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CHARACTERIZATION OF AND HORMONAL EFFECTS ON SUBCELLULAR FRACTIONS FROM XANTHOPHORES OF THE GOLDFISH CARASSIUS AURATUS L.[1]

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A method is described for the subcellular fractionation of goldfish xanthophores. The procedure produces relatively pure fractions of carotenoid droplets, pterinosomes, cytosol and what appears to be plasma membrane. The presence of a distinct pattern of proteins is shown to be associated with the carotenoid droplets. Treatment of the xanthophores with ACTH affects the buoyant density of some carotenoid droplets and stimulates the phosphorylation of a polypeptide associated with the carotenoid droplets.

INTRODUCTION

It is well established that actin, myosin and associated proteins, are present in non-muscle cells and, in conjunction with microtubules, are involved in most processes of cellular movements (1). Among these movements are those of numerous cytoplasmic organelles. In some lower vertebrate pigment cells the translocation of organelles (melanosomes, carotenoid droplets, etc.) is known to be under hormonal or neural control (2). In the goldfish xanthophore, the carotenoid droplets disperse throughout the cell upon treatment with MSH[3], ACTH, cAMP or theophylline (3).

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3. Abbreviations: ACTH, adrenocorticotropin; cAMP, adenosine 3',5'-cyclic monophosphate; MSH, melanocyte stimulating hormone; SDS, sodium dodecyl sulfate.

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In the absence of MSH or upon treatment with epinephrine, the pigment organelles aggregate in the perinuclear area. Recently, we developed a method for preparing viable, hormone-responsive xanthophores from goldfish in sufficient quantities to permit biochemical analysis (4). As a first step in studying the biochemistry of the translocation of their pigment organelles, we have prepared subcellular fractions representing most of the organelles present in xanthophores. We wish to report preliminary results characterizing the proteins and phosphoproteins of these fractions and the influence of ACTH upon them.

MATERIALS AND METHODS

Xanthophores were prepared by standard methods (4) but without the density gradient purification. The purity of these cells as judged by light microscopy was about 50%. Two aliquots (1-2 x 10^6 cells/ml) were routinely incubated in Hank's balanced saline for 30 min. at 22-25°C. To one aliquot was added 1 I.U./ml ACTH.

All sucrose solutions were buffered to pH 7.5 at 0°C with 10mM TrisHCl. Xanthophore pellets were homogenized in 20 volumes of 0.25M sucrose in a teflon/glass Duall homogenizer (at 1800 RPM for 2 min.) and centrifuged at 1000 x g for 10 min. The supernatant was brought to 1.0M sucrose and the volume was adjusted to bring the optical density at 485nm to between 3.0 and 5.0 (equivalent to 1.5 - 2.5 x 10^6 xanthophores/ml). Two ml of this sample were layered over three 2 ml zones of sucrose at 1.5M, 1.9M and 2.6M. Over this was layered 2 ml of 0.4M and 2.5 ml of 0.25M sucrose. The samples were then centrifuged for 2 hrs at 100,000 x g in an SW-41 rotor. The resulting fractions were collected by flotation and monitored by their optical density at 280 or 485nm.

SDS electrophoresis was performed according to Laemmli (5) on 10-17.5% exponential acrylamide gradients. The gels were stained with Coomassie Blue R-250 and radioactive samples were visualized by fluorography (6).

For the analysis of phosphoproteins, xanthophores were prelabeled for 15 min. in a balanced salt solution containing 500 uCi/ml of [^{32}P]-phosphate (1 Ci/mmole). After prelabeling, ACTH was added to one of two aliquots and the incubation was continued for an additional 30 min. The samples were then fractionated and separated on SDS gels. The ^{32}P incorporation into TCA-insoluble material was determined for 0.4ml fractions of the gradients (7).

Electron microscopy was performed on samples fixed in 0.5% OsO_4 and embedded in Epon. Thin sections, stained with uranyl acetate and lead citrate, were examined with a Philips 301 electron microscope.

