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Protein Synthesis in a Cell-free System from Rat Brain Sensitive to ACTH-like Peptides

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Abstract: Protein synthesis was measured using a cell-free system obtained from subcortical rat brain tissue. The concentrations of Mg^{2+} and K^+ and the amount of tissue, during both the preparation and the final assay, were critical to the incorporation of amino acids as expressed per milligram protein. Even under optimal conditions mainly elongation of growing peptide chains was measured. Behaviorally active fragments of ACTH modulated the activity of the system in a biphasic manner; i.e., at a low concentration (10^{-8} M) of ACTH a stimulation of between 10 and 70% was found; a high concentration (10^{-4} M) was inhibitory (50 to 70%). Structure-activity studies revealed that the stimulatory effect was confined to the N-terminus of the peptide (1–24), whereas the C-terminal sequence was responsible for the inhibition. The stimulation by $ACTH_{1-24}$ was dependent on Ca^{2+} and Mg^{2+}. Cyclic AMP (10^{-5} M) stimulated the amino acid incorporation too. When a similar cell-free extract was prepared from brain tissue of hypophysectomized rats, the lower *in vivo* protein synthesis in these animals was preserved in the present cell-free system. The data are discussed in terms of a possible direct intracellular effect of ACTH on brain protein synthesis. **Key words**: ACTH—Brain protein synthesis—Hypophysectomy.

NH_2-terminal sequences of adrenocorticotropic hormone (ACTH) are involved in central nervous functioning by a direct interaction with subcortical brain structures (de Wied and Gispen, 1977; Reith et al., 1977*a*). Among others, there are changes in brain RNA and protein synthesis (Schotman et al., 1976; Gispen and Schotman, 1976; Reith et al., 1977*b*), in cyclic nucleotide levels (Wiegant et al., 1979), and in the activity of a membrane-bound protein kinase (Zwiers et al., 1976; 1978; 1979) induced by ACTH. Structure-activity studies revealed that at the membrane and cyclic AMP level similar effects are brought about by the sequences 1–24, 1–16, or 5–18; smaller fragments were found to be inactive (Zwiers et al., 1978; Wiegant et al., 1979). In contrast, the changes in protein synthesis, both *in vivo* (Schotman et al., 1972) and in tissue slices (Reith et al., 1975), were also evoked by the smaller sequences of ACTH, 1–10 and 4–10; the latter are known to possess complete behavioral activity in several paradigms (de Wied and Gispen, 1977).

In view of this discrepancy in structure-activity relationship, the effects on protein synthesis at present do not seem to be mediated by cyclic AMP, as would follow from the classical second messenger concept of Sutherland (1972). Recently, however, it has been suggested that part of the action of peptide hormones may be exerted within the cell after internalization of the peptide (Goldfine, 1978; Kolata, 1978). Indeed, autoradiographic studies revealed intracellular accumulation of an ACTH analogue into subcortical neurons after i.c.v. administration of the radioactive peptide (Rees et al., 1980).

In the present study a cell-free system from brain

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is described in which the rate of amino acid incorporation is influenced by hypophysectomy and the presence of ACTH-like peptides in the incubation medium.

MATERIALS AND METHODS

Chemicals

All chemicals used were analytical grade. L-[U-^{14}C]-leucine (330 mCi/mmol and L-[U-^{14}C]phenylalanine (531 mCi/mmol were obtained from the Radiochemical Centre, Amersham, U.K., synthetic peptides from Organon-Int. B.V., Oss, The Netherlands, and creatine kinase (rabbit muscle) from Boehringer (Mannheim, GFR).

Preparation of Postmitochondrial Tissue Extract

Subcortical brain tissue was rapidly dissected from male Wistar rats of body weight 120–140 g (Gispen et al., 1972) and washed once with the ice-cold medium for homogenization. Hypophysectomized animals were of the same weight and were used 3 weeks after the transauricular surgery (Gispen et al., 1970). All following procedures were performed at 0–4°C using sterilized glassware to minimize nucleolytic and proteolytic activities. The homogenization medium consisted of 50 mM-Tris-HCl buffer, pH 7.6, 0.25 M-sucrose and KCl and $MgCl_2$. The tissue was mixed with medium and homogenized in a glass tube with a Teflon pestle (clearance 0.250 mm at 0°C), Potter-Elvehjem type, by 10 up-and-down strokes.

Optimum concentrations of Mg^{2+}, K^+ and of the tissue during homogenization were experimentally determined (see Results). The homogenate was centrifuged at 1000 *g* for 10 min, and the resulting supernatant at 20,000 *g* for 20 min (Sorvall SM-24 fixed-angle rotor). The supernatant was used as postmitochondrial extract for the cell-free incubations.

Cell-free Incubations

The incubation medium contained (final concentrations): 50 mM-Tris-HCl, pH 7.6, 6 mM-β-mercaptoethanol, 0.5 mM-ATP, 0.1 mM-GTP, 6.25 mM-creatine phosphate, creatine kinase (0.025 mg/ml), and 0.02 ml of the postmitochondrial tissue extract. As for the homogenization conditions, the optimum concentrations for K^+ and Mg^{2+} and tissue protein in the incubation mixture were experimentally determined (see Results).

After a preincubation at 37°C for 5 min under gyrotory shaking, 10 μl of the peptide solution in aquabidest., or aquabidest. alone, was added and the incorporation period was started 1 min thereafter by the addition of 1.00 μCi [^{14}C]leucine or [^{14}C]phenylalanine. Final incubation volume was 100 μl. At various times, aliquots of 20 μl were taken and mixed with 1 ml ice-cold 0.3 M-perchloric acid. To ensure quantitative precipitation of the proteins 0.05 ml bovine serum albumin (10 mg/ml) was added. The precipitate was centrifuged at 5000 *g* for 10 min, and the resulting pellet was washed with 0.3 M-perchloric acid containing 1 mM-leucine or phenylalanine respectively, at 0°C. Subsequently, the pellet was incubated with 0.5 M-perchloric acid at 90°C for 20 min. The hot residue was washed with 0.3 M cold perchloric acid followed by methanol-ether (1:1, v/v) and ether successively. This procedure removes radiolabelled free amino acid, aminoacyl-tRNA, and lipids (see Munro and Fleck, 1966). The resulting protein residue was thoroughly dried and solubilized by mixing with a tissue solubilizer (Soluene 350, Packard). After addition of a small amount of water to avoid chemoluminescence the solution was mixed with a scintillation cocktail on xylene base (Lipoluma, Lumac). Radioactivity was measured in a Berthold 8000 liquid scintillation spectrometer applying automated quench correction by the external standard ratio method. Mean efficiency for ^{14}C was 87%. The protein content of the postmitochondrial extract was measured according to Lowry et al. (1951), the RNA content according to Munro and Fleck (1966). The incorporation was expressed as d.p.m. per milligram of extracted protein.

