – methionine, minimal Eagle's medium without methionine; mod-KRB-P, modified Krebs–Ringer bicarbonate buffer without phosphate ion.

[2] The designation IPP 140 refers to its approximate molecular weight (after boiling in 1% sodium dodecyl sulfate and β-mercaptoethanol) as measured by acrylamide gel electrophoresis (Weber and Osborn, 1969). Sedimentation velocity studies (Martin and Ames, 1961) indicate that IPP 140 has a molecular weight of approximately 110,000–400,000, suggesting that the native protein is made up of subunits.

tion of this mechanism in vivo, in vitro, and in cell-free systems.

Experimental Section

Materials. The following reagents were purchased from Sigma Corporation (St. Louis, Mo.): ATP, collagenase (type II), bovine serum albumin (factor V or crystalline), epinephrine bitartrate, theophylline, sodium dodecyl sulfate, EDTA, Tris, and glycine. Penicillin, streptomycin, Hepes, phosphate-buffered saline, and MEM − methionine were obtained from GIBCO (Grand Island, N.Y.);[1] cycloheximide was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio). Radioactive chemicals and Aquasol were purchased from the New England Nuclear Corp. (Boston, Mass). Crystalline insulin (Porcine), lot no. 615-D63-10 (25.4 U/mg), and single component Porcine insulin, lot no. 615-954B-164E, were a gift from Dr. William Boomer of the Eli Lilly Co. (Indianapolis, Ind.). Cyclic nucleotides were obtained from P-L Biochemicals (Milwaukee, Wis.) and nitrocellulose filters were purchased from Schleicher and Scheull (Keene, N.H.).

Fat Cell Incubations. The relative rates of individual protein (polypeptide) phosphorylation were studied in isolated rat fat cells under in vitro conditions described previously in detail (Benjamin and Singer, 1974a). In brief, fat cells from Wistar rats (160–180 g) were prepared (Rodbell, 1964) with collagenase (0.5 mg/ml). Protein phosphorylation was measured by incubating cells at 37° (pH 7.4) in a Dubnoff shaker (60 cycles/min) in sealed flasks under 95% O_2–5% CO_2. The standard incubation medium was phosphate-free modified Krebs–Ringer bicarbonate buffer (mod-KRB-P), containing: 2.5 m*M* glucose; 1.5 m*M* Ca^{2+}; 2% dialyzed and neutralized bovine serum albumin; 0.01 *M* Hepes buffer; penicillin (100 U/ml) and streptomycin (100 μg/ml); and Phenol Red. [^{32}P]Orthophosphate (25–50 μCi/ml of incubation medium) and other agents were added as indicated. Cell isolations and incubations were performed in plastic vials and all glassware was siliconized. De novo protein synthesis was measured by incubating fat pads (or fat cells), with [^{35}S]methionine as the tracer amino acid in mod-KRB mixed 1:1 with MEM − methionine; penicillin, streptomycin, Hepes, and albumin were added as above.

Sample Preparation. After incubation, cells were collected, washed, and dissolved in lysing buffer (pH 8.5) containing 0.1 *M* glycine, 0.1 *M* NaCl, 0.01 *M* NaEDTA, 0.01 *M* β-mercaptoethanol, and 0.1% sodium dodecyl sulfate. The proteins were then precipitated with 5 vol of cold acetone and successively extracted with acetone, ethanol, chloroform–methanol (1:1), ethanol, ether–ethanol (1:1), and ether. The dried precipitate was dissolved in a buffer (pH 6.8) containing 0.05 *M* Tris, 1% sodium dodecyl sulfate, 10% glycerine, and 1% β-mercaptoethanol. The dissolved proteins were boiled for at least 5 min before taking sample aliquots for radioactivity and protein determinations (Lowry et al., 1951). Each sample and 200 μg of carrier albumin were precipitated with 10% w/v trichloroacetic acid; after boiling for 10 min, the sample was chilled and the precipitate collected on nitrocellulose filters for determining radioactivity in a liquid scintillation spectrometer. Either Bray's solution (Bray, 1960) or Aquasol was the scintillation fluid.

Gel Electrophoresis. The relative rates of individual protein (polypeptide) phosphorylation were determined by placing the acetone-precipitable (hot trichloroacetic acid insoluble) material containing approximately 2–10 × 10^3 cpm of ^{32}P per well, dissolved in pH 6.8 buffer, on top of a 5% spacer acrylamide gel (pH 6.8). Electrophoresis was performed through a 7.5% polyacrylamide–0.1% sodium dodecyl sulfate slab gel (pH 8.8; approximately 1 mm thick) or through a similar 7.5 to 30% gradient gel to resolve proteins of varying molecular weight (Laemmli, 1970; Maizel, 1971; Studier, 1972). After electrophoresis, the slabs were dried and autoradiograms were developed (Fairbanks et al., 1965) by placing the dried gel directly against Kodak BB X-ray film (Rochester, N.Y.). The relative optical density of each region of the film was determined with a double-beam scanning microdensitometer (Joyce, Loebl and Co., Ltd., Gateshead, England). Molecular weights for the various proteins were approximated by the method of Weber and Osborn (1969).

Protein Kinase Isolation and Assay. Rats (260–280 g) were either fasted for 48 hr (control group) or fasted for 36 hr and refed with 5% dextrose in their drinking water and rat chow ad libitum for 12 hr (experimental group). Approximately 10 g of fat (epididymal and lumbar gutter) was obtained from 3 or 4 rats in each group. All subsequent operations were carried out at 4°. The fat was washed in saline and then homogenized with a Teflon homogenizer, in 8 vol of 25 m*M* potassium phosphate buffer (pH 7.4), containing NaCl (0.1 *M*), $MgCl_2$ (5 m*M*), and β-mercaptoethanol (0.01 *M*). The fat cell homogenate was sonicated (3 times for 1 sec at tap 3) with a Bransom sonifier (Bransom Corp., Danbury, Conn.). The sonicated homogenate was centrifuged at 5000g_{av} for 5 min; the infranatant was centrifuged at 20,000g_{av} for 20 min, and then centrifuged at 175,000g_{av} for 1 hr. The supernatant (S_{175}) was collected carefully, leaving behind the top 2 or 3 ml (opalescent, lipid-rich layer), and filtered through fine mesh nylon stocking to remove any white fat particles still present.

