mitochondrial cristae (i.e., μm^2 of SER, RER, and mitochondrial cristae/μm^3 of cytoplasm) were estimated by conventional stereologic methods[9], using the sampling procedures previously reported[1]. The absolute amount of the various organelles in the individual glomerulosal cell was obtained by determining the average cellular volume with the same indirect approach detailed in an earlier contribution[10]. Student's *t*-test was used for the statistical evaluation of results.

Results and discussion. According to what was reported previously[1,2], both cAMP and cGMP were found to reverse, although not completely, the hypophysectomy-induced atrophy of the zona glomerulosa: in fact, the volume of cells, nuclei, mitochondrial compartment as well as the surface of SER and mitochondrial cristae display a significant increase, while the volume of lipid compartment shows a considerable decrease (Table).

The possibility that the trophic action of cGMP consists in the competitive inhibition of adrenal phosphodiesterase can be disregarded, since theophylline, while significantly enhancing the growth of intact rat zona glomerulosa (presumably by inhibiting the cAMP biologic degradation), does not exert trophic action on 11-days hypophysectomized rat glomerulosal cells, in which the ATP-adenyl cyclase system is no longer functioning[11] (Table).

The Table shows that the simultaneous chronic administration of emidoses of cAMP and cGMP induces full maintenance of the growth of hypophysectomized rat zona glomerulosa. This finding could be reasonably explained by assuming that cGMP potentiates the cAMP-action by inhibiting adrenocortical phosphodiesterase. However, the following pieces of evidence are against this possibility: 1. the doses of single cyclic nucleotide administered in the present study were very high, and 2. cGMP was not found to act sinergistically at high doses of cAMP[12].

On this basis, our data seem to be consistent with the hypothesis that cGMP, like cAMP, can act as an intracellular mediator of the trophic action of ACTH on the rat zona glomerulosa.

However, it is to be stressed that, since rat adrenal guanyl cyclase is not sensitive to ACTH[13], the challenge hypothesis that cGMP can function as an intracellular mediator of other glomerulotrophic factors (e.g., renin-angiotensin system) cannot be excluded, at present. Studies are in progress to settle this point[14].

Riassunto. Oltre il cAMP, anche il cGMP esercita azione trofica sulla zona glomerulare di ratto. Entrambi i nucleotidi ciclici però, anche se somministrati in alte dosi, non annullano completamente gli effetti dell'ipofisectomia. La somministrazione simultanea di cAMP a cGMP provoca, invece, pieno mantenimento del trofismo della zona glomerulare di ratto ipofisectomizzato. Viene discussa la possibilità che entrambi i nucleotidi ciclici intervengano nella mediazione intracellulare dell'azione glomerulotrofica dell'ACTH.

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Estrogen-Induced Phosvitin Synthesis in Cultured Chick Embryo Liver Cells

Phosvitin is a yolk protein of certain oviparous vertebrates. In birds, it is synthesized in liver of laying hens and transported in the blood to the developing oocyte[1,2]. In male and immature birds, hepatic synthesis of phosvitin can be induced by estrogen administration[3,4]. Such a system has been extensively used for studying the nature of the mechanism involved in steroid hormones regulation of specific proteins synthesis in vivo[2,5-9]. An in vitro system could improve the experimental opportunities for these investigations. We have therefore investigated the possibility of stimulating phosvitin synthesis in chick embryo liver cultures.

Materials and methods. Livers removed from 14-day old chick embryos were gently cut into small pieces, washed in Tyrode's solution and dissociated in 0.25% buffered trypsin (Difco) at room temperature for 25–30 min. The cells were then filtered through a nylon mesh, centrifuged (35 *g*, 10 min) and resuspended in 199 (Gibco, Grand Island, N.Y.) with 20% added calf serum and penicillin 200 units/ml. 5 ml of cell suspension (4×10^6 cells/ml) were put in flasks and incubated at 37 °C. Cells obtained from the same chick embryos' hearts were used as control.

Estradiol-17-β (Merck; in propylene glycol, 50 $\gamma/\mu l$) was incorporated in the medium at the final concentration of 500 γ/culture. The time schedule was recorded in Figure 1a. 45 liver cultures (25 test and 20 control ones) and 20 heart cultures (10 test and 10 control ones) were carried out and were observed daily with an inverted Reichert microscope. Slides were fixed in methanolic alcohol and stained with May-Grümwald-Giemsa.

After collection and centrifugation (35 g-10 min) the culture medium was dialized extensively against 0.9% NaCl, lyophilized, dissolve in 0.5 ml 0.9% NaCl and then examined by means of the Ouchterlony[10] double gel immunodiffusion and the immunoelectrophoretic micro-

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technique of SCHEIDEGGER[11] against antiphosvitin antiserum (anti-Ph, Calbiochem) and the anti-total adult chicken serum antiserum (anti-TACS, Colorado Serum Co). The protein P was determined on 10% trichloroacetic acid precipitates by the method of BARTLETT[12] after alkaline hydrolysis, considered specific for phosvitin[5] and the protein content according to the method of LOWRY et al.[13].

