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The effects of glucagon, phenylephrine and insulin on the phosphorylation of cytoplasmic, mitochondrial and membrane-bound proteins of intact liver cells from starved rats

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1. The effects of glucagon, insulin and phenylephrine on the phosphorylation of cytoplasmic, mitochondrial and membrane proteins were studied in intact hepatocytes from 24h-starved rats incubated with [^{32}P]P_i. A rapid cell-fractionation technique was used, followed by radioautography of the proteins separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. 2. Glucagon consistently caused a significant increase in the phosphorylation of four readily separable cytoplasmic phosphoproteins, of M_r 93000, 50000, 46000 and 20000, and a decrease in phosphorylation of a phosphoprotein of M_r 22000. Phosphorylation of the protein of M_r 46000 was also enhanced by both phenylephrine and insulin, and that of M_r 93000 by phenylephrine. 3. The phosphoprotein of M_r 22000 was not precipitated by boiling for 5min, and had a mobility identical with that of similar protein whose phosphorylation is enhanced in the adipocyte by insulin [Belsham & Denton (1980) *Biochem. Soc. Trans.* **8**, 382–383]. 4. Glucagon, but not phenylephrine or insulin, enhanced the phosphorylation of a mitochondrial protein of M_r 35000 and of four plasma- or microsomal-membrane proteins of M_r 50000, 30000, 23000 and 19000. 5. Mitochondria from glucagon-treated animals or hepatocytes phosphorylated a protein of M_r 30000 when incubated *in vitro* with [^{32}P]P_i and ADP. Phosphorylation of this protein did not occur with mitochondria from control, phenylephrine- or insulin-treated cells. 6. The significance of these hormonally induced changes in protein phosphorylation is discussed.

Protein phosphorylation and dephosphorylation is a major mechanism by which hormones may regulate intracellular processes (Rosen & Krebs, 1981). The isolated cell preparation is an ideal system for the study of such hormonal effects on protein phosphorylation, and both the adipocyte (Benjamin & Singer, 1975; Avruch *et al.*, 1976; Belsham *et al.*, 1980*a*,*b*; Hughes *et al.*, 1980) and hepatocyte (Avruch *et al.*, 1978; Garrison, 1978; Garrison *et al.*, 1979; Siess & Wieland, 1979) have been used in this way. For the hepatocyte the studies have been largely restricted to cytosolic proteins (Avruch *et al.*, 1978; Garrison, 1978; Garrison *et al.*, 1979). However, studies on isolated liver plasma membranes (Marchmont & Houslay, 1980) and subcellular fractions of adipocytes (Avruch *et al.*, 1976; Belsham *et al.*, 1980*a*,*b*) suggest that it would be of great interest to investigate the effects of hormones on the phosphorylation of membrane and mitochondrial fractions of the hepatocyte. In particular it would seem important to establish whether phosphorylation of membrane proteins might be responsible for the numerous effects of glucagon and phenylephrine on liver mitochondrial function (see Halestrap *et al.*, 1980; Halestrap, 1981, 1982; Armston *et al.*, 1982) and on microsomal calcium uptake (Bygrave & Tranter, 1978; Waltenbaugh & Friedmann, 1978; Taylor *et al.*, 1979). In the present paper we demonstrate effects of glucagon on the state of phosphorylation of a number of cytosolic, membrane and mitochondrial proteins, whereas phenylephrine and insulin appeared to affect only cytosolic proteins. The data provide no obvious solution to the mechanism by which hormones influence mitochondrial metabolism.

Abbreviations used: SDS, sodium dodecyl sulphate; Mops, 4-morpholinepropanesulphonic acid.

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Experimental

Materials

Male albino Wistar rats (300–400g body wt.) were starved for 24h with free access to water before being used for the preparation of liver cells.

All chemicals, biochemicals and radiochemicals, unless otherwise stated, were as given elsewhere (Hughes & Halestrap, 1981; Thomas & Halestrap, 1981). Percoll was from Pharmacia Fine Chemicals (London). Monospecific antiserum to acetyl-CoA carboxylase (EC 6.4.1.2) was prepared as described by Walker *et al.* (1976) and was a gift from Dr. R. J. Mayer (Department of Biochemistry, University Hospital and Medical School, Nottingham, U.K.). Protein inhibitors were from the Peptide Research Institute, Osaka, Japan.

