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Effect of Corticotropin Treatment *in vivo* on the Synthesis of a Specific Adrenal Cytosolic Protein

CHARACTERIZATION BY DUAL-LABELLING TECHNIQUE AND POLYACRYLAMIDE-GEL ELECTROPHORESIS

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The effect of corticotropin *in vivo* on total and specific protein synthesis in the adrenal was studied. Adrenal slices from control and corticotropin-treated animals were incubated with [^{14}C]- and [^{3}H]-leucine respectively, followed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of subcellular components. With this sensitive dual-labelling technique the following results were obtained. There was a general trophic effect on most adrenal proteins, but corticotropin produced a marked stimulation of a specific adrenal cytosolic protein. This protein had mol.wt. approx. 30000 and pI 5.5. Corticotropin increased the incorporation of labelled leucine into proteins within 4h, but no effect was observed before 2h and after 16h there was no further increase. These data suggest that this protein is not involved in the corticosteroidogenic action of corticotropin, but rather in the trophic action of this hormone.

It has been shown that corticotropin *in vivo* stimulates adrenal protein synthesis (Farese & Reddy, 1963; Bransome & Reddy, 1964). Whether or not this stimulation affects all the adrenal proteins or only specific proteins still remains unclear. In cultured mouse adrenocortical tumour cells (Y_1 clonal strain) corticotropin is able to increase some cytosolic proteins specifically, but in a transient way (Grower & Bransome, 1970). In the same cell line the biosynthesis of adrenodoxin (a non-haem-iron mitochondrial protein) is increased by corticotropin in the absence of any stimulation of mitochondrial protein synthesis (Kowal *et al.*, 1970; Asano & Harding, 1976). In this tumour cell line we also described in the presence of corticotropin a specific stimulation of synthesis of a small peptide (mol.wt. 3500) (Dazord *et al.*, 1977*a*) whose localization is microsomal (A. Dazord, D. Gallet & J. M. Saez, unpublished work).

However, data from experiments *in vivo* mainly deal with total protein synthesis. The involvement of a rapidly turning over protein mediating the steroidogenic action of corticotropin was postulated several years ago (Garren *et al.*, 1971; Schulster *et al.*, 1974). Nevertheless, the identity of such a protein is still unknown. By using perfused cat adrenal glands, Rubin *et al.* (1974) and Laychock & Rubin (1974) have shown that corticotropin increases the release of two proteins (mol.wt. 12500 and 70000 respectively), but the subcellular localization of these proteins was not determined.

The purpose of the present study was to investigate whether corticotropin injected *in vivo* was able to modify some specific adrenal proteins or whether its stimulatory effects concerned all the proteins synthesized in the adrenal glands. We have limited our study to microsomal and cytosolic proteins.

Materials and Methods

Chemicals

The corticotropin used in these studies was Synacthen [corticotropin-(1–24)-tetracosapeptide]. Synacthene Immediat and Synacthene Retard (tetracosapeptide–zinc) were purchased from CIBA, Rueil Malmaison, France.

L-[4-^{3}H]Leucine (sp. radioactivity 25–35 Ci/mmol) and L-[α-^{14}C]leucine (sp. radioactivity 40–55 mCi/mmol) were purchased from Commissariat à l'Energie Atomique, Gif-sur-Yvette, France. Acrylamide and *NN'*-methylenebisacrylamide of electrophoresis purity were from Polysciences Inc., Warrington, PA, U.S.A.

Animals

Male Sprague–Dawley rats, aged 2–3 months, were used in all experiments (0.2–0.3 kg body wt.). The rats were housed at 22±2°C on a 12h light/12h dark cycle. They were injected intramuscularly simultaneously with 20 μg of Synacthene Retard and

20 μg of Synacthene Immediat and killed by cervical dislocation at various times after the injection.

Radioactive leucine incorporation into adrenal slices

After removal, rat adrenal glands were carefully freed from adherent fat, placed in a vial containing 0.9% NaCl at 4°C and then cut into almost equal quarters with Castroviejo ultramicro-scissors (Robot Surgical Instrument Co., Washington, DC, U.S.A.).