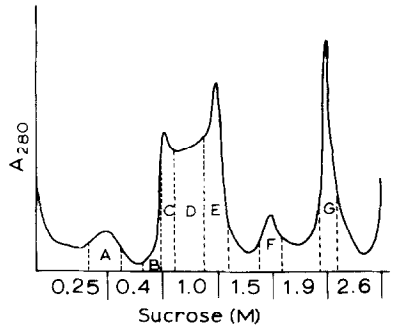

Figure 1. A typical A_{280} tracing of the sucrose gradient after centrifugation. Figures along the X-axis indicate molar concentrations of sucrose. Letters indicate individual fractions collected for further analyses: A, light carotenoids; B, heavy carotenoids; C, smooth membranes; D, cytosol; E, light membranes; F, heavy membranes; G, pterinosomes.

RESULTS AND DISCUSSION

The subcellular fractions obtained from the xanthophores are illustrated in Figure 1. Of these, four were reasonably homogenous: the carotenoid droplets (separated into two broad bands, Fig. 2) the pterinosomes (the second pigment organelle) and

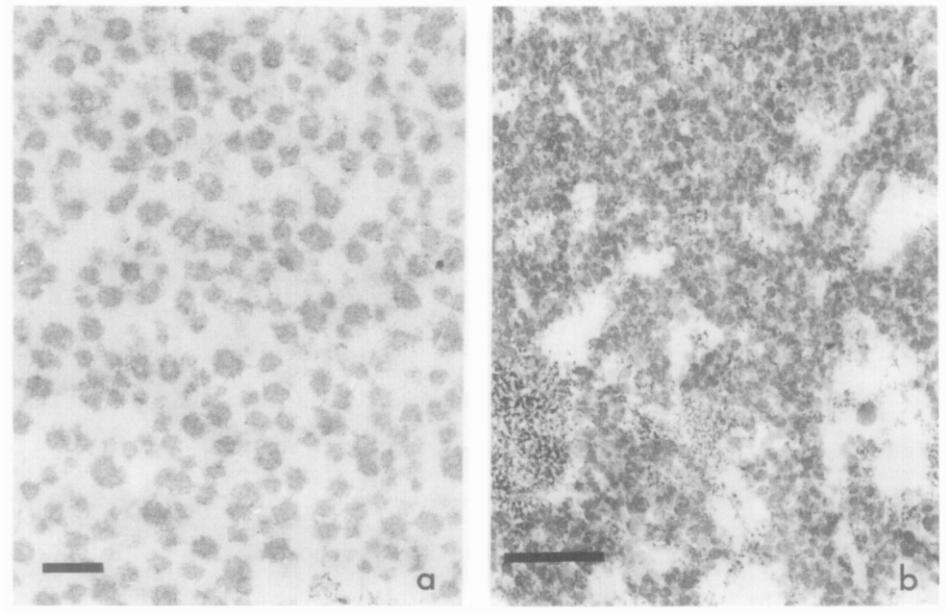

Figure 2. Electron micrographs of light (a) and heavy (b) carotenoid droplets. Bar = 0.5um.