Results. Cell growth. 24 h after the initial plating, liver cultures examined by the inverted microscope exhibited single well-isolated colonies of cells, interrupted by large cell-free spaces. Stained colonies were mainly formed by flattened parenchymal cells in monolayers, well attached to the glass bottom poligonal in shape. Some cells were binucleate. At this time the medium was changed and the test cultures were supplemented with the hormone-containing nutrient. As incubation proceeded, no difference was found between the control and the treated cultures. Cellular colonies became so confluent as to make a continuous cellular layer within 2–3 days. Epithelial-like cells are well preserved and adherent to each other. Fusiform (fibroblastic) cells appeared in monolayer after 2–3 days, near and at the edges of parenchymal cell sheets. Heart cultures grew very well and within 3–4 days had a very large populations of well spread cells, frequently triangular or stellate in shape.

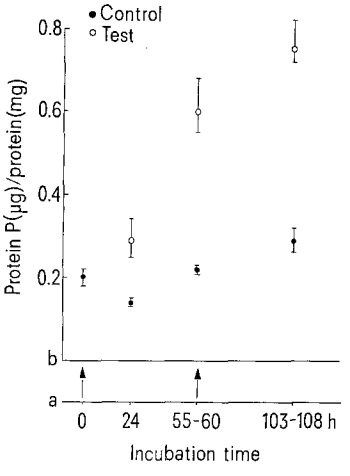

Fig. 1a). Time schedule: 0 represents the experimental starting point (24 h after plating), dotted arrow the moment of estradiol-17-β administration in test cultures and solid arrow the moment of hormone removal. b) Protein P concentration in nutrient fluids removed from liver cultures for the indicated incubation hours. Each point represents averages of 5 independent determinations, each in duplicate. Ranges give the minimum and maximum values observed.

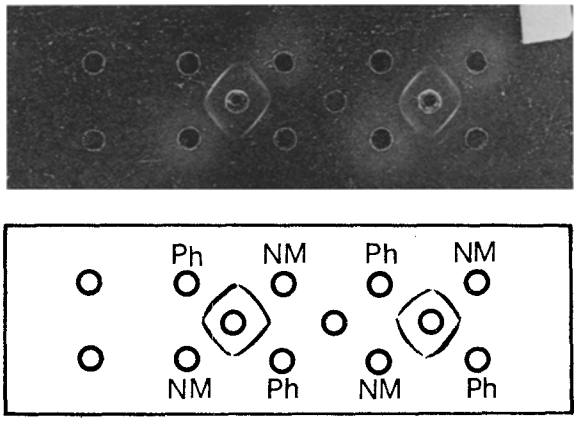

Fig. 2. Ouchterlony test; the central well contain anti-Ph antiserum; the peripheral wells are phosvitin (Ph) and preparations from treated cultures nutritional medium (NM).

Immunochemical analysis. Nutritional media obtained from control liver cultures at the following incubation times 24, 55 and 108 h after the initial plating, and checked for phosvitin by the double diffusion test, showed no reaction. On the other hand, when the same media were tested against anti-TACS antiserum using immunoelectrophoresis, at least two fractions were detected. Two serum proteins have been also demonstrated in the hormone-treated liver cultures at 55 and 108 incubation hours. A slight reaction was observed against anti-Ph antiserum in fluids from 24 h incubated test liver cultures, while a strong reaction occurred with media from cultures which were treated for 55 h and 108 h. No reaction was observed when media removed at the same incubation time from test and control heart cultures were tested both against anti-protein and anti-Ph antiserum (Figure 2).

Biochemical analysis. Data concerning protein P concentration are recorded on the graph (Figure 1b). Protein P was clearly detectable after 24 h of incubation in the liver test cultures and increases quite linearly for the other incubation period.

Discussion. Under our experimental conditions, primary cultures of chick embryonic liver cells grew very well, as demonstrated both by their morphology and ability to produce serum proteins. Estradiol 17-β induces both de novo phosvitin synthesis in liver cells grown in vitro and the secretion of this substance into the extracellular medium. The lag period in vivo (24 h)[4,5,7] is in good agreement with our observations in vitro. Phosvitin synthesis continues in vitro with increasing intensity for at least 48 h after removal of the hormone. The ability of liver cells to be affected by estrogen stimulation is already present in the embryonic period. For the estrogenic effect, neither animal integrity[14] nor organ structural maintenance are required and, therefore, the hormone directly acts upon liver cells[15].

Riassunto. È stata dimostrata la capacità di culture primarie di fegato embrionale di sintetizzare fosvitina in vitro dopo trattamento con estrogeni.

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