The adrenal quarters of corticotropin-injected and control animals were then incubated in a metabolic shaker in 1ml of Hanks medium (Hanks, 1948) supplemented with 2mg of glucose/ml, 4.25mg of Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/ml, 0.35mg of $NaHCO_3$/ml, pH 7.6. Before the incubation, the tubes were flushed for 5s with a mixture of O_2/CO_2 (19:1), then hermetically closed and shaken at 37°C for 2h in the presence of [^{3}H]leucine (200 μCi/ml) or [^{14}C]leucine (20 μCi/ml).

In the dual-labelling experiments, incubation mixtures containing [^{3}H]leucine were supplemented with the appropriate amount of non-radioactive leucine to give the same leucine concentration as used with [^{14}C]leucine. Each incubation contained the adrenals of three rats and for each experimental point two incubations were carried out. Each experiment was done at least three times.

Isolation of subcellular components

At the end of the incubation period the tubes were put in ice, rinsed twice with 2mM-Tris/HCl, pH 7.4, containing 5mM-leucine, homogenized (Polytron PT_{10}; Kinematica G.m.b.H., Luzern, Switzerland) three times every 5s at speed 5 in 3.5ml of 25mM-Tris/HCl/2mM-$MgCl_2$/0.25M-sucrose, pH 7.4. Cell debris and nuclei were removed by two low-speed centrifugations (800 g, 10min, 4°C). The resultant supernatant was also centrifuged twice (10000 g, 30 min, 4°C) to remove mitochondria. The postmitochondrial fraction was then centrifuged twice (Beckman SW 50 rotor) first for 2h then for 30min at 130000 g. Both 130000 g (referred to as microsomal preparation) pellets were pooled, rinsed in the same buffer, centrifuged once (130000 g, 30min, 4°C) and resuspended in 25mM-Tris/HCl/2mM-EDTA, pH 7.4, containing 1% (w/v) sodium dodecyl sulphate, and dialysed for 2h against the same buffer containing 0.1% (w/v) sodium dodecyl sulphate. The supernatant of the first 130000 g centrifugation (cytosolic preparation) was dialysed twice for 2h against 2 litres of 25mM-Tris/HCl, 5mM-leucine, pH 7.4, then against 2 litres of 2mM-Tris/HCl, pH 7.4, to eliminate the excess of non-incorporated radioactive leucine. The cytosolic preparations were then freeze-dried and resuspended in 1ml of 25mM-Tris/HCl, pH 7.4, containing 0.1% (w/v) sodium dodecyl sulphate.

Quantitative analysis of protein synthesis

Samples (50 μl) of the homogenate were used to measure incorporation of [^{3}H]- or [^{14}C]-leucine into trichloroacetic acid-insoluble proteins as previously described (Dazord *et al.*, 1977*a*).

Sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis

Electrophoresis was carried out as previously described (Dazord *et al.*, 1977*a*). Gel electrophoresis was carried out at pH 7.2 in gels containing 15% or 11% (w/v) acrylamide, 0.1% (w/v) *NN'*-methylenebisacrylamide, in system J 3561 as described by Neville & Glossman (1974).

The upper-reservoir buffer always contained 0.1% (w/v) sodium dodecyl sulphate. After electrophoresis, the gels were cut into 2 or 1mm slices with an Aliquogel fractionator (Gilson, Villiers-le-Bel, France). Each slice was dissolved in 0.5ml of H_2O_2 for 2h at 50°C and counted for radioactivity in 5ml of scintillation fluid [8.25g of Scintimix 1 (Koch–Light, Colnbrook, Bucks., U.K.), 0.5 litre of Triton X-100, 1 litre of toluene]. For dual-labelling technique, the dissolved gel slices were counted for radioactivity in Picofluor TM_{30} (Packard Instrument Co., Rungis, France) in a SL30 liquid-scintillation spectrometer (Intertechnique, Plaisir, France). The counting efficiency was 25% for ^{3}H and 50% for ^{14}C.

Flat-bed electrofocusing

The ampholine electrofocusing kit 2117-101 (LKB-Produkter, Bromma, Sweden) and the LKB 2117-301 multiphor basic unit were used in analytical thin-layer polyacrylamide. The LKB Ampholine PAGplates (pH 3.5–9.5) (1804-101) contained 5% (w/v) acrylamide with a cross-linkage degree of 3%. Electrophoresis was carried out according to the manufacturer's instructions at 6°C. After the formation of the pH gradient, the electrophoretic focusing was completed in about 60min. Plates were then cut into slices (15mm × 2mm), dissolved in 0.5ml of H_2O_2 and counted for radioactivity as above. Control slices of the same plate were dissolved in water and the pH was measured.