Preparation and incubation of liver cells

Rat liver parenchymal cells were prepared by the method of Berry & Friend (1969) as modified previously (Mendes-Mourão *et al.*, 1975*a*,*b*), by using collagenase and not hyaluronidase. After washing, the cells were suspended in bicarbonate-buffered saline medium, pH7.4 (Krebs & Henseleit, 1932), at a final concentration of 15mg of protein/ml, measured by the biuret reaction. Albumin (20mg/ml) was added, and the suspension was gassed briefly with O_2/CO_2 (19:1) before dilution into the incubation buffer. For protein-phosphorylation experiments 1ml of cell suspension (7.5mg of protein/ml) was incubated in low-phosphate (0.5mM) bicarbonate-buffered saline solution, containing 10mM-L-lactate and 2mM-pyruvate, in plastic vials at 37°C. At the start of the incubation 100μCi of [^{32}P]P_i/ml was added. In most experiments, after 60min hormones were added and incubation was continued for a further 10min.

Cellular fractionation

Cells were disrupted by a modification of the method of Gellerfors & Nelson (1979) described previously (Vargas, 1982). At the end of the incubation, the cell suspension was transferred into a 1.5ml centrifuge tube and centrifuged for 1s in an Eppendorf Centrifuge (5414A). The supernatant was discarded, and 1ml of buffer A (10mM-Tris/HCl, 250mM-sucrose, 5mM-EDTA, 20mM-KF, pH7.4) was added, and the cells were disrupted by sonication for 2 × 3s with the microprobe of a Branson sonicator (Dawe Instruments; Type 7532B) set at minimun power. After centrifugation at 10000*g* for 1min, the supernatant was frozen with liquid N_2 and kept at −20°C. The pellet, composed essentially of mitochondria contaminated with plasma membranes, was further purified by centrifugation in a self-forming Percoll density gradient (Belsham *et al.*, 1980*a*). Then 1ml of buffer A containing 22% (v/v) Percoll was added, and after 5min of centrifugation at 10000*g*, the purified mitochondria were collected from the bottom of the tube as described by Belsham *et al.* (1980*a*), washed again in buffer A to remove the contaminating Percoll, and dissolved in SDS-sample buffer [125mM-Tris/HCl, 20% (w/v) sucrose, 5% (w/v) 2-mercaptoethanol, 0.02% Bromophenol Blue and 7% (w/v) SDS, pH7.4] by heating at 100°C for 5min. The frozen supernatant was thawed and then centrifuged in an air-driven ultracentrifuge (Beckman Airfuge) at an air pressure of 30lb/in^2 (200kPa) for 10min, which allowed the separation of the microsomal and plasma membranes from the cytosol. The membranes were resuspended in 0.15ml of buffer A, resedimented by centrifugation in the Airfuge and dissolved in the SDS-sample buffer, described above, and the cytosolic proteins were precipitated with 10% (w/v) trichloroacetic acid and dissolved in SDS-sample buffer after washing once with 1ml of water.

Preparation and incubation of mitochondria from rat liver

Mitochondria from rat liver were obtained from control and glucagon-treated rats as described previously (Halestrap, 1975, 1978). To investigate their ability to phosphorylate proteins, mitochondria were incubated at 37°C at a concentration of 2–4mg of protein/ml in buffer B (125mM-KCl, 7mM-Tris, 10mM-Mops, 2mM-EGTA, pH7.4), to which was added 0.25mM-potassium phosphate, 5mM-succinate, 2.5mM-$MgCl_2$, 0.1mM-ADP and 100μCi of [^{32}P]P_i/ml. After 10min the incubation was terminated either by addition of trichloroacetic acid (10%, w/v, final concn.) or by centrifugation at 10000*g* for 30s in an Eppendorf 5414A centrifuge. The mitochondrial pellet or precipitated protein was immediately dissolved in SDS-sample buffer, and the supernatant proteins from the centrifugation were precipitated with trichloroacetic acid (10%, w/v, final concn.) before dissolving in SDS-sample buffer as outlined above.

Separation and detection of [^{32}P]phosphoproteins

All protein samples dissolved in SDS-sample buffer were heated at 100°C for 5min. Separation of proteins was performed by using discontinuous SDS/polyacrylamide-gel electrophoresis in 1cm tracks on 10% (w/v)-acrylamide slab gels as described previously (Belsham *et al.*, 1980*a*; Hughes & Halestrap, 1981). Electrophoresis was at 20°C for 3–4h at 30mA/slab. After electrophoresis, the gel was soaked for 1h in 20% (w/v) trichloroacetic acid plus 50% (v/v) methanol to precipitate proteins in the gels before proteins were stained with Coomassie Brilliant Blue. The gels were then laid on Cellophane and dried on boards under vacuum. Radioauto-

graphy of the gels was performed as described previously (Hughes & Halestrap, 1981), with Kodak Kodirex KT X-ray film for 2–7 days. Radioautographs were scanned at 625 nm with a Gilford densitometer linked to a Hewlett–Packard 9845S computer. Exposure of the film was such that the maximal peak absorbance did not exceed 1.0. Relative peak areas were estimated by using the graphics facility of the computer.