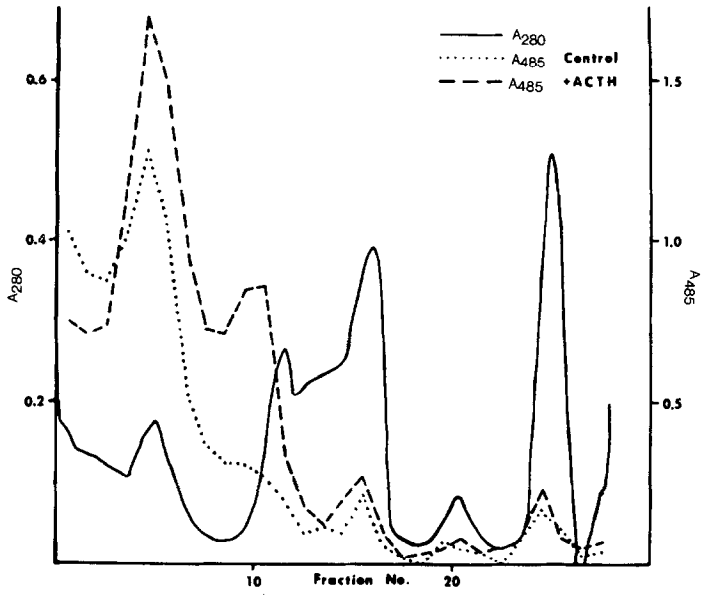

Figure 3. Distribution of carotenoid droplets (A_{485}) in the sucrose gradients. The effect of treating the cells with ACTH prior to homogenization is to increase the proportion of heavy carotenoid droplets. The tracing for A_{280} is representative of both samples.

the light membranes (tentatively identified as plasma membrane). The other fractions were more heterogenous. Contiguous with and below the heavy carotenoid droplets was a band composed primarily of smooth vesicles but also contaminated by larger membrane fragments and carotenoid droplets. The heavy membranes were composed of a heterogenous mixture of organelles and acellular debris.

The morphology of the heavy carotenoid droplets was similar to that observed in the intact cell (Fig. 2). Also, they appeared to be clustered together in groups which occasionally included membrane and nuclear fragments. In contrast, the light carotenoid droplets were irregular in outline with a diameter 3-4 times greater than droplets within an intact xanthophore. It is likely that the light droplets were formed by coalescence during isolation

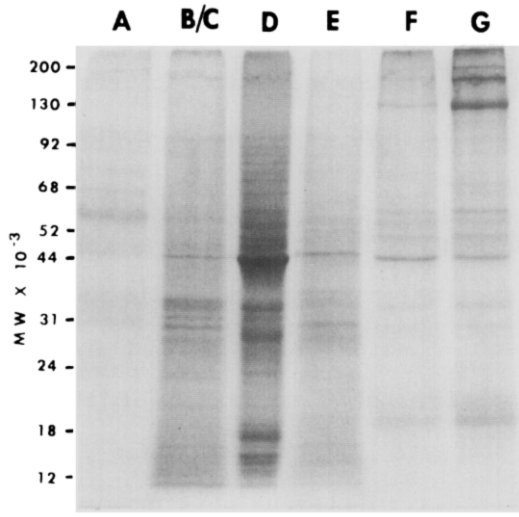

Figure 4. SDS electrophoresis of density gradient fractions. Molecular weights are indicated at left. Letters designate fractions as in Figure 1. Results are similar for untreated and ACTH treated cells.

The distribution of the carotenoid droplets between the heavy and light fractions was influenced by ACTH treatment of the cells prior to homogenization. The percentage of carotenoid droplets in the heavy fraction doubled after ATCH treatment (34% vs. 16%; Fig. 3). Considering the unusual ultrastructure of the light carotenoid droplets, it is possible that ACTH treatment increased the stability as well as the buoyant density of the droplets, resulting in a higher proportion of the smaller, heavy droplets. The properties of these two fractions are under further investigation.

The results of the protein analysis of these fractions by SDS electrophoresis are shown in Figure 4. In particular, a unique set of polypeptides were found in the light carotenoid droplet fraction. Because of their uniqueness and the small amount of protein between the fractions, it is unlikely that these polypeptides resulted from randon cross-contamination. We did not detect changes in the polypeptide profile of any fraction upon

ACTH treatment of the cells. However, only small amounts of protein (5 - 10μg) were recovered with the carotenoid droplets and there may have been differences in minor components which were not detected.

The most prominent polypeptide in the xanthophore was one of about 46K daltons, which was found in all fractions. The abundance and molecular weight of this band suggest that it may be actin. If it is actin, the association of this polypeptide with the carotenoid droplets could represent the remains of a carotenoid droplet/microfilament complex formed during translocation. However, this polypeptide was also associated with the pterinosomes, which are not subject to directed translocation, and which calls into doubt the significance of the association. Also, the amount of this polypeptide which was associated with the carotenoid droplets was uneffected by treatment of the cells with ACTH. Clearly, more work is needed to clarify the identity and possible functions of this protein.