Determination of the Specific Activity of [^{14}C]Leucine and [^{14}C]Phenylalanine

Aliquots of the cold, acid-soluble fraction of the incubation mixture were taken for the determination of the actual specific activity of [^{14}C]leucine or [^{14}C]phenylalanine during the incubation. In this case, the 0.3 M-perchloric acid did not contain added nonradioactive amino acids. Amino acids were separated using a Technicon TSMII amino acid analyser with one column (42 × 0.6 cm) filled with Durrum DC-6A resin. Elution was performed with 0.3 M-lithium citrate buffers containing 4% thiodiglycol and 0.3% Brij-35 at pH 3.30 for 100 min and at pH 4.15 for 50 min. This procedure gave a good resolution of the neutral amino acids. The amount of leucine was quantified using *o*-phthalaldehyde and β-mercaptoethanol, and fluorescence detection (Roth and Hampai, 1973). ^{14}C-labelled norleucine served as an internal standard. Fluorescence and radioactivity were determined in the same sample after stream-splitting.

RESULTS

Characterization of the Cell-free System for Amino Acid Incorporation

From preliminary data (not shown) and those reported by others (Liu et al., 1975; Campagnoni and Harris, 1977) it was clear that amino acid incorporation into proteins of a cell-free brain extract is highly dependent on Mg^{2+} and K^+ ions. In addition, in our hands, the optimum concentrations for Mg^{2+} and K^+ were different in the extraction medium as compared with those in the incubation medium. Fig. 1 shows that the highest activity was obtained with 2 mM-Mg^{2+} and 25 mM-K^+ in the added extraction medium. In the case of a 1:2 (w/v) ratio of tissue to medium this resulted in final concentrations in the extract of 3 mM-Mg^{2+} and 62 mM-K^+ using the values for the endogenous Mg^{2+} and K^+ concentrations of 95 and 5 μmol per g fresh tissue, as reported by Tower (1976).

The sensitivity of the assay-system to Mg^{2+} and

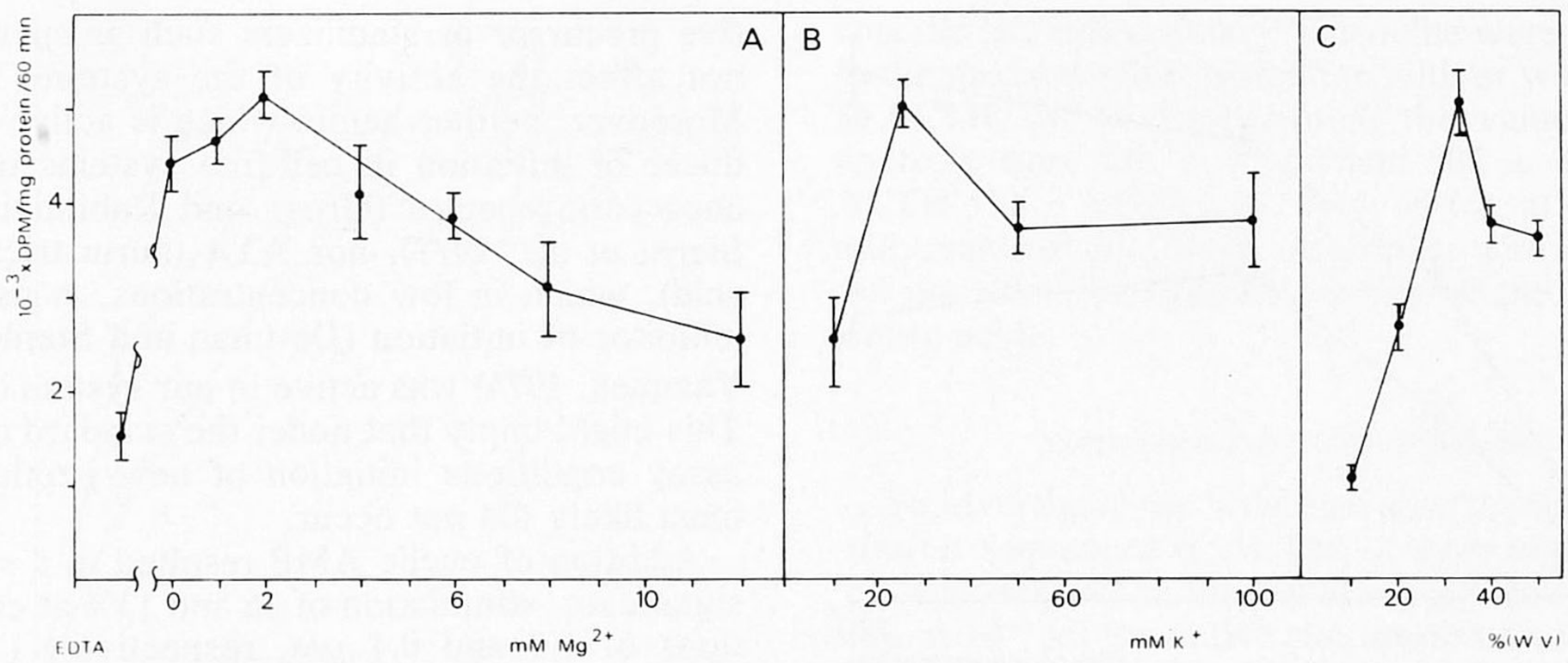

FIG. 1. The influence of Mg^{2+} **(A)**, K^+ **(B)** and of the tissue concentration (% of wet weight per total volume) during the extraction **(C)** on the activity of the resulting extract. Ion and tissue concentrations were varied in the extraction medium as indicated on the horizontal axes, one at a time, using the optimum concentrations for the other two conditions. For the final ion concentrations in the extract the implication of the endogenous tissue concentration has to be considered (see text). The activity of the extract for leucine incorporation is tested using the optimal conditions for incubation according to Fig. 2 (12 mM-Mg^{2+}, 100 mM-K^+, 120 μg protein) and 1 μCi [U-^{14}C]leucine. Bars represent the mean ± S.E.M. of three incubations.

K^+ is shown in Fig. 2, A and B. Highest leucine incorporation over 60 min was obtained at a concentration for Mg^{2+} of 12 mM and for K^+ between 70 and 200 mM (in further experiments 12 mM Mg^{2+} and 100 mM K^+ were used routinely). Thus, to obtain maximum activity in the incubation the Mg^{2+} concentration had to be raised considerably compared to that in the extract.