Plasma corticosterone

Corticosterone was measured by radioimmunoassay as described by Saez *et al.* (1977).

Protein assay

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results

Electrophoretic profile of adrenal cytosolic and microsomal proteins of control and corticotropin-treated rats: single-labelling experiments

The responsiveness of rat adrenals to corticotropin *in vivo* was controlled by measuring the plasma corticosterone values, which usually show a 4–5-fold increase. The incorporation of [^{3}H]leucine *in vitro* into trichloroacetic acid-insoluble proteins from adrenals *in vivo* treated with corticotropin is always significantly stimulated (according to different experiments: 1.5–3-fold compared with controls) (see below, Table 1). Fig. 1 shows the incorporation of [^{3}H]-leucine into different cytosolic (Fig. 1*a*) and microsomal (Fig. 1*b*) proteins as analysed by sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis. There are several peaks of cytosolic proteins both in basal and in corticotropin-stimulated conditions. The peak of R_F 1 is composed mainly of free radioactive leucine, which could not be completely eliminated by dialysis. All the peaks of protein are increased to almost the same extent (about a 2-fold increase) after corticotropin treatment, except for peak R_F 0.74. In 10 experiments, the rate of incorporation of [^{3}H]leucine into this peak compared with controls was always more pronounced than in the other cytosolic proteins from corticotropin-treated adrenals. On the other hand four peaks of protein were clearly marked in the microsomal fractions, but after corticotropin treatment the proportional increase was not significantly different.

Dual-labelling experiments

Adrenal slices from corticotropin-treated animals were incubated with [^{3}H]leucine, whereas those from control animals were incubated with either [^{3}H]- or [^{14}C]-leucine. When the samples were ready for electrophoresis the cytosolic proteins labelled with [^{3}H]leucine (from control or corticotropin-treated animals) were mixed with the same amount of cytosolic proteins from adrenals of control animals that had previously been incubated with [^{14}C]leucine. As expected (Fig. 2), corticotropin produces an overall increase of the ^{3}H/^{14}C ratio in each slice. When 11% (w/v) acrylamide gels were used, a much greater increase in the 0.76 relative mobility area was observed. There was also an increase at the dye front, which was also noted in control conditions. This increase reflects the fact that we used ten times more [^{3}H]leucine than [^{14}C]leucine, and that non-incorporated [^{3}H]leucine is not completely eliminated by dialysis. To avoid the proximity of the 0.76 relative mobility area and of the free leucine, further electrophoresis was performed in 15% (w/v) polyacrylamide gels (Fig. 2). In this system the specific protein that showed increased synthesis had R_F about 0.60 (Fig. 2*a*).

Conversely, corticotropin did not stimulate syn-

Table 1. *Time course of the stimulating effects of corticotropin on plasma corticosterone, total adrenal protein synthesis and specific synthesis of protein E*

Following the time intervals after corticotropin injection (single injection) and 4h after the last injection (repeated injections) the animals were killed and the adrenal slices incubated either with [^{3}H]leucine (corticotropin and controls) or with [^{14}C]leucine (controls only) as indicated in the Materials and Methods section. Each experimental point was determined twice (^{3}H/^{14}C ratio is the mean of two assays). The values given for protein E are the top of the peak. For the rest of the gel the mean ^{3}H/^{14}C ratio was calculated for several gel slices above and below the stimulated area. Other values were determined for each point three times (six determinations): values are means ± S.D.

Time intervals after corticotropin injection (single injection) (h)	Corticosterone (ng/ml of plasma)	[^{3}H]Leucine incorporation into trichloroacetic acid-precipitable proteins of adrenal homogenates (c.p.m./μg of protein)	^{3}H/^{14}C ratio	
			Remaining gel	Protein E
0	74± 8 (6)	440± 80 (6)	1.1	1.2
2	221±17 (6)	590± 40 (6)	1.1	1.4
4	280±20 (6)	1028±100 (6)	2	3.8
8	317±25 (6)	1000±140 (6)	1.8	2.6
16	120±10 (6)	800±100 (6)	1.6	1.8
Time intervals after corticotropin injections (repeated injections every 12h) (h)				
0	80±10 (6)	360±40 (6)	1.3	1.3
4	373±50 (6)	600±60 (6)	1.9	4.4
16; 4	435±60 (6)	600±50 (6)	1.9	4.4
28; 16; 4	579±72 (6)	580±36 (6)	1.9	3.4