Results

The technique described in the Experimental section for cell fractionation of hepatocytes exposed to [^{32}P]P_i allowed preparation of cytoplasmic, membrane and mitochondrial fractions containing phosphorylated proteins within 15 min of cell disruption. Densitometric scans of radioautographs showing phosphorylated cytosolic, membrane and mitochondrial proteins separated by SDS/polyacrylamide-gel electrophoresis are shown in Figs. 1(*a*)–1(*c*). Results for cells exposed to glucagon (0.1 μM) are also included, and several changes in the phosphorylation pattern can be seen. In some experiments samples of cytosolic, membrane and mitochondrial proteins from glucagon-treated hepatocytes were run on the same slab gel to allow direct assessment of the contamination of one fraction by another. The scans indicated very little cross-contamination, a result expected from previous enzyme-marker experiments (Vargas, 1982). In all phosphorylation experiments cells were incubated with [^{32}P]P_i for 1 h before hormone addition, since it was established in preliminary experiments that protein phosphorylation with ^{32}P had reached equilibrium by this time. It was also established that the cells maintained good rates of gluconeogenesis from L-lactate after this period of incubation (4–6 nmol of glucose/min per mg of cell protein) and that glucagon and phenylephrine caused at least a 30% stimulation of this rate.

Phosphorylation of cytosolic protein

The effects of glucagon (0.1 μM), phenylephrine (20 μM), insulin (0.1 μM) and dibutyryl cyclic AMP (20 μM) on the phosphorylation of cytosolic protein are summarized in Table 1. Fig. 1(*a*) shows that glucagon induced increases in the phosphorylation of proteins of apparent M_r 123000, 93000, 84000, 50000, 46000, 41000, 39000 and 20000 and a decrease in the phosphorylation of a protein of M_r 22000. Results are only given in Table 1 for proteins of apparent M_r 93000, 50000, 46000, 22000 and 20000, since these were the proteins whose changes were most easily resolved on the gels on a regular basis. Only the phosphorylation of the protein of M_r 46000 was also increased consistently with both phenylephrine and insulin, and the protein

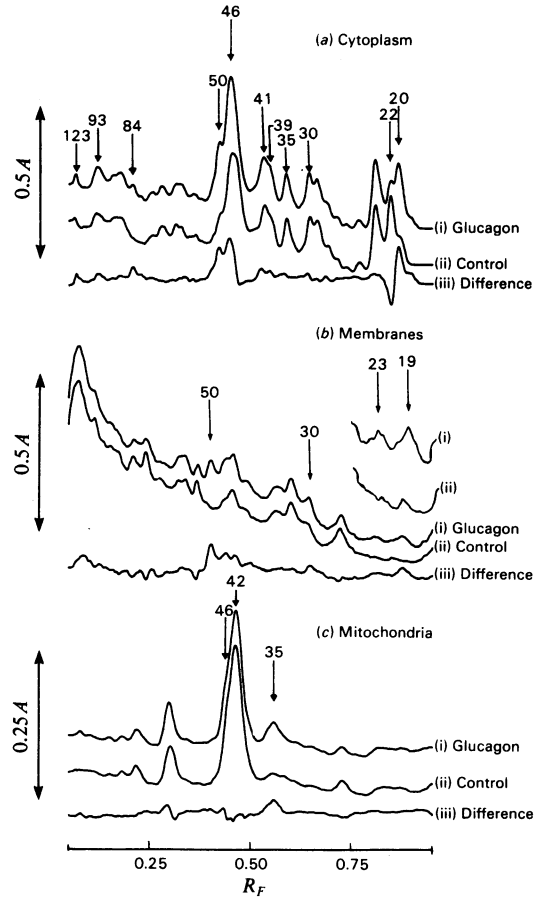

Fig. 1. *Densitometric scans of radioautograms showing the effects of glucagon on the phosphorylation of cytoplasmic membrane and mitochondrial proteins*

Rat liver cells were incubated with continuous shaking in Krebs/bicarbonate buffer containing [^{32}P]phosphate for 1 h before addition of hormone. After a further 10 min the cells were rapidly broken by sonication, fractionated, and samples were prepared for electrophoresis on 10%-polyacrylamide slab gels as described in the Experimental section. The loading of each track was carefully matched for protein concentration, and was equivalent to 0.2 mg of original liver-cell protein for the supernatant fraction and approx. 5 times this amount for membrane and mitochondrial fractions. Typical scans (at 625 nm) of radioautographs from gels are shown for cytoplasmic (*a*), membrane (*b*) and mitochondrial (*c*) fractions from control (ii) and glucagon (0.1 μM)-treated (i) cells. Difference scans (glucagon − control) are shown in (iii). In (*b*) the insert shows the last portion of the scan with the sensitivity amplified 5-fold. The M_r values of peaks ($\times 10^{-3}$), estimated by using standard molecular-weight markers (Hughes & Halestrap, 1981), are indicated. Further details are given in the Experimental section.