Fairly high tissue-to-medium ratios are commonly used in recently described procedures for the preparation of cell-free extracts for protein synthesis (Fellous et al., 1973; van der Mast et al., 1977; Liu et al., 1978). In our system, a ratio of 1:2 resulting in a 33% homogenate led to the most active extract (Fig. 1, C). From this extract, an equivalent of 120 μg protein showed maximum leucine incorporation per milligram protein in the assay system of 100 μl (Fig. 2, C). Under these conditions, the incorporation of leucine is almost linear over 60 min of incubation, levelling off thereafter. Phenylalanine continued to be incorporated until at least 120 min (Fig. 3). Specific activities of leucine and phenylalanine in the precursor pools were determined by amino acid analysis at 30 and 60 min of incubation (see Methods). Using 1 μCi of the radioactive precursors at both incubation times the specific activity of leucine was about 540 d.p.m./pmol and of phenylalanine about 930 d.p.m./pmol.

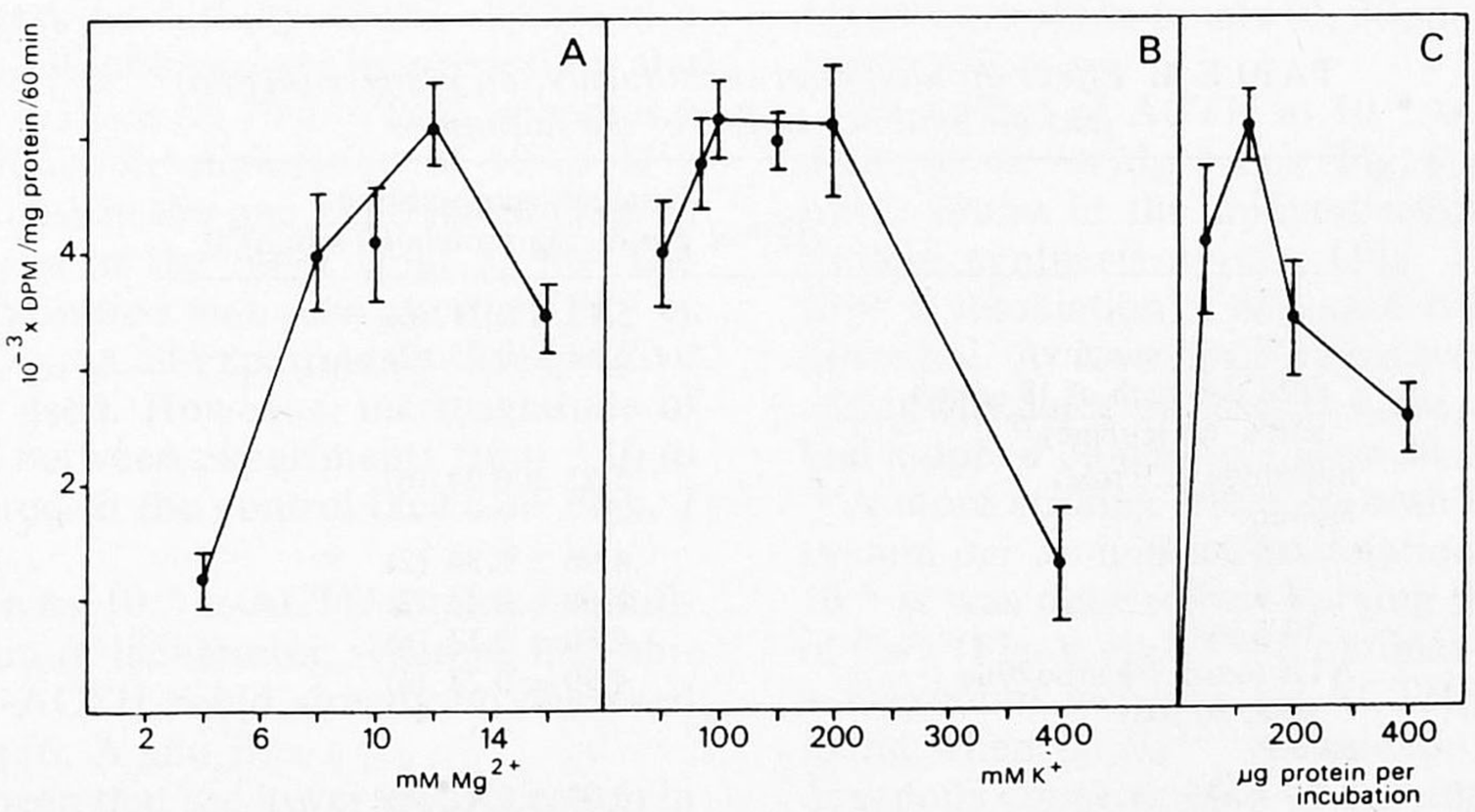

FIG. 2. The influence of Mg^{2+} **(A)** and K^+ **(B)** and the amount of tissue extract (expressed as micrograms protein) **(C)** in the incubation medium on the activity for protein synthesis. Ion concentrations and the amount of tissue were varied separately while the other parameters were kept at their optimum. The activity was tested after isolation of the extract under the optimum conditions (2 mM-Mg^{2+}, 25 mM-K^+, and 30% tissue) of Fig. 1 using 1 μCi [U-^{14}C]leucine. Bars represent the mean ± S.E.M. of three incubations.