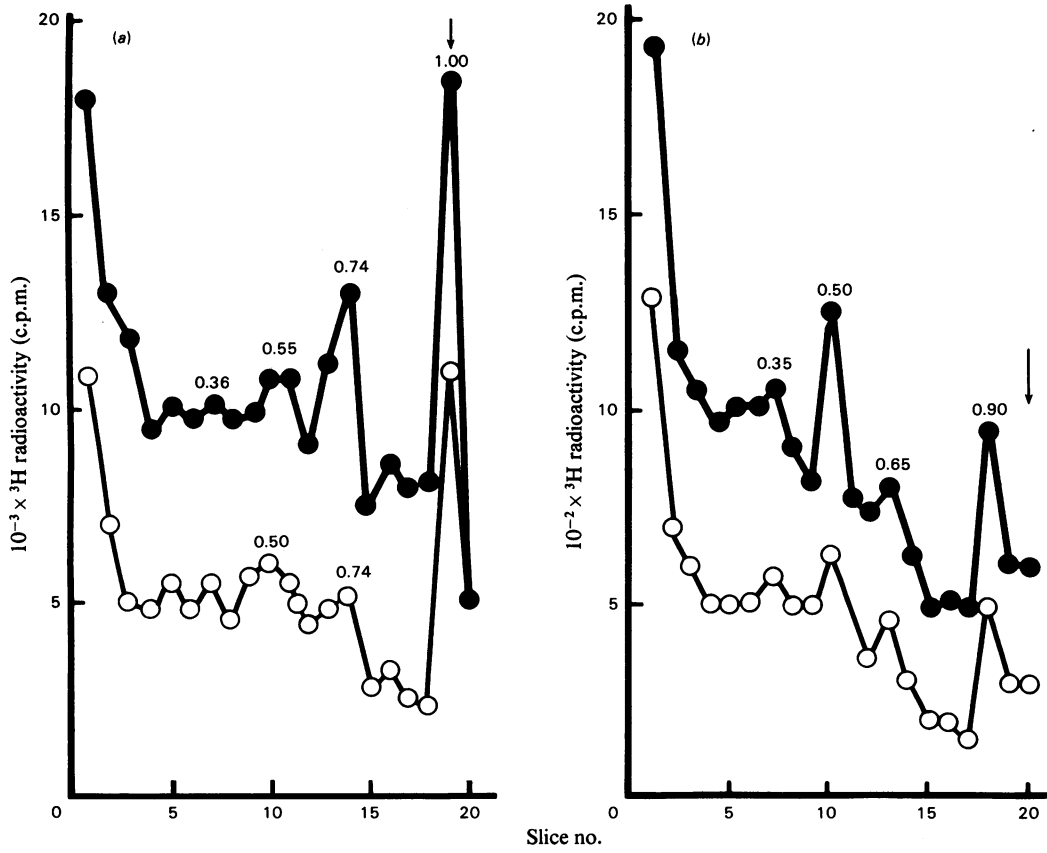

Fig. 1. *Electrophoretic profile of adrenal cytosolic (a) and microsomal (b) proteins from controls* (○) *and corticotropin-treated rats* (●)

Rats were injected with 20μg of Synacthene Immediat and 20μg of Synacthene Retard, and killed 4h later. Adrenal slices from controls and treated animals were incubated for 2h in Hanks medium in the presence of [^{3}H]leucine (200μCi/ml). Subcellular fractionation was carried out as described in the Materials and Methods section. Cytosolic and microsomal proteins (100μg of each) were subjected to 0.1% (w/v) sodium dodecyl sulphate/11% (w/v) polyacrylamide-gel electrophoresis. The gels were cut into 2mm slices, and the radioactivity was counted. The R_F values of the main peaks are indicated. The arrow marks the position of the dye on each gel.

thesis of any specific microsomal protein, although the total protein synthesis was stimulated by the hormone (Fig. 2*b*).