The S_{175} proteins which precipitate between 0 and 65% saturated ammonium sulfate solution were collected, washed, dissolved in 3–4 ml of phosphate-buffered saline (without Ca^{2+}, but with 0.01 *M* β-mercaptoethanol added), and then dialyzed against the same buffer for 18 hr. This concentrated S_{175} protein solution, which contains both protein kinase activity and the endogenous substrates, was used for the protein kinase assays (Walsh et al., 1968; Wray et al., 1973; Erlichman et al., 1974). Varying amounts of protein were incubated at 30° with approximately 10 μCi of [γ-^{32}P]ATP (specific activity, 21.6 Ci/mmol in a buffer (pH 7.4) containing potassium phosphate (50 m*M*), NaCl (0.1 *M*), $MgCl_2$ (10–12 m*M*), and β-mercaptoethanol (0.01 *M*). (Theophylline (1–5 m*M*) and NaEGTA (0.3 m*M*) slightly inhibit this assay; these agents did increase the rate of endogenous protein phosphorylation in a cruder assay, using fresh fat cell homogenates for both substrate and kinase activity.)

After incubation, aliquots were added to cold water (about 0.5 ml) containing ATP (0.25 μmol) and albumin (250 μg). Cold trichloroacetic acid (10%, w/v) was then added and the resulting precipitates were boiled in a water bath for 5 min, chilled, and collected on cellulose filters which had been presoaked with phosphate-containing buffer and ATP. Background counts per minute, using boiled protein samples in the same assay, were less than 8% of the total radioactivity incorporated. As noted for other tissues (Wray et al., 1973; Roses and Appel, 1973; Eipper, 1974), when assayed with low levels of endogenous substrate, protein kinase activity was not linear for time periods longer than 1 min. With an increasing amount of substrate, the ac-

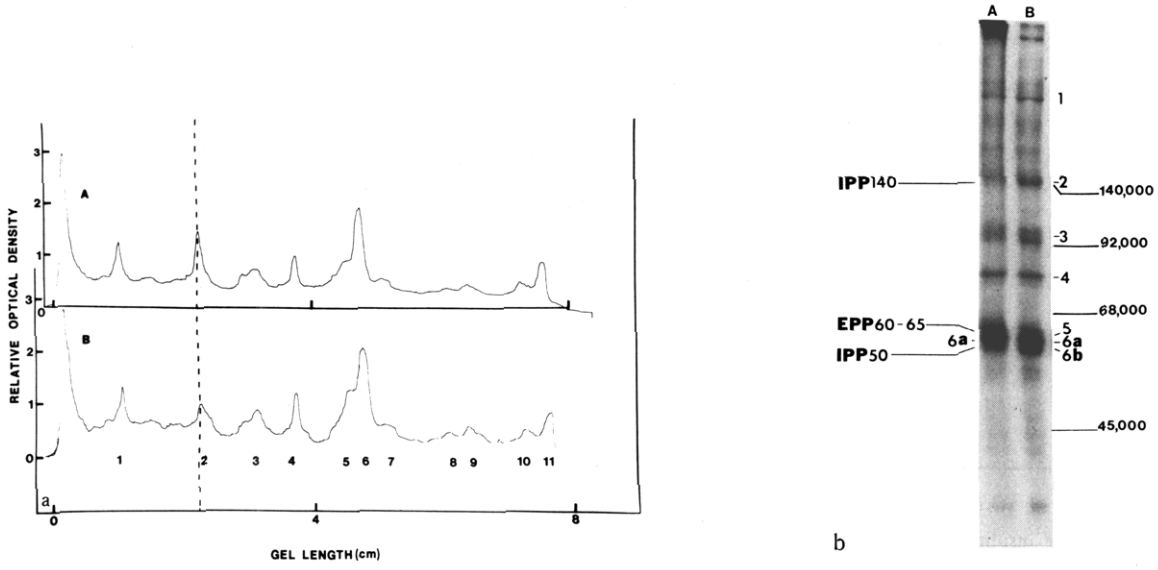

FIGURE 1: Effect of insulin on the phosphorylation of fat cell protein. (a) Densitometric tracing. Fat cells obtained from 1 rat (150 g) were incubated for 10 min at 37° in a total volume of 2 ml of mod-KRB-P (see Experimental Section) with $^{32}P_i$ (final concentration, 25 μCi/ml). Insulin (100 μU/ml) was added to one preparation and the incubations continued for an additional 50 min. Approximately 1 mg of protein was recovered from the cells obtained from 0.5 g of fat pad tissue. The [^{32}P]protein (counts per minute per microgram of protein) incorporated into the acetone-precipitable, lipid-free, hot Cl_3CCOOH-insoluble fractions was: (A) insulin treated, 623; (B) control, 307. Samples were placed in wells on a 5% spacer, 7.5–30% acrylamide, slab gel for electrophoresis (18 hr at 60 V). Approximately equal amounts of radioactivity (corrected counts per minute) were placed on the gel: (A) 9700; (B) 10,200. Actual counts per minute placed on this gel and all others to be described were 10–50% of the corrected (for decay) values. Exposure times of autoradiograms were 2–10 days. The relative optical densities of this and other autoradiograms were determined with a double-beam, scanning microdensitometer; full densitometer deflection, 3.0 optical density (OD) units. Panels have been aligned for vertical comparison and the major phosphorylated bands designated 1–11. Note the preferential stimulation of phosphorylation of a protein fraction (band 2, dashed line) by insulin. (b) Autoradiogram (different preparation than a); fat cell incubation with $^{32}P_i$, 15 min; insulin addition, 50 μU/ml. Incubation continued for 30 min. Radioactivity incorporated (counts per minute per microgram of protein): (A) control, 209; (B) insulin treated, 408. Approximately equal amounts of radioactivity were placed in wells on a gel 4.5% spacer, 7.5% acrylamide (see a) for electrophoresis (at 100 V for 4.5 hr) and autoradiography: (A) 6950 cpm; (B) 6210 cpm. Estimates of the molecular weights of individual protein bands were made by comparing the mobilities of protein of known molecular weight (boiled in pH 6.8 lysing solution) run on the same gel from which representative autoradiograms were obtained: *Escherichia coli* β-galactosidase, subunit mol wt 140,000; rabbit muscle phosphorylase *a*, 92,000; bovine serum albumin, 68,000; ovalbumin, 45,000; ribonuclease, 12,000. Note on this gel, ovalbumin and ribonuclease would be run off the gel. Radioactivity (percent ± standard deviation; number of experiments in parentheses) associated with each band (Ueda et al., 1973) is shown:

	Band Number				
	1	2	3	4	5–7
Control (16)	12.9 ± 4.5	10.5 ± 1.8	10.6 ± 2.1	9.5 ± 3.3	56.5 ± 5.4
Insulin (16)	11.8 ± 4.4	17.4 ± 2.1*	9.7 ± 2.5	8.5 ± 2.5	52.6 ± 5.6
Dibutyryl Cyclic GMP (5)	11.4 ± 1.3	8.6 ± 0.4	11.0 ± 1.5	9.6 ± 1.5	59.2 ± 1.7

where the asterisk indicates $P < 0.001$ (unpaired t test for difference between insulin and control groups).

tivity was linear for 3–4 min. Protein kinase activity increased linearly with protein concentration up to at least 1.4 mg/ml.