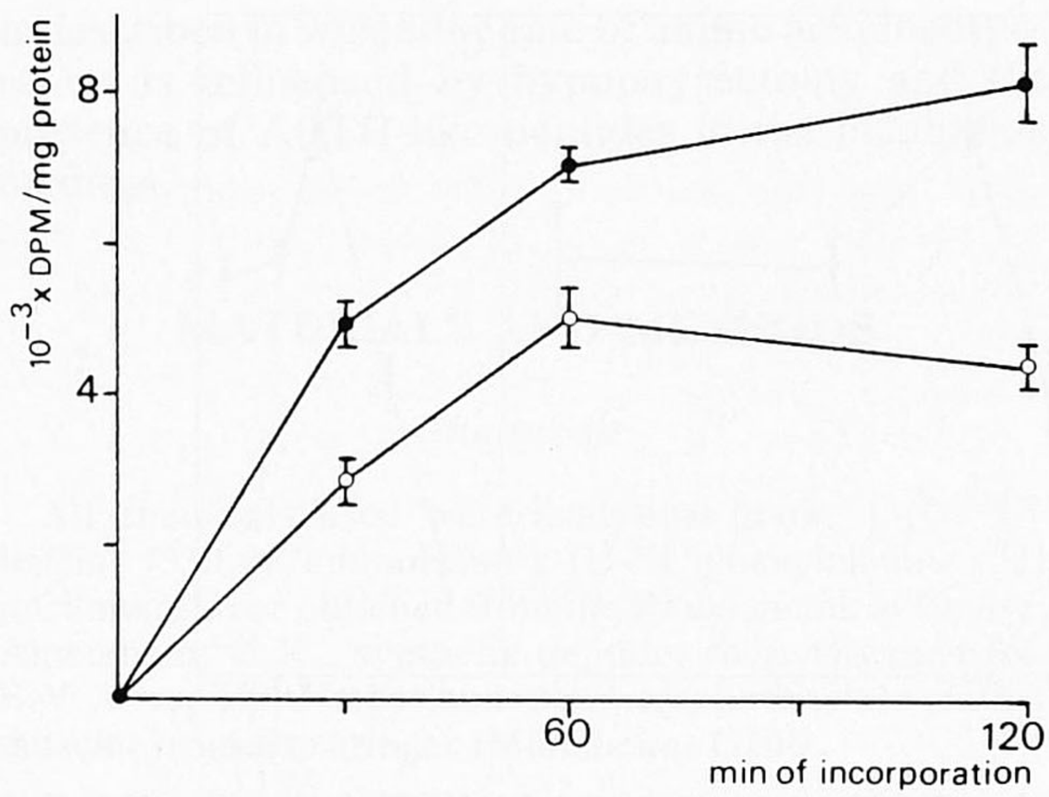

FIG. 3. Time curve of the incorporation of [^{14}C]leucine (○——○) and [^{14}C]phenylalanine (●——●) by the cell-free brain extract. The optimal conditions for the isolation of the extract and for the assay of the activity were used as indicated in Figs. 1 and 2. Bars represent mean ± S.E.M. of six incubations.

These data are indicative of a constant specific activity for both precursors for at least 60 min of incubation.

From the above-mentioned data, incorporation rates could be calculated, being 10 pmol of leucine and 8 pmol of phenylalanine/mg protein/h. Expressed per microgram RNA—the brain extract contained 47 μg RNA/mg protein—the incorporation values for leucine and phenylalanine are 0.22 and 0.16 pmol, respectively. According to the calculation used by van der Saag and de Greeff (1979), for the present system one arrives at an approximate translation rate of 7 amino acid residues per ribosome per hour.

Addition of amino acids other than the radioactive precursor or stabilizers such as spermine did not affect the activity of the system (Table 1). Moreover, neither hemin which is active as an inducer of initiation in cell-free systems of several eucaryotic species (Gross and Rabinovitz, 1972; Sierra et al., 1977), nor ATA (aurin tricarboxylic acid), which in low concentrations, is used as an inhibitor of initiation (Dettman and Stanley, 1973; Vazques, 1974) was active in our system (Table 1). This might imply that under the standard or control assay conditions initiation of new peptide chains most likely did not occur.

Addition of cyclic AMP resulted in a small, but significant, stimulation of 16 and 13% at concentrations of 1.0 and 0.1 μM, respectively (Table 1). Thus, the present system might be susceptible to regulation via changes in cyclic AMP concentration.

Effect of Hypophysectomy

After hypophysectomy, the depletion of trophic hormones leads to deficiencies in metabolic processes in peripheral tissues such as liver (Korner, 1968). In brain also, a decrease in RNA and protein synthesis and polysomal aggregates was observed *in vivo* (Gispen et al., 1970; Schotman et al., 1972; Reith et al., 1978) and in tissue slices (Reith et al. 1975). It seemed worthwhile to establish whether such a deficiency in brain protein synthesis would also be expressed in the cell-free extract used at present. Indeed, a relative decrease in the incorporation of leucine of 20 to 30% was observed similar to that reported for *in vivo* and slice conditions. The decrease was manifest throughout the total period of incorporation (Fig. 4). Amino acid analysis revealed that the endogenous amount of leucine in the

TABLE 1. *Effect of additional substituents, an inducer (hemin) and an inhibitor (ATA) of the initiation*

	Leucine incorporation (10^{-3} × d.p.m./mg protein/60 min/μCi)
Control	5.12 ± 0.31 (6)
Amino acid mixture (125 μM each of 19 amino acids, no leucine)	5.34 ± 0.75 (2)
Spermine (2.0 mM)	5.25 ± 0.24 (6)
Hemine	
0.5 μM	4.96 ± 0.34 (2)
1.0 μM	5.60 ± 0.43 (2)
10.0 μM	4.80 ± 0.13 (6)
ATA (aurin tricarboxylic acid, 50 μM)	4.90 ± 0.23 (6)
cAMP	
0.1 μM	6.04 ± 0.42 (6)[a]
1.0 μM	6.22 ± 0.68 (6)[a]
10.0 μM	5.36 ± 0.13 (6)

Figures are mean ± S.E.M.; number of incubations given in brackets.
[a] $2p < 0.05$ (Student's t-test) compared with control.

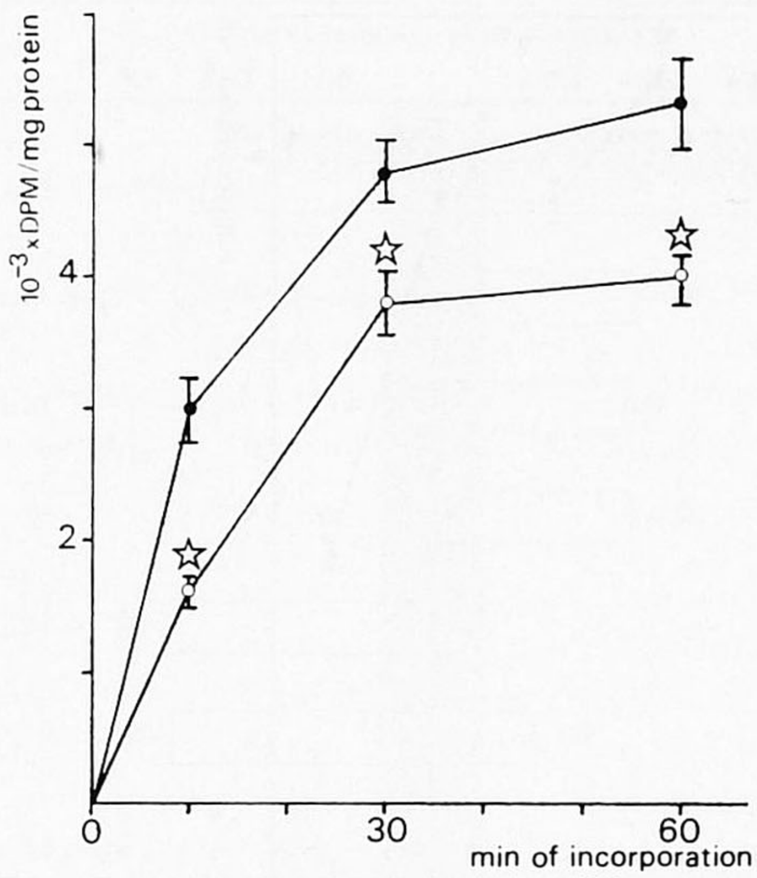

FIG. 4. The effect of hypophysectomy; cell-free extracts were prepared from subcortical tissue of sham-operated and hypophysectomized rats, 3 weeks after surgery. The extraction and incubation procedure used the optimal conditions as indicated by Figs. 1 and 2. Bars represent mean ± S.E.M. of six animals, being either sham-operated (●——●) or hypophysectomized (○——○). Asterisk denotes $2p < 0.01$ by Student's *t*-test.

brain tissue of hypophysectomized rats was not significantly different from that of intact rats, i.e. 40 ± 2(12) and 37 ± 3(12) pmol/mg wet tissue, respectively.