Table 1. *Effects of glucagon, phenylephrine, insulin and dibutyryl cyclic AMP on the phosphorylation state of selected cytoplasmic phosphoproteins*

Hepatocytes from starved rats were incubated with [^{32}P]P_i and hormones before rapid subcellular fractionation and preparation of protein samples for SDS/polyacrylamide-gel electrophoresis as described in the Experimental section. The extent of phosphorylation of proteins was calculated from the peak areas of the scanned radioautograms. Results are expressed as the means ± S.E.M. for five experiments on separate cell preparations; statistical significance was calculated by using a paired Student's *t* test: *$P < 0.001$; **$P < 0.025$.

	Phosphorylation of protein from hormone-treated cells (% of control)			
M_r of phosphoprotein	Glucagon (0.1 μM)	Dibutyryl cyclic AMP (20 μM)	Phenylephrine (20 μM)	Insulin (0.1 μM)
93000	398 ± 37*	176 ± 14*	139 ± 9*	132 ± 15
50000	356 ± 50*	245 ± 26*	140 ± 27	121 ± 21
46000	197 ± 15*	197 ± 23*	159 ± 13*	155 ± 7*
22000	43 ± 8*	60 ± 7*	98 ± 6	95 ± 8
20000	175 ± 9*	174 ± 25**	128 ± 12	120 ± 31

of M_r 93000 also showed some enhancement of phosphorylation after phenylephrine treatment. The results shown in Fig. 1(*a*) and Table 1 are similar to the results of others (Avruch *et al.*, 1978; Garrison, 1978; Garrison *et al.*, 1979), and discussion of the possible identity of these peaks is included in those papers and will not be repeated here.

However, one observation would appear to be unusual, the decrease in phosphorylation of a protein of M_r 22000. We are aware of only one other report of glucagon causing a decrease in phosphorylation of a protein of similar molecular weight (Knowles, 1981). The inability of most previous workers to detect this change (Avruch *et al.*, 1978; Garrison, 1978; Garrison *et al.*, 1979; Siess & Wieland, 1979) may reflect the different SDS/polyacrylamide-gel-electrophoresis conditions used in their experiments, where more emphasis was placed on higher-molecular-weight proteins, and resolution of proteins of M_r less than 25000 was not attempted. We have used 5% polyacrylamide gels to look at such proteins of higher subunit molecular weight, but have not detected any consistent hormonally induced changes in the phosphorylation state of bands which might correspond to acetyl-CoA carboxylase and ATP citrate lyase as detected by others (Witters *et al.*, 1979; Alexander *et al.*, 1979; Janski *et al.*, 1979; Witters, 1981). However, the best-documented effects of glucagon on the phosphorylation of these enzymes has come from studies using starved/re-fed rats, and not from starved rats as used in the present studies and those of Garrison (1978). For acetyl-CoA carboxylase we have used a specific antibody to the enzyme to immunoprecipitate any enzyme-bound ^{32}P (Brownsey *et al.*, 1979), but have still been unable to detect significant changes in the overall phosphorylation state of the enzyme at a variety of hormone concentrations and times of exposure to hormones.

With glucagon detailed time courses and dose–response curves were performed (results not shown), and the observed changes in phosphorylation of the proteins shown in Table 1 were essentially the same as those of phosphoproteins of similar subunit molecular weight reported by Avruch *et al.* (1978). Insulin (0.1 μM) was only capable of reversing the phosphorylation induced by glucagon at the lowest effective concentrations of this hormone (1 nM), and, in agreement with Avruch *et al.* (1978), no additional phosphorylated proteins were apparent, as might be predicted from the studies *in vitro* by Marchmont & Houslay (1980).

