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Biochem. J. (1967) **104**, 8c

The Relationship between Neurophysin and the Soluble Proteins of Pituitary Neurosecretory Granules

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(*Received* 24 *April* 1967)

The polypeptide hormones, oxytocin and vasopressin, have been isolated from pituitary posterior lobes as a hormone–protein complex by several workers. van Dyke and his associates isolated from fresh tissue an apparently homogeneous protein with mol. wt. 30000. It possessed oxytocic, pressor and antidiuretic activities in the same ratios as they occur in the gland (van Dyke, Chow, Greep & Rothen, 1942). Later Acher and his co-workers isolated similar material from acetone-desiccated tissue (Acher, Manoussos & Olivry, 1955; Acher, Chauvet & Olivry, 1956). The hormones were dissociated from the complex by precipitation of the protein constituent, neurophysin, with trichloroacetic acid. The hormone–protein complex dissociates in dilute acid, and this property has been used to isolate neurophysin entirely free from biological activity by gel filtration in 0·1 N-formic acid on Sephadex G-25 (Hope, Schacter & Frankland, 1964; Frankland, Hollenberg, Hope & Schacter, 1966). The neurophysin, isolated from acetone-desiccated glands, consisted of several constituents separable by electrophoresis. Seven protein fractions have now been isolated by column-chromatographic procedures (Hope & Hollenberg, 1966; Hollenberg & Hope, 1967); all but one was found to bind the polypeptide hormones. The amino acid analyses of the fractions with binding capacity were similar to one another and to the analysis reported by Block & van Dyke (1952) for the hormone–protein complex isolated from fresh tissue (van Dyke *et al.* 1942).

Surprisingly, neurosecretory granules isolated from the posterior lobes of bovine pituitary glands contain only two soluble proteins (Dean & Hope, 1966). Since the components absent from neurosecretory granules could not be detected in any other subcellular fraction, it was evident that the composition of neurophysin changed during the isolation procedure (Dean & Hope, 1967).

The possibility that the two soluble proteins of neurosecretory granules are partially degraded by the activity of a proteolytic enzyme(s) has now been studied. Since extracts of bovine pituitary posterior lobes have proteolytic activity (Adams & Smith, 1951; Ramachandran & Winnick, 1957; La Bella, Reiffenstein & Beaulieu, 1963; Dean & Hope, 1967), we examined extracts of pituitary posterior lobes in media of different pH values for proteolytic activity and for the protein constituents by starch-gel electrophoresis. A procedure suitable for the isolation of the soluble proteins of the neurosecretory granules from acetone-desiccated pituitary tissue is described.

Methods and materials. Acetone-desiccated powder of bovine pituitary posterior lobes (1·8 i.u. of oxytocic activity/mg.) was extracted by suspending the powder (2·5 g./100 ml.) in a suitable medium. After 18 hr. at 4° the insoluble material was removed by centrifugation. The supernatant was dialysed against distilled water and freeze-dried.

Zone electrophoresis was done in horizontal starch gels according to the method of Smithies (1955) in the buffer system described by Ferguson & Wallace (1961) with a starch concentration of 15 g./100 ml. of buffer. Protein samples (50 μl. of 5 mg./ml.) were dissolved in the same buffer used to prepare the gel. Protein was stained with 0·05% Nigrosine.

Proteolytic activity was assayed by a modification of the method described by Anson (1938). The

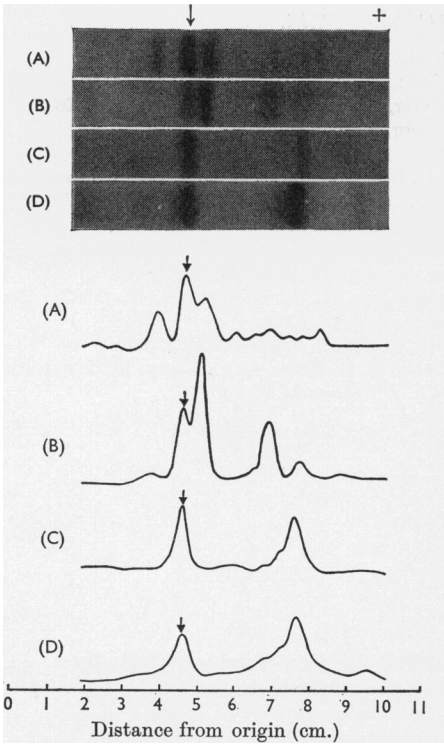

Fig. 1. Electrophoresis of the protein constitutents of protein–hormone complexes isolated from various sources. (A) Protein isolated from fresh-frozen bovine pituitary posterior lobes by the procedure of van Dyke *et al.* (1942). (B) Protein isolated from acetone-desiccated powders of bovine pituitary posterior lobes by the procedure of Acher *et al.* (1956). (C) Protein isolated from acetone-desiccated powders of bovine pituitary posterior lobes treated with acid (0·1 N-HCl) to destroy proteolytic activity. (D) Soluble proteins from purified neurosecretory granules isolated from bovine pituitary posterior lobes.

incubation medium contained 2·0% crystalline bovine haemoglobin, 0·35 N-acetic acid and 4 mM-$(NH_4)_2SO_4$ and was adjusted to pH 3·5 with N-NaOH. A sample (0·2 ml.) and the substrate solution (2·0 ml.) were incubated together for 1 hr. at 37°. The reaction was stopped with 2·0 ml. of 10% (w/v) trichloroacetic acid solution.