Characteristics of the corticotropin-induced radioactive protein

The nature of the cytosolic corticotropin-stimulated peak of protein was demonstrated by experiments with Pronase. Incubation in the same test tube of cytosols from control ([^{14}C]leucine) and corticotropin-treated rats ([^{3}H]leucine) in the presence of Pronase (100μg/ml) resulted in the disappearance of most ^{3}H- and ^{14}C-labelled proteins and specially the peak of R_F 0.60, with a parallel dramatic increase of radioactivity in the area of free leucine (results not shown). Fig. 3 shows the results obtained when the ^{3}H/^{14}C ratio was calculated.

In the same experiment we showed that alkaline hydrolysis (90min incubation of the cytosolic proteins in the presence of 0.3M-NaOH at 37°C) did not affect either the ^{3}H content of cytosolic protein or the ^{3}H/^{14}C ratio (results not shown). Thus it is unlikely that this corticotropin-stimulated peak of material is a Leu-tRNA^{Leu}.

All the data so far mentioned were from labelling experiments. Fig. 4 shows the protein pattern of the gels after fixation and staining with Coomassie Blue. Stained gels were cut manually into 2mm slices and the ^{3}H/^{14}C ratio was determined to see the exact correspondence of the stained bands with the highest ^{3}H/^{14}C ratio observed after corticotropin stimulation.

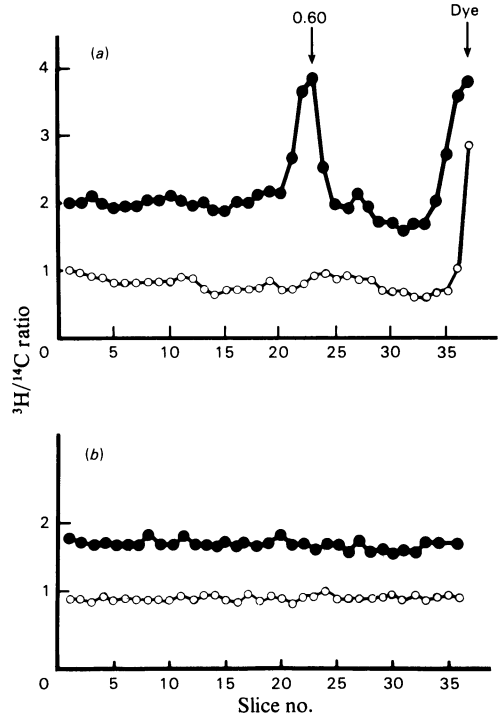

Fig. 2. *$^3H/^{14}C$ ratios of the labelled proteins from controls (○) and corticotropin-treated rats (●) in adrenal cytosolic (a) and microsomal (b) fractions*

Adrenals from controls were incubated separately with either [^{3}H]- or [^{14}C]-leucine. Cytosolic and microsomal proteins were prepared as described in the Materials and Methods section. The same amount of proteins (100μg) labelled with [^{3}H]- or [^{14}C]-leucine were co-electrophoresed in 15% (w/v) polyacrylamide gels in the presence of 0.1% (w/v) sodium dodecyl sulphate. Adrenals from corticotropin-treated rats were incubated with [^{3}H]leucine, and then cytosolic and microsomal proteins were co-electrophoresed with those of controls whose incubations had been carried out with [^{14}C]leucine. The gels were cut into 1mm slices, and the $^3H/^{14}C$ ratio was determined in each slice.

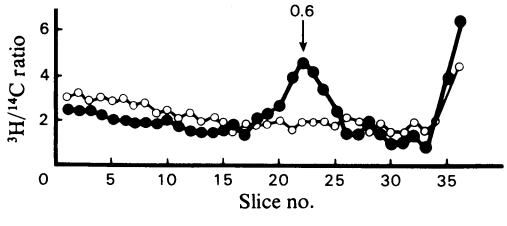

Fig. 3. *Effect of Pronase on the profile of labelled cytosolic adrenal proteins*

Corticotropin-treated cytosolic proteins (100μl; 100μg of protein) labelled with [^{3}H]leucine were incubated with 100μl of control cytosolic proteins labelled with [^{14}C]leucine and 20μl of Pronase (5mg/ml) at 37°C for 30min and then subjected to 15% (w/v) polyacrylamide gels in the presence of 0.1% (w/v) sodium dodecyl sulphate (●). Control gels (○) (no Pronase treatment of the cytosolic proteins) were run simultaneously. The gels were cut into 1mm slices and the $^3H/^{14}C$ ratio was determined.