Results

(*A*) *Protein Phosphorylations.* Neither insulin (24 experiments) nor epinephrine (11 experiments) changed the overall rate of $^{32}P_i$ incorporation into total fat cell phosphoprotein. However, in each paired experiment (Figure 1), physiological levels of insulin distinctly increased the incorporation of $^{32}P_i$ into one discrete region of the gel (insulin-phosphorylated protein; IPP 140), and those of epinephrine increased the incorporation of $^{32}P_i$ into another region of the gel [epinephrine-phosphorylated protein; EPP 60–65 (Benjamin and Singer, 1974a)]. Representative densitometric tracings are shown in Figure 1a, and the radioactivities (percent ± standard deviation) associated with each band are summarized in the legend. Insulin stimulated the phosphorylation of band 2 (IPP 140) and decreased the relative incorporation of radioactivity into band 5 (EPP 60–65). In preparations with very good electrophoretic resolution (7.5% acrylamide gels), band 6 is consistently resolved into two components (Figure 1b). This autoradiogram (Figure 1b) also demonstrated that insulin increased the relative phosphorylation of band 6b (bands 8 to 11 have been run off the gel). This insulin-phosphorylated protein has a molecular weight of approximately 50,000 (IPP 50). To validate the autoradiographic method of data analysis we compared the densitometric tracings of the autoradiograms of control and experimental samples with the actual radioactivity profiles obtained by slicing the gels and assaying the radioactivity content of each slice (Ueda et al., 1973); both methods of analysis (densitometric tracings and radioactivity profiles) gave similar results.

(*B*) *Protein Synthesis.* The apparently increased rate of IPP 140 phosphorylation could be due to insulin increasing this protein's turnover rate. Since insulin increases the rate of total fat cell protein synthesis (Carruthers and Winegrad, 1962), we measured IPP 140 synthesis (relative to other proteins) by incubating tissue with [^{35}S]methionine (1–4 hr). As expected, insulin increased the rate of overall protein synthesis, but did not increase the de novo rate of IPP 140 synthesis (Figure 2A). However, insulin markedly increased the relative synthesis of two different proteins/

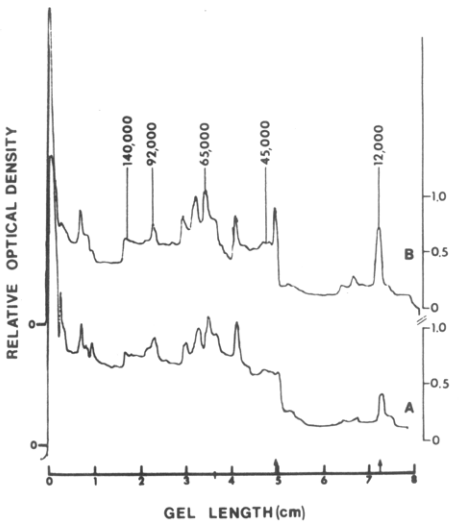

FIGURE 2: Effect of insulin on [^{35}S]methionine incorporation into newly synthesized fat cell proteins. Fat pads from a 150 g rat were incubated for 10 min in 2 ml of buffer (pH 7.4) containing: 1:1 mod-KRB–methionine-free minimal Eagle's medium; 0.02 *M* Hepes; 2% albumin; and insulin (100 μU/ml, as indicated). [^{35}S]Methionine (specific activity, 180 Ci/mmol) was added (12 μCi/ml), and the incubations were continued for 2 hr. Radioactivity incorporated (counts per minute per microgram of protein): (A) control, 4521; (B) insulin treated, 7792. Radioactivity (corrected for decay) placed on a gradient gel: (A) 1.9×10^5 cpm; (B) 2.1×10^5 cpm. Full densitometer deflection: 1.6 OD units. The mean ± SD radioactivities incorporated (counts per minute per microgram of protein per hour) were 1583 ± 334 for the controls and 4385 ± 1638 for the insulin-treated preparations ($N = 4$). After staining with Coomassie dye, their mobilities were compared with protein standards run on the same gel (mobility vs. molecular weight plots are not valid with gradient gels).

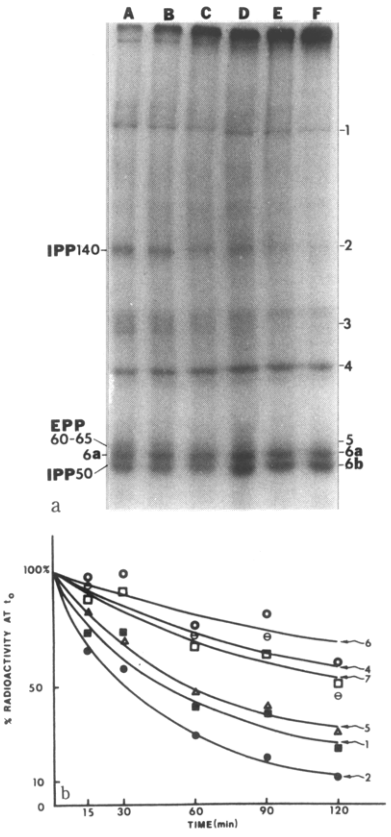

FIGURE 3: (a) The dephosphorylation of fat cell protein; autoradiogram. Fat cells were prepared from 2 g of fat obtained from 2 rats (150 g) and were incubated in 2 ml of mod-KRB–P with insulin (50 μU/ml) and $^{32}P_i$ (25 μCi/ml) for 30 min. Potassium phosphate (final phosphate concentration, 8 m*M*) was added at zero time (t_0) and the incubations were continued for varying periods of "cold chase" (t_m, minutes). Radioactivities (counts per minute per microgram of protein) incorporated at each time point (t_m, min) were: (A) t_0 = 809; (B) t_{15} = 556; (C) t_{30} = 666; (D) t_{60} = 273; (E) t_{90} = 207; (F) t_{120} = 188. Radioactivities (counts per minute) placed on the gel were: (A) 5942; (B) 6018; (C) 5977; (D) 5978; (E) 4622; (F) 5963. Note the variable quantity of radioactivity at the origin; the amount of radioactivity actually placed in well D is probably greater than that measured. (b) Dephosphorylation of individual protein bands; densitometric tracings of autoradiograms (such as in a). At t_0 the percent radioactivity of each band was (1) 10.3 (■); (2) 17.0 (●); (3) 9.6; (4) 15.2 (⊖); (5) 17.8 (△); (6) 24.4 (○); (7) 5.5 (□). The percent radioactivity in each band at each time point (t_m) of the cold chase was corrected for total protein dephosphorylation. Corrected percent radioactivity in band n at t_m = uncorrected % radioactivity of band n at $t_m \times$ [(cpm/μg of protein at t_m)/(cpm/μg of protein at t_0)] × 100. The results of three separate experiments are presented (mean percentages). Note that the values for band 3 were not included. The estimated rate of dephosphorylation for band 3 was similar to those of bands 1, 2, and 5, but since the band 3 density on the X-ray film approached background during the cold chase (a), the values were considered unreliable.

polypeptides of about 40,000 and 12,000 daltons, respectively (4 fat pad experiments).