The decrease in the phosphorylation of a protein of M_r 22000 induced by glucagon was unexpected and was of particular interest in view of the increase in phosphorylation of a protein of the same molecular weight induced by insulin in the adipocyte (Belsham *et al.*, 1980*a*,*b*, 1982; Belsham & Denton, 1980; Blackshear *et al.*, 1982). The adipocyte phosphoprotein is unusual in that it is soluble in 2% (w/v) trichloroacetic acid and not precipitated by boiling for 5 min. In Fig. 2 we demonstrate that the phosphoprotein from the hepatocyte also remains in solution on boiling and has the same mobility as the adipocyte phosphoprotein on SDS/polyacrylamide-gel electrophoresis. This suggests that the two phosphoproteins may be very similar. Furthermore, in both cases coincident with changes in the phosphorylation of the protein of M_r 22000, reciprocal changes occurred in the phosphorylation of a protein of M_r 20000, which also remained in solution after boiling (see also Belsham *et al.*, 1982). However, two observations suggest that the protein of M_r 22000 from liver and fat-cells may not be identical. Firstly, the liver phosphoprotein was largely, although not completely, precipitated by 2% (w/v) trichloroacetic acid. Secondly, in the adipocyte adrenaline increases the phosphorylation of this

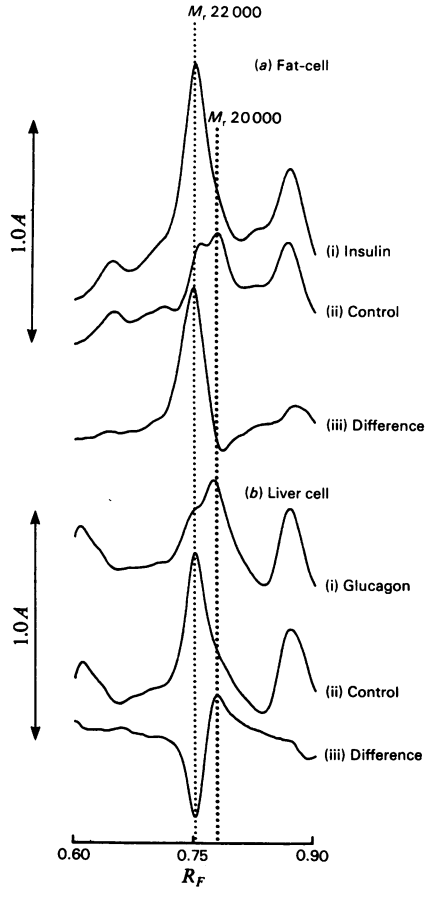

Fig. 2. *Densitometric scans showing the effects of hormones on the phosphorylation of proteins of M_r 22000 and 20000 in boiled cytoplasmic extracts of liver and fat-cells*

Cytoplasmic proteins were derived from fat-cells as described by Belsham *et al.* (1980*a*, 1982) and from liver cells as described in the Experimental section after incubation with [^{32}P]phosphate. The extracts were heated for 5min at 100°C in the presence of 0.5% trichloroacetic acid, the denatured protein was sedimented by centrifugation (1000*g*-min) and the supernatant proteins were recovered by precipitation with trichloroacetic acid (15%, w/v). The proteins were then separated by SDS/polyacrylamide-gel electrophoresis and the derived radioautograph was scanned. Portions of the scans are shown for control (ii) and insulin (10nM)-treated (i) fat-cells in (*a*) and control (ii) and glucagon (0.1μM)-treated (i) liver cells in (*b*). Difference scans of (i)−(ii) are shown in (iii) in each case. The experiment shown is typical of three such experiments.

protein (Belsham & Denton, 1980) whereas in the hepatocyte glucagon, dibutyryl cyclic AMP and phenylephrine cause a decrease in phosphorylation (Table 1).

Phosphorylation of membrane proteins

The data of Fig. 1(*b*) indicate that glucagon enhances the phosphorylation of four membrane-bound proteins of M_r 50000, 30000, 23000 and 19000. In six experiments on different cell preparations the mean increase in phosphorylation of the protein of M_r 30000 was 148±6% (±S.E.M.; $P<0.001$ by paired t test). The other three phosphoproteins were only visible after glucagon treatment, and so changes could not be expressed in percentage terms. Neither phenylephrine nor insulin (in the presence or absence of glucagon) had any effect on the phosphorylation of these proteins or any other phosphoproteins. Comparison of the mobilities of these phosphoproteins with phosphoproteins found in the cytoplasmic and mitochondrial fractions run on the same gel confirmed that the identities of the four phosphoproteins affected by glucagon treatment were different from any cytosolic or mitochondrial phosphoproteins. It should be noted that no attempt was made to separate plasma and microsomal membranes in these experiments.