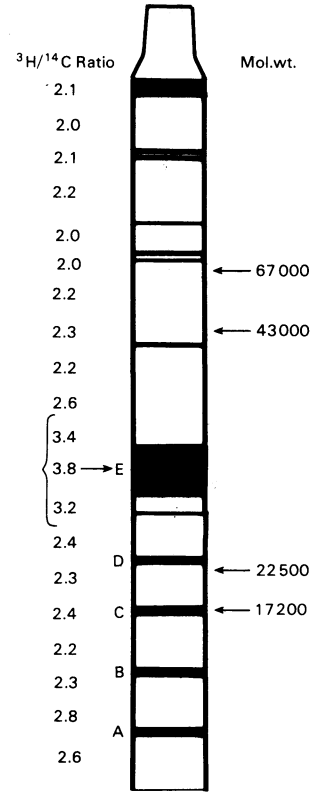

Fig. 4. *Protein pattern of adrenal cytosolic proteins*

Corticotropin-treated cytosolic proteins (100μg) labelled with [^{3}H]leucine were co-electrophoresed with the same amount of control cytosolic proteins labelled with [^{14}C]leucine. The gels (15%, w/v, polyacrylamide) were stained with Coomassie Blue: the gels were incubated in the staining solution (80mg of Coomassie Blue, 14ml of acetic acid, 75ml of methanol, 65ml of water) for 12h and then destained in a solution containing 0.2 litre of methanol, 0.28 litre of acetic acid, 3.25 litres of water. Coloured gels were cut manually and the $^3H/^{14}C$ ratio was determined (left-hand-side of the gel). The mobilities of known standard molecular weight are indicated on the right-hand-side of the gel.

This highest value was consistently found in a rather broad band called band E (whose R_F was between 0.50 and 0.60 and mol.wt. approx. 30000). The molecular weight was estimated by comparison with the mobilities of known molecular-weight standards: bovine serum albumin (67000), ovalbumin (43000), placental lactogen (22500) and myoglobin (17200).

To determine the pI of the protein E, we tried to purify it by preparative gel electrophoresis. We used the same system as described in the Materials and Methods section, but the gel tubes had a 14mm diameter (instead of 0.6mm). The gels were sliced, and from each slice a small piece was cut to calculate the $^3H/^{14}C$ ratio. The protein from the remainder of the slice with the highest $^3H/^{14}C$ value was used for further electrophoresis in exactly the same system, except that dialysis tubing was attached at the end of the gel tube. Electrophoresis was carried out long enough to collect the protein in the dialysis tubing. It was then extensively dialysed against water and freeze-dried.

In this way we were able to purify protein E, as confirmed by further analytical gel electrophoresis (Fig. 5). This pure preparation was used to check the pI of protein E by flat-bed electrofocusing on LKB PAGplates over a pH gradient from 3.5 to 9.5, as described in the Materials and Methods section. Fig. 6 shows that the pI of protein E is 5.5. In this system, the sodium dodecyl sulphate that was bound to the protein and was not eliminated by dialysis was clearly dissociated from the protein during the migration and precipitated as two bands at pH 3 and 3.6. Similar findings have been reported by others (Tuszynski *et al.*, 1977; Danno, 1977).

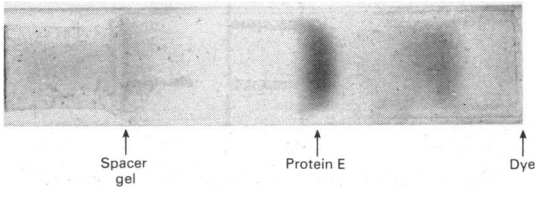

Fig. 5. *Polyacrylamide-gel electrophoresis of the purified protein E*

Cytosolic proteins (3 times the amount of that electrophoresed in Fig. 4) were electrophoresed in preparative gel tubes (14mm diameter, see the text) in the J 3561 gel system. The gels were sliced; a part of each slice was counted for radioactivity and protein from the slice with the highest $^3H/^{14}C$ value was further electrophoresed and eluted by the same preparative system (see the text). After dialysis and freeze-drying a sample of the eluate was electrophoresed in 15% (w/v) polyacrylamide gel and stained with Coomassie Blue.