We also measured both insulin-induced protein phosphorylation and new protein synthesis in the presence of cycloheximide (125 μg/ml) (Korner, 1966). Insulin (25 μU/ml) stimulated the incorporation of $^{32}P_i$ into IPP 140, even when protein synthesis was 95% inhibited. Cycloheximide alone had no apparent effect on fat cell protein phosphorylation. Therefore, our $^{32}P_i$ tracer studies predominantly measured insulin-induced changes in side-chain metabolism (phosphorylation), which were not secondary to changes in protein synthesis.

(*C*) *Protein Dephosphorylations.* The insulin-induced IPP 140 phosphorylation can be explained if insulin increases the activity of an insulin-dependent phosphatase which stimulates the turnover of IPP 140 phosphate groups. There may be such an active fat cell protein phosphatase both in whole cell isolates (Figure 3) and in broken cell preparations. When fat cells were incubated (for 45 min) with $^{32}P_i$ (50 μCi/ml) and insulin (50 μU/ml), homogenized, and then separated by centrifugation into a membrane-free supernatant and a membrane-rich pellet, only 25% of the radioactivity ([^{32}P]protein/μg of protein) of the initial homogenate was found with the proteins of the supernatant or membrane fractions: total cell phosphoprotein, 455; membrane phosphoprotein, 106; supernatant phosphoprotein, 18. Significantly, ^{32}P-lbeled IPP 140 was found primarily with the membrane-free supernatant (S_{175}) while ^{32}P-labeled EPP 60–65 and IPP 50 were found primarily with the membrane fraction.

Dephosphorylation was studied directly by incubating fat cells from fed rats with ^{32}P and insulin for 30 min (six experiments). Unlabeled potassium phosphate was then added (zero time), and the incubations were continued for varying durations of "cold chase". The specific activity of the fat cell [^{32}P]protein (counts per minute per microgram of protein) at each time was expressed as a percent of the control value obtained at zero time. The overall rate of fat cell phosphoprotein dephosphorylation is relatively slow, with an approximate half-time of 60–90 min. The percents (cpm ± SD) remaining were 88 ± 10 at 15 min, 82 ± 8 at 30 min, 54 ± 11 at 60 min, 50 ± 18 at 90 min, and 35 ± 7 at 120 min.

However, the dephosphorylation rates of individual phosphoproteins may vary widely, with the overall rate being an

average of rapid and slow individual rates. Therefore, the turnover rates of individual phosphoprotein phosphate groups were analyzed at each time point of the cold chase experiments. Approximately equal counts per minute from each sample were analyzed to compare the results autoradiographically (Figure 3a). The rate of IPP 140 dephosphorylation was more rapid than that of any of the other fat cell phosphoproteins, i.e., radioactivity was lost more rapidly from region 2 (IPP 140) than from any other region of the gel. These studies were conducted with insulin-stimulated tissue, although a control was always included to demonstrate an insulin-induced increase in IPP 140 phosphorylation. We could not show that the insulin-stimulated rate of IPP 140 phosphate group turnover is different from that in untreated cells because there is too little radioactivity incorporated into IPP 140 in the absence of insulin.

To approximate the dephosphorylation rate of each phosphoprotein (regions 1–7), the amount of radioactivity of each band was calculated from the densitometric tracings and expressed as a percent of the total radioactivity on the gel (adjusted for the loss of radioactivity at each time point of the chase). These results (Figure 3b) confirm that there is a spectrum of phosphoprotein dephosphorylation rates; IPP 140 turns over most rapidly while the most radioactive band (band 6) loses radioactivity least rapidly. Note that the rate of dephosphorylation of band 6b (IPP 50), the phosphorylation of which is increased by insulin, was not significantly greater than that of other proteins.

(*D*) *Hormone Interactions.* Our previous study (Benjamin and Singer, 1974a) reported (a) that epinephrine increased the phosphorylation of region 5 (~60,000–65,000 daltons; epinephrine phosphorylated protein, EPP 60–65); (b) that submaximal doses of insulin enhanced the phosphorylation of IPP 140 either in the absence or in the presence of epinephrine (10^{-7}–10^{-6} *M*); and (c) that insulin decreased both the submaximal epinephrine-induced phosphorylation of EPP 60–65 and the unstimulated level of phosphorylation associated with this region (e.g., Figure 1). These interrelationships were further examined in the present study by incubating fat cells with $^{32}P_i$ and some of the various agents (dibutyryl cyclic AMP, dibutyryl cyclic GMP, lithium ion, epinephrine, and insulin) which affect the adenylate cyclase–cyclic AMP system.

Compared to paired controls (100%), pharmacological doses of dibutyryl cyclic AMP (10^{-4} *M*; $N = 11$) probably increased the incorporation of $^{32}P_i$ into total cell phosphoprotein (142 ± 38%), whereas lithium ion (25 m*M*; $N = 5$) decreased $^{32}P_i$ incorporation (64 ± 12%). These results are similar to those shown in the autoradiograms and densitometric tracings (Figure 4). Dibutyryl cyclic AMP markedly stimulated the phosphorylation of EPP 60–65 (Figure 4a), but did not mimic the insulin effect on IPP 140 phosphorylation. Lithium ion inhibited both the endogenous and the epinephrine-stimulated phosphorylation of EPP 60–65 (Figure 4b), but not that stimulated by dibutyryl cyclic AMP (Figure 4a). (Lithium ion also inhibited the phosphorylation of region 4, the significance of which is totally unknown.) In contrast to dibutyryl cyclic AMP (10^{-4} *M*), dibutyryl cyclic GMP (5×10^{-4} to 5×10^{-6} *M*) had no discernible effect on fat cell protein phosphorylation ($N = 4$). Insulin (50 μU/ml) did stimulate phosphorylation of IPP 140 in the presence of either cyclic nucleotide in each experiment. These findings are consistent with lithium inhibiting the hormone-activated adenylate cyclase system (Dousa and Hechter, 1970; Singer and Rotenberg, 1973), while dibutyryl cyclic AMP acts on the protein kinase system distal to the site of lithium inhibition.