Phosphorylation of mitochondrial proteins

Previous studies on the phosphorylation of mitochondrial proteins in the rat liver or hepatocyte have not demonstrated effects of hormones on the phosphorylation of specific proteins (Zahlten *et al.*, 1972; Siess & Wieland, 1979). However, the data in Fig. 1(*c*) demonstrate that of several mitochondrial proteins phosphorylated in the intact hepatocyte one minor band of M_r 35000 showed increased phosphorylation with glucagon. This protein was unlikely to be a cytoplasmic protein contaminating the mitochondrial fraction, since after Percoll-density-gradient centrifugation less than 3% of the lactate dehydrogenase was present in the mitochondrial pellet, and this distribution was not affected by glucagon treatment. In addition, no phosphoprotein of M_r 35000 that changed with glucagon treatment was present in the cytoplasm. In 11 experiments on separate batches of cells the stimulation in the phosphorylation of this mitochondrial protein induced by glucagon (mean ± S.E.M.) was 264±34%. No effect of either phenylephrine or insulin was seen on the phosphorylation of this protein. The major phosphoproteins of the liver mitochondria were of M_r 42000 and 46000 (appearing as a shoulder on the M_r-42000 peak in most gels) and correspond to the decarboxylase subunits of pyruvate dehydrogenase and 2-oxo acid dehydrogenase complexes respectively (Hughes & Halestrap, 1981). No significant changes in the phosphorylation of these phosphoproteins were observed with any hormone treatment. However, these proteins are intramitochondrial and are therefore exposed to conditions allowing phosphorylation and dephosphoryla-

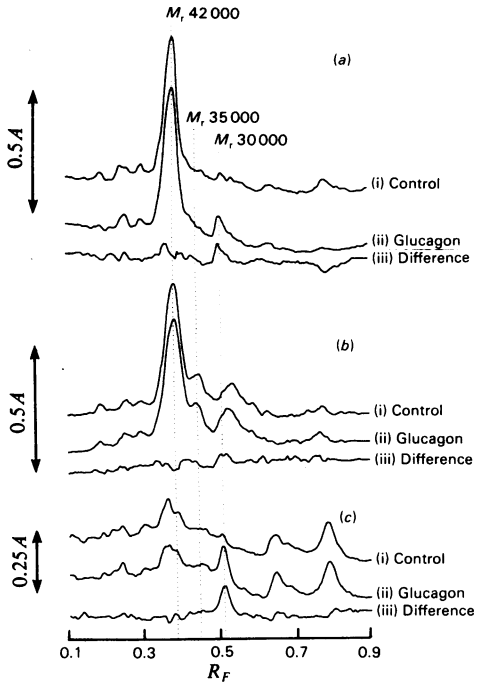

Fig. 3. *Effect of glucagon pretreatment of rats on the phosphorylation of liver mitochondrial proteins in vitro*

Mitochondria from control or glucagon-treated rats were incubated in KCl medium containing [^{32}P]-phosphate (0.25mM), ADP (0.1mM) and succinate (5mM) for 10min at 37°C. Mitochondria were either precipitated with 10% (w/v) trichloroacetic acid (*a*) or sedimented by centrifugation (*b*), and the remaining supernatant proteins were precipitated with 10% (w/v) trichloroacetic acid (*c*) before separation of proteins by SDS/polyacrylamide-gel electrophoresis. Densitometric scans are shown for mitochondria from control (i) and glucagon-treated (ii) animals, and the difference scans (ii)–(i) are shown in (iii). The data given are typical of six such experiments. Further details are given in the Experimental section.

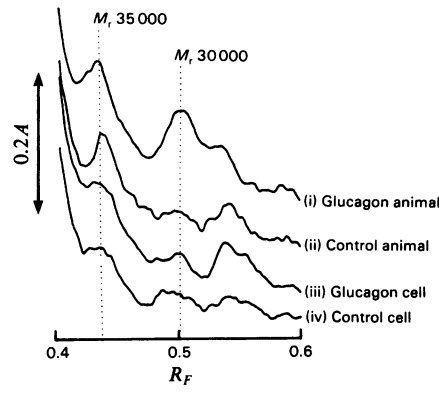

Fig. 4. *Effect of glucagon treatment of rat liver cells on the phosphorylation in vitro of a mitochondrial protein of M_r 30000*

Mitochondria were prepared from hepatocytes by sonication after incubation of the cells for 1h without hormone and then a further 10min in the presence or absence of glucagon (0.1μM). Mitochondria were incubated in KCl medium containing [^{32}P]phosphate (0.25mM), ADP (0.1mM) and succinate (0.5mM) for 10min; the protein was then precipitated with 10% (w/v) trichloroacetic acid and proteins were separated by SDS/polyacrylamide-gel electrophoresis. For comparison, densitometric scans of radioautographs are shown for liver mitochondria from control (ii) and glucagon-treated (i) animals incubated as described in the legend to Fig. 3 as well as for control (iv) and glucagon-treated (iii) cells. Only the relevant portion of each scan is shown to allow the use of an expanded absorbance scale. The experiment shown is typical of three such experiments.

tion during the preparation of the mitochondrial fraction.