Influence of $ACTH_{1-24}$

$ACTH_{1-24}$ was added to the incubation mixture in a concentration range of 10^{-4} to 10^{-9} M. The incorporation of either [^{14}C]leucine in one experiment (Fig. 5, A) or [^{14}C]phenylalanine in another (Fig. 5, B) was measured after 60 min of incubation. $ACTH_{1-24}$ changed the activity of the system in a biphasic way; i.e., it inhibited the incorporation at a concentration of 0.1 mM by 70 and 50%, whereas a smaller, but significant, stimulation of 10 to 11% was found at 0.1 μM in the one experiment (Fig. 5, B) and at 0.01 μM in the other (Fig. 5, A). The increase in incorporation was seen at either 10^{-7} or 10^{-8} M of $ACTH_{1-24}$ in all experiments, irrespective of the precursor used. However, the magnitude of the effect varied between experiments from +10 to +70% as compared to the control (see also Figs. 7 and 8).

The stimulation by 10^{-8} M-ACTH attained significance after 60 min of incubation, whereas the inhibition by 10^{-4} M-ACTH could already be observed after 30 min (Fig. 6, A and B).

It could have been that the lower incorporation in the presence of 10^{-4} M-ACTH was the result of a decrease in the specific activity of the radioactive precursor, caused, for example, by an increase in protein breakdown. To check for this possibility, specific activities of the [^{14}C]leucine were measured in the medium after incubation with or without 10^{-4} M-$ACTH_{1-24}$. In d.p.m./pmol, the values for the controls were 536 ± 17 (4) and 508 ± 42 (4), for ACTH 553 ± 49 (4) and 558 ± 46 (4), at 30 and 60 min, respectively. Thus, incubation with $ACTH_{1-24}$ did not alter the specific activity of the precursor amino acids.

Structure-Activity Studies

To investigate the structure-activity relationship, shorter sequences of $ACTH_{1-24}$ were assayed (Fig. 7). With respect to the inhibitory effect of the peptide at 10^{-4} M, the active site appeared to be within the sequence 11–24, as the peptides, 11–24 and 1–24, were active and 1–10 was inactive (Fig. 7, *left*). The stimulation at 10^{-8} M, however, apparently is encoded for within the 4–10 sequence. For the peptides 1–16, 1–10, and 4–10 all stimulated the leucine incorporation by about 20%, whereas the sequence 11–24 was inactive (Fig. 7, *right*).

A mixture of amino acids equivalent to $ACTH_{1-24}$ equimolar to 10^{-4} M was tested and found to be inactive (Fig. 7, *left*).

Dependence on Mg^{2+} and Ca^{2+} Ions

The stimulation by ACTH at 10^{-8} M was dependent on Mg^{2+} and Ca^{2+} ion concentrations. The incorporation data of this experiment were subjected to a two-way analysis of variance per time point and treatment group. In Fig. 8 mean incorporation values over all time points are shown. The mean values are higher in the case of the Mg^{2+} curve compared to that for Ca^{2+}. In the case of the Mg^{2+} curve mean values were obtained from four time points (0, 30, 60, and 90 min), whereas for Ca^{2+} the mean was taken from three points (0, 30, and 60), resulting in lower values.

The effect of ACTH at 10^{-8} M showed a similar dependence on Mg^{2+} ions (Fig. 8, *left*) as was previously found in the optimalisation of the cell-free protein synthesis system (Fig. 2, A). At 16 mM-Mg^{2+} a stimulation of 14%, and at 10 mM of 44% was observed. At lower Mg^{2+} concentrations no ACTH effect was detected, and at 4 mM, the incorporation had stopped completely after 30 min.

A more striking effect on both the activity of the system *per se* and its modulation by $ACTH_{1-24}$ at 10^{-8} M was obtained on varying the concentrations of Ca^{2+} (Fig. 8, *right*). An optimal basal activity and a maximum increase by $ACTH_{1-24}$ (+76%) were found when no Ca^{2+} was added. Assuming an endogenous concentration of 2.2 μmol per g fresh tissue (Tower, 1969), the effective concentration at "0 mM" in Fig. 8 will be 150 nM. At higher Ca^{2+} concentrations, a stimulation by ACTH was also observed, although at 2 mM both the basal activity and