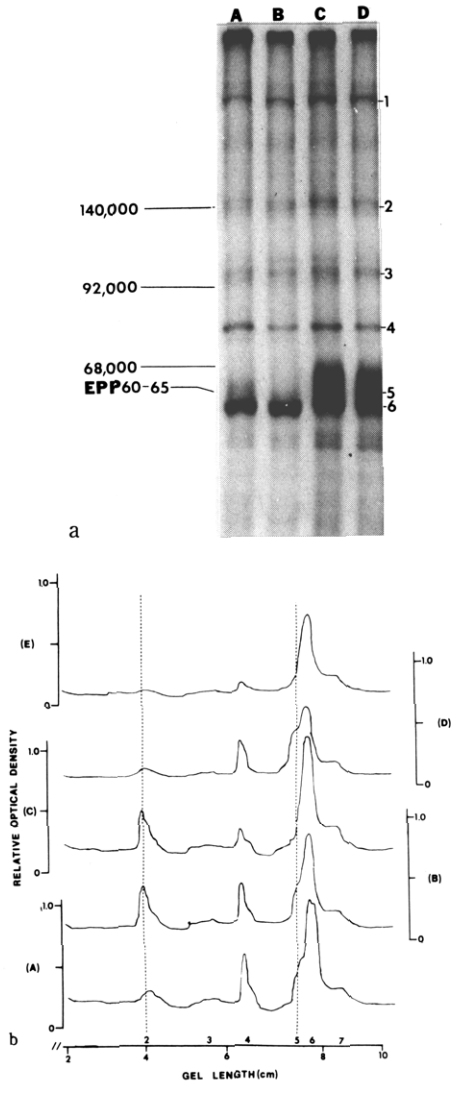

FIGURE 4: (a) Effects of dibutyryl cyclic AMP and lithium ion on protein phosphorylation; autoradiogram. Dibutyryl cyclic AMP (final concentration, 5×10^{-4} *M*) was added after 15 min of incubation of cells (control) and lithium chloride (25 m*M*) with $^{32}P_i$ (25 μCi/ml) and the incubations were continued for an additional 30 min. Radioactivity incorporated (counts per minute per microgram of protein): (A) control, 693; (B) lithium, 491; (C) dibutyryl cyclic AMP, 751; (D) lithium plus dibutyryl cyclic AMP, 690. Radioactivity (counts per minute) placed in each well for electrophoresis (as in a): (A) 4860; (B) 4200; (C) 5020; (D) 4190 (note that the increased total counts per minute on gel C increased the density of all bands). (b) Effects of lithium ion on epinephrine- and insulin-induced protein phosphorylations; densitometric tracings. Fat cells were incubated in mod-KRB–P or in mod-KRB–P containing lithium chloride (25 m*M*) for 10 min; $^{32}P_i$ (25 μCi/ml) was then added and the incubations were continued for 15 min. The incubations were continued with the indicated hormones for an additional 60 min. Radioactivity (counts per minute) placed in each well for electrophoresis: (A) control, 4770; (B) insulin (100 μU/ml), 5110; (C) insulin plus lithium chloride (25 m*M*), 4140; (D) epinephrine (10^{-6} *M*), 4330; (E) epinephrine plus lithium chloride, 4180. The OD scales for curves A, C, and E are on the left, and those for B and D are on the right. NOTE THE PREFERENTIAL STIMULATION OF PHOSPHORYLATION OF SOME PROTEIN FRACTIONS (band 2 in curves B and C and band 5 in curve D; dashed lines). There is also an inhibition of phosphorylation in some protein fractions (bands 4 and 5 in curves C and E); full densitometric deflection, 1.6 OD unit.

The results also suggest (a) that insulin stimulates an insulin-specific protein kinase, which affects the phosphoryl-

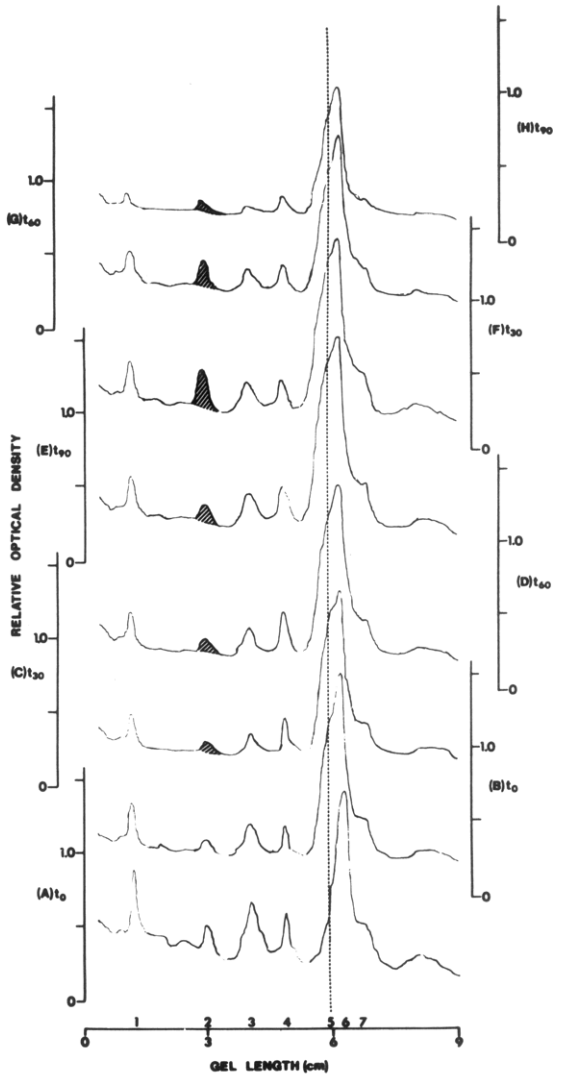

FIGURE 5: IPP 140 phosphorylation; effects of insulin and prior stimulation of protein kinase by dibutyryl cyclic AMP; densitometric tracings. All fat cell preparations (A–H) were incubated for 15 min with $^{32}P_i$ (50 μCi/ml) in mod-KRB-P with lithium chloride (25 mM). Dibutyryl cyclic AMP (5×10^{-4} M) was added to flasks (B–H). All incubations (A–H) were continued for an additional 30 min. Unlabeled potassium phosphate (final phosphate concentration, 8 mM) was added, and 5 min later insulin (50 μU/ml) was added to flasks (F–H). The radioactivities incorporated (counts per minute per microgram of protein) at each time of the "cold chase" after insulin addition (t_0) were: control: (A) t_0, 439; dibutyryl cyclic AMP: (B) t_0, 501; (C) t_{30}, 564; (D) t_{60}, 369; (E) t_{90}, 282; insulin plus dibutyryl cyclic AMP: (F) t_{30}, 456; (G) t_{60}, 333; (H) t_{90}, 350. Radioactivity (counts per minute) placed in each well for electrophoresis: (A) 14,160; (B) 14,490; (C) 15,420; (D) 12,750; (E) 12,640; (F) 15,940; (G) 15,860; (H) 15,560. The relative optical density scales for curves A, C, F, and G are on the left and those for B, D, F, and H are on the right of the figure. The dashed vertical line passes through band 5 and the shaded portions of band 2 in curves C–H align the respective paired samples for the "cold chase". Full densitometer deflection 1.6 OD units.