In Figs. 3 and 4 data are shown on the phosphorylation of isolated mitochondria incubated with [γ-^{32}P]ATP. This ATP was synthesized *in situ* by the mitochondria, from ADP and [^{32}P]P_i participating in oxidative phosphorylation, and complete labelling was achieved within 15s of mitochondrial addition. No differences in the specific radioactivity of the [γ-^{32}P]ATP formed by control or glucagon-treated mitochondria were observed. Several phosphoproteins were apparent and again, as expected from previous studies (Hughes & Halestrap, 1981), the most prominent were peaks corresponding to pyruvate dehydrogenase and 2-oxo acid dehydrogenase. Whether derived from whole livers (Fig. 3) or hepatocytes (Fig. 4), a protein of M_r 30000 showed phosphorylation only when the animals or cells were pretreated with glucagon. Phenylephrine treatment was ineffective. This protein was quite distinct from the phosphoproteins of M_r 35000 whose phosphorylation was enhanced by glucagon in the intact cell. Nor did it appear to be derived from the M_r-35000 protein by proteolysis, since the presence of a variety of proteinase inhibitors such as pepstatin, antipain and leupeptin at 20μg/ml during preparation and incubation of the mitochondria did not alter the mobility of the phosphoprotein on polyacrylamide-gel electrophoresis. The nature of this M_r-30000 phosphoprotein was further investigated in the experiments shown in Fig. 3. After incubation of mitochondria with [γ-^{32}P]ATP, the mitochondria were rapidly sedimented by centrifugation and both the supernatant and mitochondrial pellet subjected to SDS/polyacrylamide-gel elec-

trophoresis. The phosphoprotein of M_r 30000 was found in the supernatant, whereas, as expected, the major intramitochondrial phosphorylated proteins pyruvate dehydrogenase and 2-oxo acid dehydrogenase (Hughes & Halestrap, 1981) were associated with the mitochondrial pellet. Thus the M_r-30000 phosphoprotein appears to be loosely attached to the mitochondria and easily dissociated from the outer membrane in KCl medium. If the experiment was performed in sucrose medium, the phosphoprotein remained attached to the mitochondria after centrifugation (results not shown). The phosphoprotein of M_r 35000 observed in mitochondria from hepatocytes was not lost from the mitochondria if they were washed in KCl medium (results not shown). Some of the phosphoproteins washed off the mitochondria derived from whole livers may be plasma and microsomal contaminants. However, for the M_r-30000 protein this seems unlikely, since the same band was found in mitochondria from hepatocytes subjected to purification with Percoll (Fig. 4). No phosphorylation of a protein of M_r 30000 was apparent in control mitochondria even with addition of large quantities of purified catalytic subunit of cyclic AMP-dependent protein kinase.

Discussion

The data presented in Fig. 1(*a*) and Table 1 confirm and extend the results of others on the effects of glucagon, phenylephrine and insulin on the phosphorylation of cytoplasmic proteins of the hepatocyte (Avruch *et al.*, 1978; Garrison, 1978; Garrison *et al.*, 1979; Knowles, 1981). The present experiments were performed on hepatocytes from 24h-starved rats, as were those of Garrison and coworkers, rather than on starved/re-fed rats as used by Avruch *et al.* (1978) and in studies on the effects of hormones on the phosphorylation state of lipogenic enzymes (Witters *et al.*, 1979; Alexander *et al.*, 1979; Janski *et al.*, 1979; Witters, 1981). A distinct feature of the results reported here is that they demonstrate that glucagon increases phosphorylation of a protein of M_r 20000 and decreases the phosphorylation of a protein of M_r 22000. These two phosphoproteins exhibit the unusual property of remaining in solution after boiling for 5min. It is possible that glucagon does not actually decrease the phosphorylation of the protein of M_r 22000, but may cause its migration to another cellular location. We have been unable to find any direct evidence for this. Alternatively glucagon could enhance lysosomal proteolytic activity (Hopgood *et al.*, 1980; Ballard, 1980) and cause selective degradation of this protein. This might be responsible for the increase in phosphorylation of the M_r-20000 protein induced by glucagon treatment (Fig. 1*a*; Table 1). Fat-cells also contain two phosphoproteins of M_r 22000 and 20000, which are not precipitated by boiling (see Belsham *et al.*, 1980*b*, and Fig. 2). Moreover, insulin increases the phosphorylation of the former while decreasing that of the latter. These reciprocal changes are reversed by subsequent addition of anti-insulin serum (Belsham *et al.*, 1982). However, the relationship between these proteins in liver and fat-cells has not been established. It should be noted that the fat-cell proteins are soluble in 2% (w/v) trichloroacetic acid, whereas those from the liver are not. Furthermore, both adrenaline and phenylephrine result in increased phosphorylation of the protein of M_r 22000 in fat-cells (Belsham *et al.*, 1980*b*, 1982; G. J. Belsham & R. M. Denton, unpublished work), whereas phenylephrine and glucagon cause a decrease in phosphorylation of this protein in liver cells (Table 1).