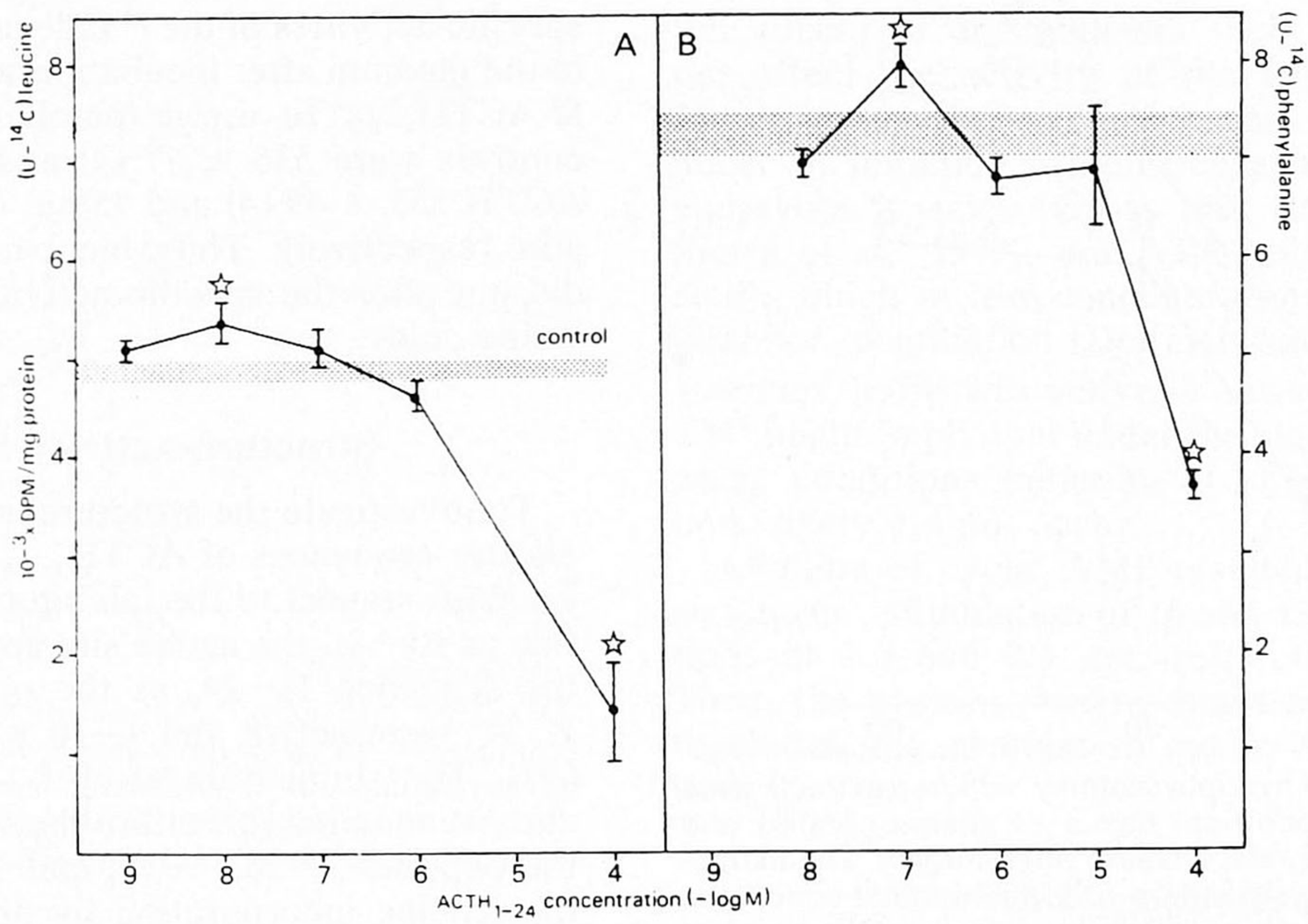

FIG. 5. Dose-response curves for the effect of $ACTH_{1-24}$ on the activity for amino acid incorporation of the cell-free extract. $ACTH_{1-24}$ was added to the incubation mixture after 4 min of preincubation. Optimum conditions for preparing tissue extract and incubation were used (see Figs. 1 and 2 and Table 1). One minute after the addition of the peptide, 1 μCi of [U-^{14}C]leucine **(A)** or [U-^{14}C]phenylalanine **(B)** was added. The incubation was terminated after 60 min. Bars represent mean ± S.E.M. of six incubations. Asterisk denotes $2p < 0.05$ by Student's *t*-test.

the peptide effect were greatly diminished, and in both cases the incorporation ceased after 30 min of incubation. In the presence of EGTA the incorporation of the system was limited to 30 min, and stimulation by ACTH was completely abolished.

DISCUSSION

The present study was undertaken to investigate the possibility that neuropeptides in general and ACTH-like peptides in particular modulate brain

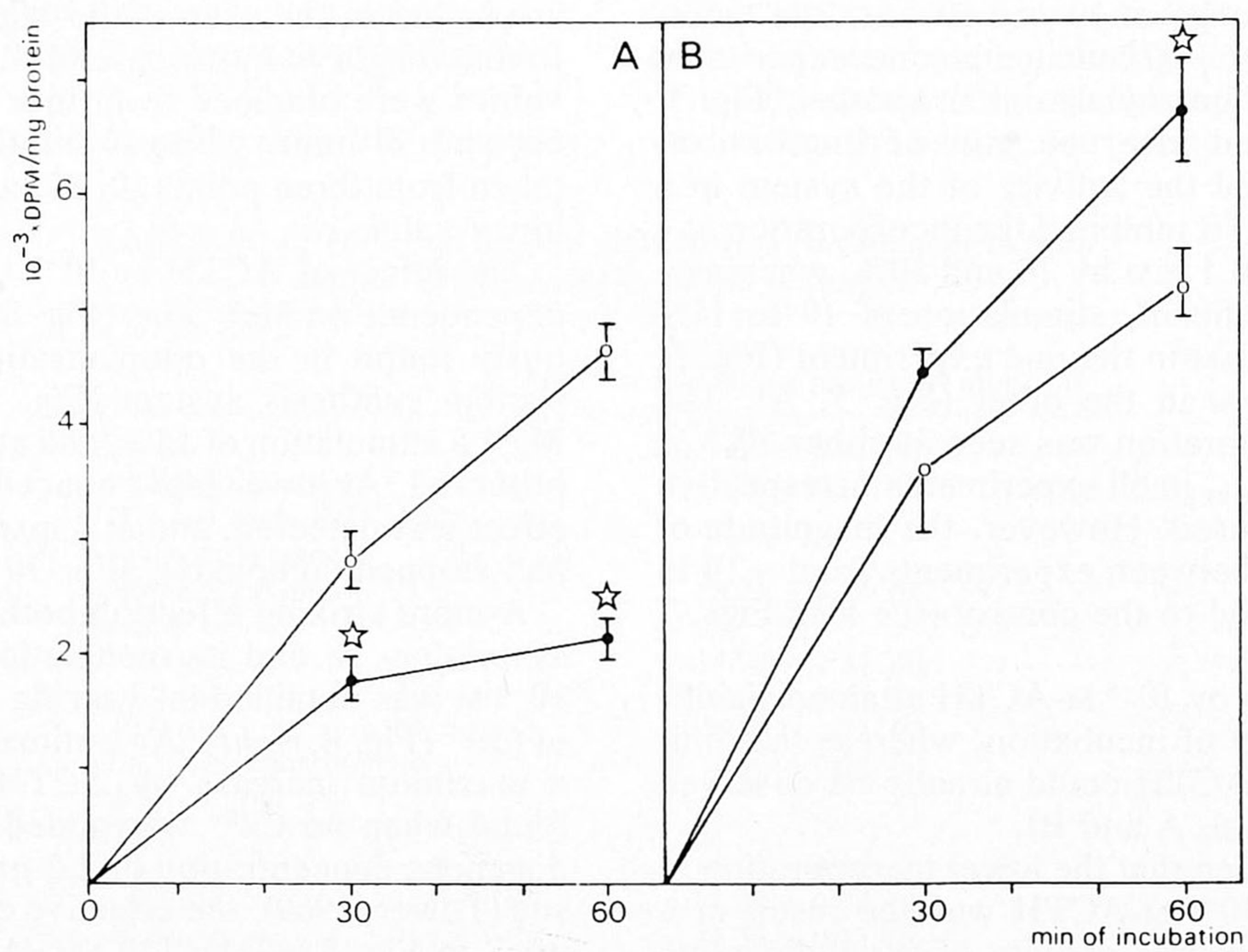

FIG. 6. The effect of $ACTH_{1-24}$ at a concentration of 0.1 mM **(A)** and of 0.01 μM **(B)** on the leucine incorporation over 30 and 60 min of incubation. Optimum conditions for the extraction and assay procedure were used as indicated by Figs. 1 and 2. Bars represent mean ± S.E.M. of six incubations with (●——●) or without (○——○) $ACTH_{1-24}$. Asterisk denotes $2p < 0.05$ by Student's *t*-test.