The ^{32}P incorporation profile of the density gradients is shown in Figure 5. ACTH induced a four-fold increase in ^{32}P incorporation in the light carotenoid droplets. The other quantitative differences, involving the heavy membranes and pterinosomes, were not reproducible and therefore of unknown significance. A qualitative effect of ACTH treatment was seen when the phosphoproteins were analyzed on SDS gels (Fig. 6). ACTH induced the phosphorylation of a polypeptide of about 56K daltons present in both the light and heavy carotenoid droplets. Figure 6 also shows several labeled bands visible in the untreated heavy carotenoid droplets which are absent in the ACTH treated sample. Since, these bands are seen in the fraction immediately below the heavy carotenoid droplets in the ACTH treated sample, they

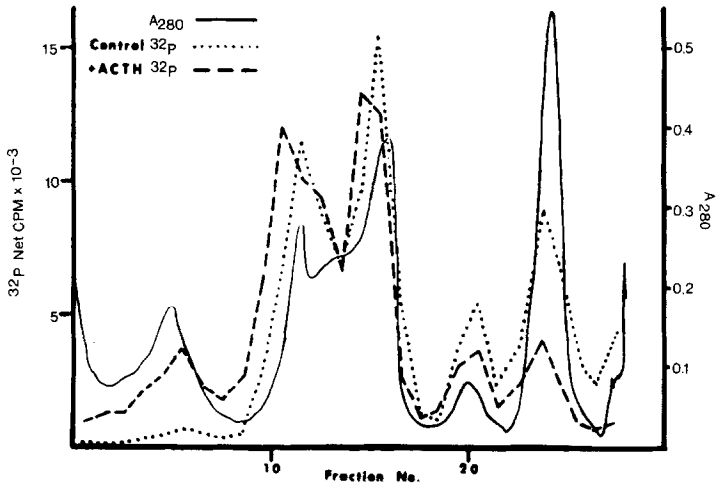

Figure 5. ^{32}P incorporation into subcellular fractions with and without treatment with ACTH. Increased incorporation in both carotenoid fractions is evident.

probably are the result of cross-contamination between these two fractions, the extent of which could vary from one sample to another. The other fractions were too heavily labeled to detect small differences in all regions. However, there appeared to be an increase in the phosphorylation of a 16K dalton polypeptide located primarily in the cytosol. Separation of the

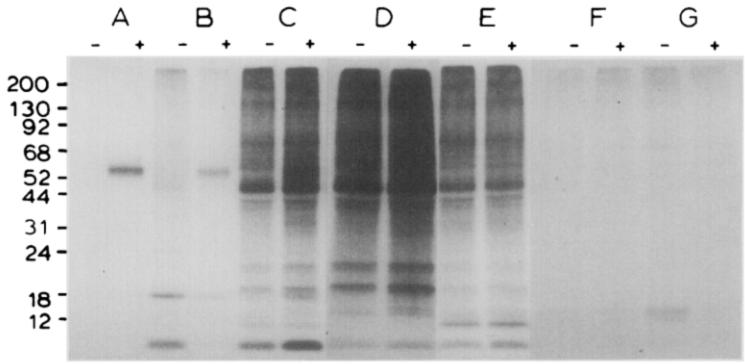

Figure 6. SDS electrophoresis of phosphoproteins. "+", cells treated with ACTH prior to homogenization; "-", untreated control; other labels as in Figure 4.

phosphoproteins on two dimensional gels should allow a more detailed analysis.

These results demonstrate two effects of ACTH treatment on carotenoid droplets: the buoyant density and perhaps the stability of some droplets are increased and one polypeptide associated with the droplets is phosphorylated. The latter indicated that ACTH acts on these cells, at least in part, through the activation of protein kinase by cAMP, a known agonist of ACTH in this system (3). The presence of a unique set of proteins and the specific phosphorylation of one of them suggest that some component(s) of the carotenoid droplets may be involved in the control and/or mechanism of their own translocation.

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