The stimulation by glucagon of the phosphorylation of four plasma membrane or microsomal membrane proteins of M_r 50000, 30000, 23000 and 19000 has not been reported previously, although other workers have performed related experiments. Blat & Loeb (1971) have reported that glucagon treatment of the whole rat enhanced the phosphorylation of a liver ribosomal protein of unspecified molecular weight. It has also been shown that isolated liver plasma membranes (Marchmont & Houslay, 1980; Kobayashi & Ozawa, 1981) or microsomal membranes (Behar-Bannelier & Murray, 1980) incubated with [γ-^{32}P]ATP and Mg^{2+} show many phosphorylated proteins on SDS/polyacrylamide-gel electrophoresis. Two of the plasma-membrane phosphoproteins, of M_r 23000 and 20000, show enhanced phosphorylation in the presence of 100mM-phosphate (Kobayashi & Ozawa, 1981). It is possible that these correspond to the phosphoproteins of M_r 23000 and 19000 seen in the present experiments. Marchmont & Houslay (1980) have reported that two integral proteins of the plasma membrane of M_r 140000 and 80000 are phosphorylated in a cyclic AMP-stimulated manner, whereas peripheral proteins of M_r 52000, 28000 and 14000 are only phosphorylated in the presence of both cyclic AMP and insulin. Our data fail to demonstrate changes in the phosphorylation of any phosphoproteins that might correspond to these integral or peripheral membrane proteins occurring in the intact cell. However, at the specific radioactivity of ^{32}P used in the present experiments and the low concentration of these proteins present in plasma membranes (Houslay & Marchmont, 1981), we cannot be certain whether or not we would expect to detect such changes.

In skeletal muscle two proteins involved in the transport of Ca^{2+} across sarcolemmal and sarcoplasmic-reticulum membranes may be phosphorylated and activated in response to adrenergic stimula-

tion (Katz, 1979; LePeuch *et al.*, 1980; Rinaldi *et al.*, 1981). The sarcoplasmic-reticulum protein phospholamban has a subunit of M_r 11000 which often runs as a dimer of M_r 22000 on gels (LePeuch *et al.*, 1980), whereas the plasmalemmal protein has an apparent M_r of 23000. Thus the phosphoproteins of M_r 23000 seen in the present experiments may well be one of these two proteins and may account for the increase in the Ruthenium-Red-insensitive transport of Ca^{2+} into liver microsomal preparations from glucagon-treated rats (Bygrave & Tranter, 1978; Waltenbaugh & Friedmann, 1978; Taylor *et al.*, 1979).

The stimulation of the phosphorylation of a mitochondrial protein of M_r 35000 by glucagon may be related to the numerous effects of this hormone on liver mitochondrial function (Halestrap *et al.*, 1980; Halestrap, 1981, 1982; Armston *et al.*, 1982). However, α-adrenergic agents such as phenylephrine cause similar changes in mitochondrial function without affecting the phosphorylation of this protein. We (Halestrap, 1981, 1982; Armston *et al.*, 1982) and others (Siess *et al.*, 1981) have suggested that changes in the mitochondrial phospholipids may account for such changes in mitochondrial function. It is known that α-adrenergic agents, but not glucagon, induce enhanced phosphatidylinositol turnover in the mitochondrial fraction of hepatocytes (Kirk & Michell, 1981), and this may be related to any change in membrane disposition caused by these hormones. The phosphoprotein of M_r 35000 might be responsible for the parallel change in membrane disposition caused by glucagon.

Incubation of mitochondria *in vitro* with extramitochondrial [γ-^{32}P]ATP caused phosphorylation of a protein of M_r 30000 only in liver mitochondria from glucagon-treated animals or cells (Figs. 3 and 4). This protein was lost from the mitochondria into the medium during incubation in KCl- but not in sucrose-based media (Fig. 3). There would appear to be many parallels between the proteins of M_r 35000 and 30000 phosphorylated *in situ* and *in vitro* respectively. We cannot explain any relationship between these two proteins other than that a proteinase may be active during incubations of mitochondria *in vitro* which is insensitive to the proteinase inhibitors used.

Note Added in Proof (Received 12 July 1982)

Since submitting this paper it has come to our attention that Garrison *et al.* (1981) have also observed that glucagon causes the dephosphorylation of a M_r-22000 cytoplasmic protein.

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