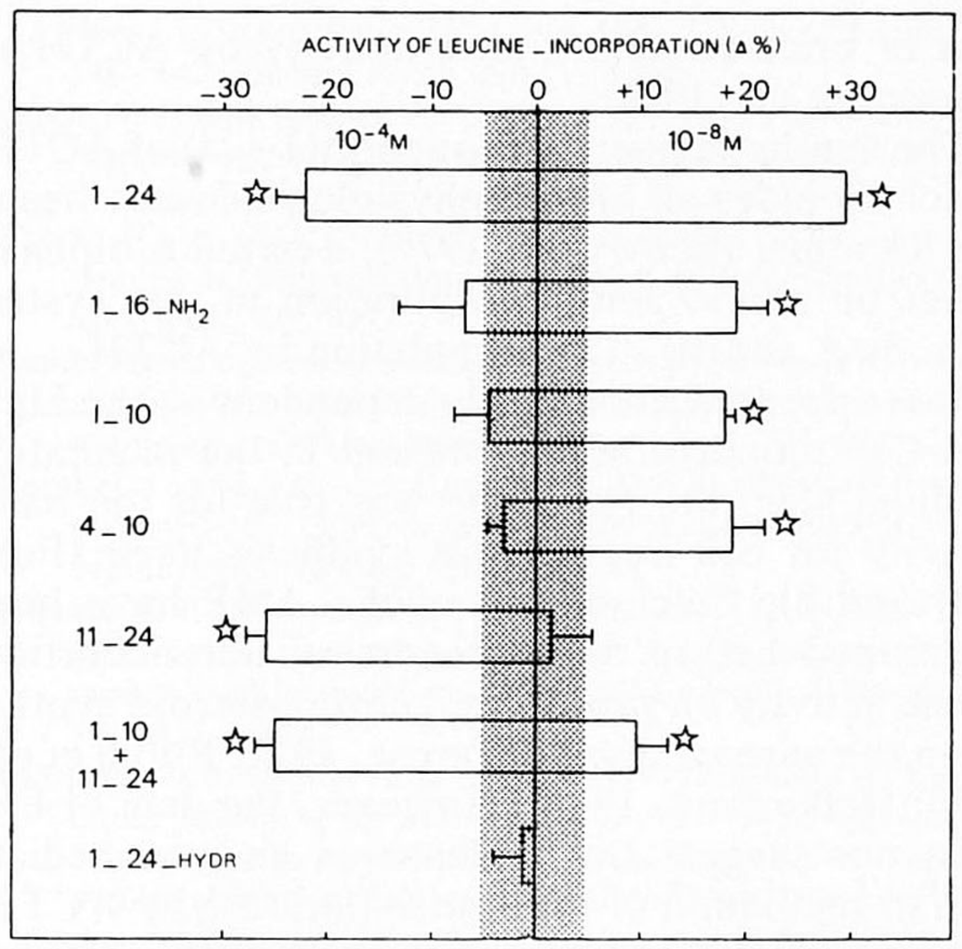

FIG. 7. Structure-activity relationship within $ACTH_{1-24}$ for the inhibitory (10^{-4} M, *left*) and stimulatory (10^{-8} M, *right*) effect on leucine incorporation. The activity was determined under optimal conditions as indicated by the Figs. 1 and 2. The effects were expressed as percentage change from the basal activity; bars and shaded area represent mean ± S.E.M. of six incubations. Asterisk denotes $2p < 0.05$ by Student's *t*-test.

protein synthesis by a direct interference with translational processes within the cell. For this purpose we used a system designated by others as PMS (postmitochondrial supernatant), S_{20} (supernatant after differential centrifugation at 20,000 *g*) or cell-free extract (Widelitz et al., 1976; Liu et al., 1975; 1978; Sierra et al., 1977). This choice was made for the following reasons: (1) similar assay systems have been fruitfully used in other tissues in the unravelling of regulatory principles in translation, the heme-regulated translation of globin in reticulocytes being the most salient example (Gross and Rabinowitz, 1972; Ranu et al., 1976); (2) at present, none of the procedures used for further purification of components of the translational machinery in brain have led to a regulatory principle that is likely to be a candidate for regulation by hormones or neuropeptides *in vitro*.

In our hands, careful optimalization of both the extraction procedure and the incubation conditions for the cell-free extract appeared to be essential to maintain the sensitivity to hormonal regulation (Figs. 1, 2, and 7). The optimum concentrations found for K^+ and Mg^{2+} (Figs. 1 and 2) differ significantly from those reported for crude brain extracts by others (Fellous et al., 1973; Liu et al., 1975; Widelitz et al., 1976), but are rather similar to those reported by Zomzely-Neurath and Roberts (1972), who used a system reconstituted from purified ribosomes and pH 5 enzymes. This is interesting because these authors postulated that changes in ion concentrations were part of a mechanism by which brain protein synthesis rates are related to functional states of the neuron.