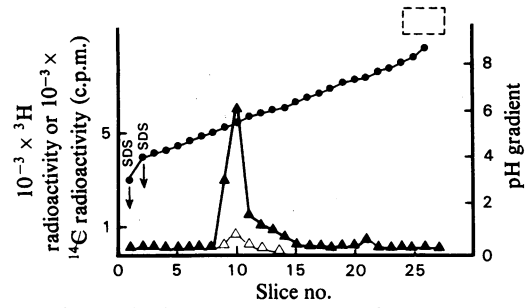

Fig. 6. *Flat-bed isofocusing of purified protein E*

Purified protein E (see Fig. 5) was subjected to flat-bed electrofocusing as described in the Materials and Methods section. Plates were then cut into thin slices to determine the amount of ^3H (▲) and ^{14}C (△) present and the pH (●). The sample application point is indicated by broken lines. Abbreviation: SDS, sodium dodecyl sulphate.

Time course of the corticotropin-stimulated synthesis of protein E (Table 1)

After a single injection of corticotropin [Synacthene Immediat (20 μg) plus Synacthene Retard (20 μg)] maximum stimulation of synthesis of protein E was observed after 4h. After 8h the stimulation, although obvious, diminished. For the first 2h and then after 16h, the stimulation if present is very small. It is noteworthy that in fact the time course of stimulation of this specific protein E is distinct from that of both corticosteroidogenesis and protein synthesis. On the other hand, repeated injections of corticotropin maintained this increased synthesis of protein E.

Discussion

Corticotropin action on the adrenal cortex is in fact multiple: stimulation of steroidogenesis and of RNA synthesis (Farese, 1968; Imrie *et al.*, 1965), stimulation of protein synthesis (Bransome & Reddy, 1964; Farese, 1971*a*,*b*), inhibition of protein degradation (Canick & Villee, 1974; Dazord *et al.*, 1977*b*) and stimulation of DNA synthesis (Farese & Reddy, 1963; Imrie *et al.*, 1965; Saez *et al.*, 1977).

As far as protein synthesis is concerned (and if one omits data obtained from the established mouse adrenal tumour-cell line Y_1) stimulatory action of corticotropin has been observed mainly *in vivo*. The administration *in vivo* of cycloheximide during corticotropin stimulation indicates that a specific protein with a short half-life may be essential for steroidogenesis (Garren *et al.*, 1971). However kinetic studies carried out with perfused isolated adrenal cells (Lowry & McMartin, 1974; Schulster

& Jenner, 1975) strongly suggest that corticotropin is not able to induce the synthesis of this protein, but rather exerts a post-translational activation.

Although corticotropin stimulates corticosterone production *in vitro*, we and likewise Ferguson *et al.* (1967), Halkerston *et al.* (1964), Morrow *et al.* (1967) and Farese & Prudente (1977) were unable to show stimulation by corticotropin *in vitro* of adrenal protein synthesis in slices from normal adrenals. Therefore to further elucidate the effects of corticotropin on adrenal protein synthesis we used a combined system *in vivo* and *in vitro*.

From our data it is obvious that corticotropin action *in vitro* on protein synthesis in normal rat adrenal glands actually involves two mechanisms, a general trophic effect that seems to affect most of the adrenal proteins and a specific stimulation of a cytosolic protein whose mol.wt. is approx. 30000 and pI 5.5. Our data (lack of effect up to 2h) suggest that this protein like the lutropin-induced protein (Janszen *et al.*, 1977; Janszen *et al.*, 1978) is not involved in the corticosteroidogenic action of corticotropin. It seems therefore reasonable to hypothesize that this protein could mediate the trophic effects of the hormone or merely be the expression of it. In this respect corticotropin administration *in vivo* has been shown to stimulate the enzymic adrenal activities of DNA polymerase and thymidine kinase (Masui & Garren, 1970), of adrenal RNA polymerase (Fuhrman & Gill, 1974) and of ornithine decarboxylase (EC 4.1.1.17) (Levine *et al.*, 1973, 1975). However, all these data only deal with enzymic activities, and not with synthetic rates. Further, these enzymic activities were measured independently of total protein synthesis, and therefore it is not really clear whether these aforementioned results are specific to corticotropin or only the expression of the general trophic action of this hormone.

To conclude, we have shown that corticotropin specifically stimulates the synthesis of a cytosolic adrenal protein that apparently does not seem to be involved in the acute steroidogenic action of the hormone. The exact nature and the role of this protein as well as the mechanism by which corticotropin exerts its stimulatory effect on this synthesis are unknown.

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