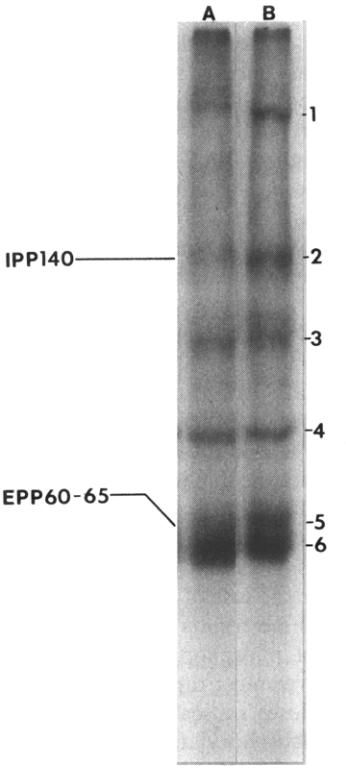

FIGURE 6: Effect of refeeding on fat cell protein phosphorylation; autoradiogram. Fat cells from fasted and refed rats were incubated with $^{32}P_i$ (50 μCi/ml) for 45 min. Radioactivity incorporated (counts per minute per microgram of protein in 45 min): (A) fasted, 475; (B) refed, 430. Approximately 10,000 cpm was placed in wells on the gel for electrophoresis. In four separate experiments, the mean radioactivities incorporated (counts per minute per microgram of protein) were: (A) fasted, 575; (B) refed, 430.

ation of specific proteins and is separate from the adenylate cyclase system, and/or (b) that activation of the insulin-specific protein kinase is proximal to insulin's effects on the cyclic AMP-dependent protein kinase system. To test these two suggestions, fat cells were incubated with $^{32}P_i$ after cyclic AMP dependent protein kinase was first stimulated with dibutyryl cyclic AMP. Lithium ion was present to suppress stimulation of adenylate cyclase (Dousa and Hechter, 1970). After a short "cold chase" with unlabeled phosphate ion, insulin's effects on fat cell protein phosphorylation were determined (three experiments). Dibutyryl cyclic AMP stimulated the phosphorylation associated with gel region 5 (EPP 60–65; cf. Figure 4), but produced relatively little phosphorylation of region 2 (IPP 140; Figure 5, A–E). However, after the addition of unlabeled phosphate ion, insulin (F–H) markedly increased IPP 140 phosphorylation (region 2, Figure 5, F). In addition, the "cold chase" produced a rapid loss of radioactivity associated with IPP 140 (region 2, Figure 5, G and H), confirming its rapid dephosphorylation. There was little relative loss of radioactivity associated with either EPP 60–65 (region 5) or with IPP 50 (region 6b), but there was loss of radioactivity associated with regions 1 and 3 confirming the findings in Figure 3b). Thus, even under conditions of excess P_i, of stimulated cyclic AMP dependent protein kinase, and of suppressed adenylate cyclase stimulation, insulin still increases the incorporation of ^{32}P into a specific phosphoprotein (IPP 140). The results suggest (a) that insulin specifically enhances the phosphorylation of IPP 140 by a mechanism separate from the adenylate cyclase–cyclic AMP system and (b) that the proximate phosphate donor is not P_i.

(*E*) *Endogenous Protein Phosphorylations* (*Fasting and Refeeding*). Our in vitro observations (on fat cells from fed rats) may be related to normal phosphoprotein physiology by comparing in vivo conditions of starvation (associated with decreased insulin levels) to those of refeeding (associated with increased insulin levels); IPP 140 phosphorylation should be greater in cells obtained from refed rats than in those obtained from starved rats. A group of eight (150–