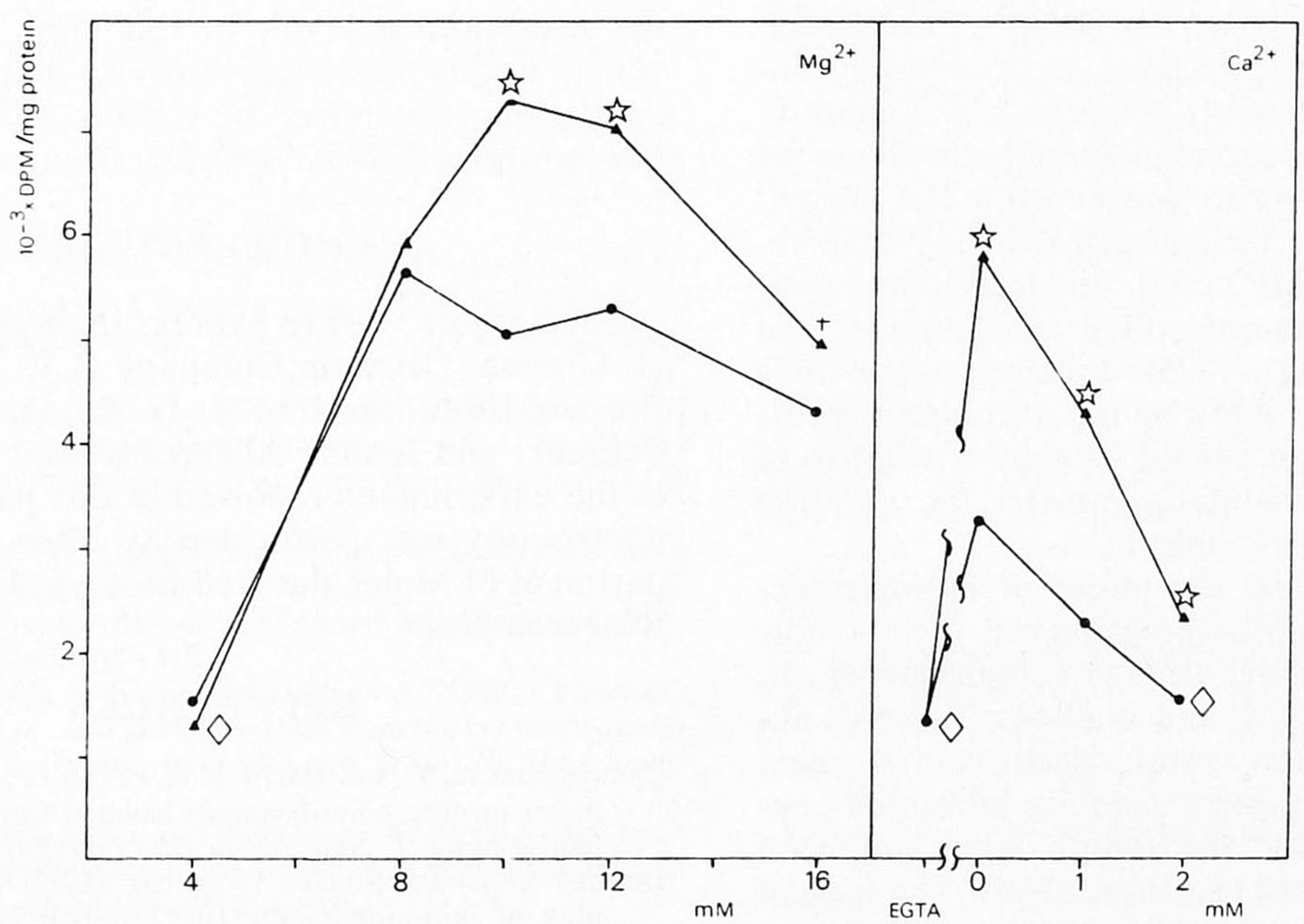

FIG. 8. The influence of the concentration of Mg^{2+} and Ca^{2+} during the incubation on leucine incorporation in the presence of 10^{-8} M ACTH (▲——▲) vs control (●——●). Incubations (100 μl) were made for each point seven times. Samples (20 μl) of the incubations were assayed for the amounts of [^{14}C]leucine incorporated into polypeptide chains after 0, 30, 60, and 90 min for the Mg^{2+}, and after 0, 30, and 60 min for the Ca^{2+} curve. Two-way analysis of variance was performed on the data. Each point in the figure resembles the mean incorporation over the three (Mg^{2+}) or two (Ca^{2+}) time points. $^*p < 0.01$; $\dagger p < 0.05$, significance for the effect of ACTH (10^{-8} M) compared with control at that ion concentration. The incorporation of leucine increased significantly in time ($p < 0.01$) except when indicated as ◇. In those cases no significant increase in incorporation was observed after 30 min of incubation.

As starting material, a 30% homogenate might not have been the most favourable for obtaining subcellular fractions by differential centrifugation. Nevertheless, the resulting postmitochondrial supernatant appeared to consist mainly of ribosomes, endoplasmic reticulum, and some larger, empty membrane fragments when it was inspected by electron microscopy (not shown). Moreover, the apparent absence of synaptosomal structures (e.g. vesicles with transmitters, postsynaptic densities) makes it highly unlikely that eventual changes in the protein synthesis, *in vitro* brought about by peptides, are mediated by transmitter release. The absence of lysosomal structures is in accordance with the lack of major proteolytic activity as concluded from the incorporation kinetics for leucine and phenylalanine (Fig. 3). This makes the use of such an extract advantageous over that of homogenates, in which proteolysis exceeds synthesis after only 10 min of incubation (Holopainen and Oja, 1977).

The translation rate of the present extract system was calculated to be approx. 0.12 amino acid residues per ribosome per minute. Although reinitiation is not likely to occur (Table 1), this rate is about 20 times as high as the one reported by Gilbert et al. (1972). Van der Saag and de Greeff (1979) recently reported a rate of 0.5–2.0 residues, but they used a reconstituted system from neonatal mouse brain together with poly U-stimulated initiation. Nevertheless, the incorporation rate in a cell-free extract is still rather low, when compared to average rates of brain protein synthesis *in vivo* (Reith et al., 1978). However, regulatory principles dependent on endogenous hormone levels (in the case of hypophysectomy, Fig. 4) and second messenger mechanisms (cAMP effect, Table 1) are preserved in the present cell-free system. For reticulocytes, it has been reported that cyclic AMP could, under certain conditions, reverse hemin-controlled inhibition of the initiation (Giloh et al., 1975). Further conclusions about a role of cyclic AMP on initiation in the present system from brain cannot be drawn because of the lack of an experimental parameter for initiation in the present system (Table 1).

The data concerning the effect of hypophysectomy on the cell-free incorporation rate (Fig. 4) corroborate similar findings *in vivo* (Schotman et al., 1972; Reith et al., 1978) and in slices (Reith et al., 1975) and in a cell-free system (Dunn and Korner, 1966). In peripheral tissues a similar hormonal control mechanism at the translation level had been previously put forward (Korner, 1968). The lack of effect of prior hypophysectomy on subsequent cell-free incorporation rates in brain tissue as was reported by Frankel and van der Laan (1972) might be caused by the freezing step in their procedure. A complete loss of hormonal regulatory mechanisms by such procedures, leaving the basal activity however unchanged, has been reported for the modulation of brain protein kinase activity by $ACTH_{1-24}$ (Zwiers et al., 1976).

The synthetic peptide sequence (1–24) of ACTH, which includes all known physiological activities of the hormone (Schwyzer, 1977), exerted a biphasic effect on amino acid incorporation in this system (Fig. 5, A and B). The stimulation by $ACTH_{1-24}$ at 10^{-8} M appeared to be highly dependent on the Mg^{2+} and Ca^{2+} concentrations present in the incubation medium (Fig. 8). The same was true for the basal activity for cell-free protein synthesis itself (Figs. 2, B and 8). Calcium and cyclic AMP have been mentioned before as mediators of adrenocorticotropic activity on protein and corticosteroid synthesis in the adrenal cortex (Farese, 1971; Rubin et al., 1972; Halkertson, 1975). However, the data of Fig. 8 do not suggest Ca^{2+} release as an intermediate step in regulation of the translation rate by ACTH, because at Ca^{2+} concentrations above the optimum the stimulation of protein synthesis by ACTH still remained.

The active sequence of ACTH, 4–10, responsible for the increase in the activity of the cell free system at low concentrations (Fig. 7) was also the active principle in the effects on avoidance learning (De Wied, 1969; De Wied and Gispen, 1977) and protein synthesis *in vivo* and in slices (Schotman et al., 1972; Reith et al., 1975) before. This opens the possibility that those long-term effects of ACTH as neurohormone (Gispen et al., 1979) are mediated by an interaction of small fragments, as $ACTH_{4-10}$, at the translational level. In this way, the data presented in this paper may serve as a start in answering the question posed by Kolata (1978): "Polypeptide hormones: What are they doing in cells?"

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