Results and discussion. The protein extracted from fresh tissue with 0·01 N-H_2SO_4 by the procedure used by van Dyke *et al.* (1942) had as many as eight electrophoretically distinct constituents (Fig. 1A) and the protein extracted from acetone-desiccated powder with 0·01 N-H_2SO_4 by the procedure used by Acher *et al.* (1956) contains six such constituents (Fig. 1B). Although two major constituents are common to both preparations, the densitomer tracings show that their relative intensities are different. When the soluble proteins of the neurosecretory granules were separated by electrophoresis under the same conditions only two bands could be detected (Fig. 1D). It can be seen that the band with the lower mobility (marked with an arrow) corresponds to the most conspicuous band in the electrophoretogram of the protein from fresh tissue. In the protein prepared from acetone-desiccated glands it was present but did not constitute the major protein constituent; in fact, the major protein component of the protein prepared by the method of Acher *et al.* (1956) was absent from the granules altogether. The second granular protein has a higher electrophoretic mobility, and it could be detected in only trace amounts in both of the two preparations of neurophysin.

The methods for the isolation used by van Dyke *et al.* (1942) and by Acher *et al.* (1956) are similar in that the extraction and precipitation procedures are carried out at about pH 4. This pH is close to the optimum for the activity of the lysosomal cathepsins of liver tissue.

The cathepsin activity in the pituitary-posterior-lobe acetone-desiccated powder with haemoglobin as substrate was optimum at pH 3–4; little activity was detectable at pH 7, and at pH 1·5 the activity was irreversibly destroyed. When an extract of acetone-desiccated powder in 67 mM-sodium phosphate buffer, pH 7·4, was submitted to electrophoresis, it produced only two constituents, which were identical in position with the two proteins of the neurosecretory granules. However, on subsequent adjustment of the pH to 3·9 and precipitation of the hormone–protein complex there was an increase in the number of electrophoretically distinct components. In particular, the major component of neurophysin isolated by the method of Acher *et al.* (1956) appeared. Presumably the proteins of the neurosecretory granules are not degraded in neutral solution, but are subject to the action of a cathepsin at pH 3–4. An extract of desiccated tissue made at pH 1·5 in 0·1 N-HCl, to destroy the activity of proteolytic enzymes, gave an electrophoretic pattern indistinguishable from that produced by the proteins of purified neurosecretory granules. Further, the hormone–protein complex precipitated from this extract at pH 3·9 produced an electrophoretic pattern identical with that of the soluble proteins from the neurosecretory granules. Of the two neurosecretory granule proteins, the one with the higher electrophoretic mobility is particularly susceptible to degradation at pH 3·9, even at 4°, since it was present in only small amounts in both hormone–protein complexes prepared by the previously published methods. These findings now make it possible to isolate the native soluble proteins of the neurosecretory granules in large

amounts from a readily available source, namely the acetone-desiccated powder of bovine pituitary posterior lobes. The formation of artifacts during the isolation procedure may be avoided by destroying the activity of the lysosomal enzyme cathepsin.

The work was supported by a research grant from the Medical Research Council.

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Biochem. J. (1967) **104**, 10c

Regulation of Adenylate Deaminase from Ehrlich Ascites-Tumour Cells by a Phosphonate Analogue of Adenosine Triphosphate

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(*Received* 24 *April* 1967)

The catalytic activities of many enzymes are modified by AMP and ATP (for review see Atkinson, 1966) and it has been suggested that the AMP/ATP ratio is a major factor in control of metabolic processes (Krebs, 1964). However, this ratio is subject to changes brought about by phosphorylation and by the action of adenylate kinase. For studies of natural and artificial regulation of enzymic activity in crude extracts from cells there is a need for compounds that simulate the regulatory properties of AMP and ATP but are not substrates of kinases that would change the concentrations of these compounds in multi-enzyme systems. The present communication describes the extraction from Ehrlich ascites-tumour cells of an adenylate deaminase (AMP aminohydrolase, EC 3.5.4.6) that is activated by ATP, inhibited by GTP and by high concentrations of ATP, and has an absolute requirement for univalent cations. The catalytic activity of this deaminase is modified by AMP-PCP* (the $\beta\gamma$-methylene analogue of ATP; Myers, Nakamura & Flesher, 1963) in a manner closely resembling the modification of activity by ATP.

* Abbreviation: AMP-PCP, *O*-adenylyl methylenediphosphonate.

Materials and methods. AMP-PCP was isolated as the barium salt (Myers *et al.* 1963) and converted into the sodium salt by treatment with chelating resin Chelex-100 (Na^+ form) (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) The ATP content, measured with glucose 6-phosphate, NADP and glucose 6-phosphate dehydrogenase, was less than 1% on a molar basis. Other nucleotides were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Extracts were prepared from ascites-tumour cells as described by Murray (1966); 5mM-glutathione was added to the 50mM-tris–chloride buffer in which the cells were disintegrated. The supernatant obtained by centrifuging for 20min. at 20000*g* was fractionated at 1° with $(NH_4)_2SO_4$ and material precipitating between 0·28 and 0·40 saturation (Dixon, 1953) was dissolved in 5mM-glutathione–50mM-tris–chloride buffer, pH 7·3 (2·5ml./ml. of packed cells), and dialysed against three portions of the same solution for a total of 18hr. After centrifuging at 100000*g* for 30min. the supernatant (about 10mg. of protein/ml.) was used for assays of adenylate deaminase activity. Enzyme kept at − 15° for 4 weeks lost no activity. The specific activity in