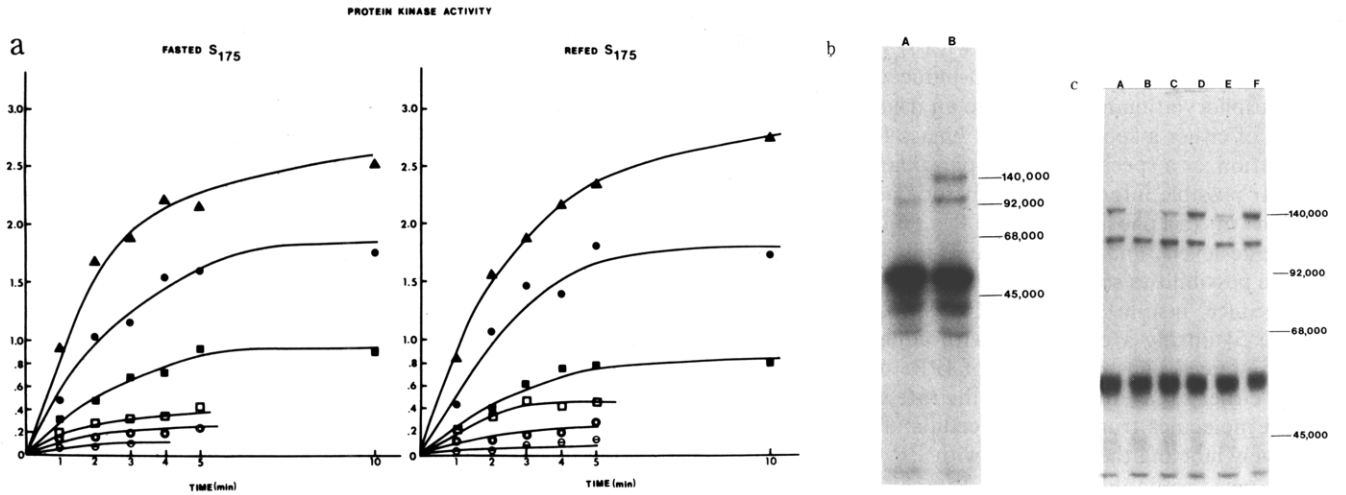

FIGURE 7: Comparisons of protein kinase and endogenous substrate activities (protein kinase assay). (a) Time course. Fat cell supernatants from fasted and refed rats were prepared and incubated in a protein kinase assay. Each time point is the mean value (picomoles of ^{32}P from ATP transferred to endogenous S_{175} protein) of two separate experiments, each performed in duplicate; specific activity of [γ-^{32}P]ATP = 21.6 Ci/mmol, 10 μCi was added to each 0.5-ml assay. Total amounts of protein (micrograms) S_{175} added to assay system (total volume, 0.5 ml) for fasted and refed, respectively, were: (⊖) 24, 25; (○) 49, 49; (□) 122, 123; (■) 243, 246; (●) 486, 492; (▲) 730, 738. (b) Autoradiograms (starved vs. refed). Supernatant fractions (concentrated S_{175}; 240 μg of protein) which were obtained from fat pads of fasted and refed animals were incubated in 0.5-ml protein kinase assays. After 5 min of incubation at 30°, 0.5 ml of lysing buffer (pH 8.5 with mercaptoethanol and sodium dodecyl sulfate) was added and the mixture was heated at 60° for 5 min. After chilling, 4 vol of acetone was added. The precipitate was collected by centrifugation and processed as described for fat cell protein. Radioactivity incorporated in 5 min of [^{32}P]protein (counts per minute per microgram of protein): (A) fasted, 111; (B) refed, 126. Radioactivity (counts per minute) placed on the gel: (A) 2020; (B) 2320. Sample electrophoresis: 18 hr at 60 V. These results were duplicated on three separate fasted and refed preparations. Several new bands appeared in this concentrated assay and have been designated by letters. The physiological and electrophoretic behavior of the band labeled 140,000 was identical with that of band 2 (IPP 140) in whole cell preparations. (c) Autoradiogram (insulin vs. control). Fat pads from five fed rats were divided into two equal groups, each incubated in 15 ml of mod-KRB for 20 min; one group was also treated with insulin (100 μU/ml), and the other served as the control. Control group (360 μg of protein) and insulin-treated group (360 μg of protein) S_{175} supernatants (N = 3) were incubated in protein kinase assays with the indicated additions: control, sub-groups: (A) no additions; (B) $CaCl_2$ (5×10^{-5} M); (C) cyclic AMP (10^{-7} M); insulin treated, sub-groups: (D) no additions; (E) $CaCl_2$ (5×10^{-5} M); (F) cyclic AMP (10^{-7} M). Radioactivity: ^{32}P incorporated (counts per minute per microgram of protein) in 5 min: (A) 219; (B) 198; (C) 185; (D) 140; (E) 113; (F) 141. Radioactivity (counts per minute) placed on the gel: (A) 4600; (B) 4850; (C) 5070; (D) 4950; (E) 4580; (F) 5140.

180 g) rats were fasted for 36 hr; half were then refed for 12 hr while the remainder (controls) continued to fast. In each case (four experiments) the rate of endogenous IPP 140 phosphorylation after incubation with $^{32}P_i$ was greater in fat cells obtained from refed rats than in those obtained from starved animals (Figure 6). In refed animals (compared to fasted) the increased phosphorylation of region 5 (EPP 60–65) is consistent with an increased endogenous rate of fat cell lipolysis (Jungas and Ball, 1963). Furthermore, IPP 140 phosphorylation was increased by the addition of insulin to the fasted cells (not shown in this figure), proving that these cells were capable of responding to insulin.

(*F*) *Protein Kinase Assay.* To demonstrate either an increase in protein kinase activity or a change in specific protein phosphorylation, a high-speed, concentrated supernatant was prepared from fat cells of both fasted and refed rats and used in a typical protein kinase assay with [γ-^{32}P]ATP (Benjamin and Goodman, 1969; Walsh et al., 1968; Wray et al., 1973; Erlichman et al., 1974). The total protein kinase activities of starved and refed animals were the same (Figure 7a). However, an increase in the specific phosphorylation of a protein–polypeptide with a similar electrophoretic mobility to IPP 140 was observed in the S_{175} fraction from the refed animals (Figure 7b), with no alteration in the stained protein profiles. Cyclic AMP (10^{-7} M) did not increase the phosphorylation of IPP 140 (not shown), and mixing samples from refed and fasted animals did not prevent IPP 140 phosphorylation. These results indicate that neither an inhibitor of IPP 140 phosphorylation nor an increase in the activity of a specific phosphatase for IPP 140 is present. Furthermore, incubation of similar samples with [γ-^{32}P]GTP demonstrated that the rate of protein phosphorylation was only about 10% of the value obtained using ATP; the pattern of protein labeling with [γ-^{32}P]GTP mimicked that of [γ-^{32}P]ATP in the supernatants from both fasted and refed rats. Extensive GTP–ATP interconversion may be present (Kuo, 1974).

Since insulin addition to fat cells or fat pads increased IPP 140 phosphorylation, the S_{175} fractions from the insulin-treated and control fat pads were used as both the receptor protein and protein kinase containing fractions. IPP 140 phosphorylation was specifically increased in the samples from tissue which had been treated with insulin (100 μU/ml for 20 min, N = 3). In addition, while cyclic AMP (10^{-7} M) had no effect on specific protein phosphorylation, $CaCl_2$ (5×10^{-5} M) addition markedly inhibited IPP 140 phosphorylation (Figure 7c).

Discussion

Our studies of fat cell protein phosphorylation have demonstrated (a) that insulin induces the phosphorylation of IPP 140 both in vivo and in vitro; (b) that this phosphorylation is most likely mediated by a mechanism separate from the adenylate cyclase–cyclic AMP system; (c) that insulin and epinephrine have reciprocal actions on different protein phosphorylations; (d) that epinephrine's actions are duplicated by dibutyryl cyclic AMP; (e) that one likely immediate intracellular phosphate donor to IPP 140 is ATP; and (f) that the IPP 140 phosphorylation patterns were the same in preparations of both whole cells and supernatant extracts in a reconstituted protein kinase assay. Since insu-

lin does not selectively increase the synthesis of any proteins-polypeptides which migrate with IPP 140 on gel electrophoresis (Figure 2), the insulin-induced increase in IPP 140 phosphorylation may be due to an insulin-induced stimulation of either a specific protein kinase for IPP 140 phosphorylation, or a specific IPP 140 phosphatase.

Other possible interpretations include insulin-induced alterations in: (a) phosphate or ATP metabolism; (b) cyclic nucleotide levels; and (c) nucleotide interconversion. Each of these possibilities seems unlikely.

(a) Since insulin increased IPP 140 phosphorylation (Figure 5) during a cold chase with added phosphate ion, insulin-induced phosphorylation of IPP 140 is not likely to be due either to insulin changing the rate of influx of radiolabeled molecules from the extracellular fluid or to insulin selectively labeling the ATP pool with ^{32}P.

(b) Since insulin increased IPP 140 phosphorylation both with prior and with concurrent stimulation (dibutyryl cyclic AMP or epinephrine) of cyclic AMP dependent protein kinase (Figure 5), and under conditions of suppressed adenylate cyclase activity (Figures 4 and 5), changes in cyclic nucleotide levels do not appear to be a likely interpretation.

(c) Since insulin increased IPP 140 phosphorylation over a wide range of incubation times (2–120 min), nucleotide interconversion seems unlikely. If insulin increased the specific activity of an immediate phosphate donor for IPP 140, the labeling of an immediate precursor molecule for IPP 140 phosphorylation should have increased with time, even in the absence of insulin, but such was not observed. Moreover, the reproduction of the results from whole cell preparations in a reconstituted subcellular protein kinase assay system cannot be explained by a hypothetical change in a fixed (experimentally) [γ-^{32}P]ATP pool.

Since in many systems lithium is thought to act by inhibiting epinephrine and other hormone-activated adenylate cyclases (Dousa and Hechter, 1970; Singer and Rotenberg, 1973), it is not surprising that lithium inhibits the phosphorylation of EPP 60–65 by submaximal amounts of epinephrine, or that lithium does not block the phosphorylation of EPP 60–65 by dibutyryl cyclic AMP. The facts that lithium does not inhibit IPP 140 phosphorylation and that both submaximal amounts of epinephrine and dibutyryl cyclic AMP have little or no effect on IPP 140 phosphorylation strongly suggest that the phosphorylation of IPP 140 is not mediated by the adenylate cyclase–cyclic AMP system.

Insulin and epinephrine are well known physiological antagonists both in vivo and in vitro. Our results confirm this antagonism both for EPP 60–65 (Figure 3a)[3] and for IPP 50.[4] The turnover rate of the IPP 50 phosphate groups is not very different from that of the other fat cell phosphoproteins, whereas that of IPP 140 is very much more rapid. These interrelationships demonstrate that insulin's actions may eventually result in the phosphorylation of a protein (IPP 140 or IPP 50) or the dephosphorylation of a protein (EPP 60–65). Similarly, epinephrine's actions through the adenylate cyclase system may result in protein phosphorylation (EPP 60–65) or dephosphorylation (IPP 50). Finally, insulin may stimulate the phosphorylation of a protein which is affected by the adenylate cyclase system (IPP 50) or a protein which is not related to the adenylate cyclase system (IPP 140). Although many hormone alterations take place in the fasting–refeeding experiments, our studies of endogenous IPP 140 and EPP 60–65 phosphorylation in fasted and refed fat cell samples (Figure 6) demonstrate that the rates of phosphorylation vary in a predictable way with physiology.

The in vitro demonstration of a specific hormone–substrate phosphorylation is only a tentative indication that the system functions in normal in vivo hormone regulation. The insulin-induced IPP 140 phosphorylation meets all of the criteria that can be met in order to establish a relationship between a specific protein phosphorylation and a particular hormone effect (Krebs, 1973), without knowing the protein's function in vivo. If the concentration of IPP 140 is 0.01 to 0.1% of the total fat cell protein (estimated from stained protein patterns), then the stoichiometry in our subcellular system approaches 1 mol of phosphate from ATP to 1 mol of IPP 140.[5] This suggests that IPP 140 may be a specific substrate for hormone-mediated protein phosphorylation.

Much is known about the activation and regulation of the cyclic AMP dependent protein kinase system by cyclic AMP (e.g., Walsh and Ashby, 1973; Langan, 1973; Walsh and Krebs, 1973), but little is known about the activation and regulation of the putative insulin-dependent protein kinase system. Although we have shown that there is an insulin-activated protein kinase and a phosphate receptor protein (IPP 140) in the cytosol, they could both be weakly bound membrane components. The mechanism by which insulin interaction at the cell membrane affects such a soluble protein kinase is unknown: insulin may act either (a) on a "second messenger" system which is different from but analogous to the cyclic AMP system (Rasmussen, 1970; McMahon, 1974) or (b) on a membrane catalytic system which activates a membrane-bound, insulin-specific protein kinase in equilibrium with the protein kinase in the cytosol. The rapid phosphorylation and dephosphorylation of IPP 140 and its separation from the cyclic AMP dependent system are consistent with IPP 140 being either (a) an insulin-sensitive protein kinase (phosphotransferase) or (b) a protein whose function is regulated by an insulin-sensitive protein kinase.

Although many details of the fat cell protein phosphorylations are incomplete, our findings support the hypothesis that many hormone responses are mediated by specifically controlled protein kinases, which phosphorylate unique substrates (Larner and Sanger, 1965; Langan, 1969) and which may or may not be cyclic AMP mediated. Thus, precise hormonal controls of specific protein kinase activity

[3] EPP 60–65 may be a subunit of hormone-activated fat cell lipase because (a) fat cell lipase has a similar molecular weight (Egelrud and Olivecrona, 1972; Bensadoun et al., 1974) and (b) lipase activity may depend on enzyme phosphorylation, which may be regulated, in part, by a cyclic AMP dependent protein kinase (Butcher et al., 1968; Manganiello et al., 1971; Huttunen and Steinberg, 1971).

[4] Insulin and epinephrine have reciprocal effects on IPP 50 phosphorylation (unpublished observations). In contrast to IPP 140, the phosphorylation of IPP 50 by insulin is antagonized by both epinephrine and dibutyryl cyclic AMP, suggesting that the adenylate cyclase–cyclic AMP system is important in the control of IPP 50 phosphorylation. The phosphorylation of IPP 50 differs from that of EPP 60–65 in that the roles of insulin and epinephrine on the phosphorylation are reversed: insulin increases while epinephrine decreases IPP 50 phosphorylation.

[5] Calculation: 700 μg (of S_{175} concentrate) $\times$ 0.05% (mid-range relative protein concentration) = 0.35 μg (IPP 140), which is about 2.5 pmol of IPP 140. Since approximately 20–40% of the radioactivity in the protein kinase assay is associated with IPP 140 phosphorylation, about 1 pmol of phosphorus from ATP in the assay was transferred to 2.5 pmol of protein.

and of specific substrate phosphorylation may be common mechanisms in the regulation of cellular enzyme activity.

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