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Changes in the pattern of non-specific esterases from the liver of the anuran, Rana esculenta, during spontaneous metamorphosis

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Embriologia. — *Changes in the pattern of non-specific esterases from the liver of the anuran,* Rana esculenta, *during spontaneous metamorphosis* (*). Nota di MASSIMO LANCIERI e VIRGILIO BOTTE, presentata (**) dal Socio G. MONTALENTI.

RIASSUNTO. — Sono state studiate le forme multiple di esterasi aspecifiche del fegato di girini di *Rana esculenta* durante la metamorfosi, sia mediante electrofocusing che elettroforesi su gel di amido. Il pattern enzimatico tipico della post-metamorfosi compare nel fegato prima del climax. Questo fenomeno potrebbe rientrare tra quelli di preadattamento.

Marked changes in the structural organization and functions of several organ systems of the amphibians tadpoles occur during metamorphosis. The beginning of these processes may be identified, at times, in phases that precede the climax, even by many weeks (cf. Tata, 1971). In other words, the tadpole is progressively preparing itself for a terrestial life.

Within the framework of these phenomena the study of some enzymes appears particularly relevant since it sometimes shows the existence of preadaptation processes. The enzymes involved in ureotelism, in fact, begin to be synthesized and could be active at stages when the tadpole is still linked to ammonotelism (Frieden, 1967; Botte and Delrio, 1969; Cohen, 1970; Fieden and Just, 1970; Tata, 1971).

Moreover, it is possible that many enzyme modifications during metamorphosis reflect gradual changes in their composition with the appearance of forms with different physico-chemical properties. On the basis of this hypothesis we have studied the electrophoretic pattern of non-specific esterases whose multiplicity is very common in animal tissues. It has been supposed that this characteristic could be correlated with the environments, depending on the types and amount of substrate available (Kojima *et al.*, 1970).

The non-specific esterases (EC 3.1.1.) have been extracted from the liver, as this organ undergoes a structural reorganization during metamorphosis to rapidly adapt to the different type of adult metabolism (Tata, 1871). We have used groups of *Rana esculenta* tadpoles at the developmental stages 25, 26, 30, 31 and 33, according to the seriation tables of Witschi (Witschi, 1965). The livers were removed from the animals, perfused with a physiological solution for amphibia to eliminate blood and then homogenized with distilled water (10 mg of fresh tissue in one ml of water). The homogenate was frozen and thawed 3–4 times and then centrifuged at 15,000×g. The

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supernatants were used directly for the fractionation of the enzymes by isoelectric focusing or by starch-gel electrophoresis. The protein content was evaluated according to the method of Lowry *et al.* (Lowry *et al.*, 1951).

Isoelectric focusing experiments were carried out according to the method of Bours (Bours, 1971) on thin layers in the pH range 3.5 to 9.0. Five per cent acrylamide gel solution containing 2% "Ampholine" carrier ampholite (LKB, Sweden) was used. The "Ampholines" utilized had a pH range from 3 to 9, and this was prepared by mixing "Ampholines" with pH ranges from 3–5, 5–7, 7–9 in equal proportions. Plates (16×23 cm) were cast as usual (Brahma and Starre, 1976). The distance between the electrodes was 13 cm. 15 µl of each supernatant sample (corresponding to 40–60 µg of proteins) were adsorbed on strips of Whatmann 3MM paper (10×2 mm) that were then deposited on thin layers. Isofocusing was carried out for 20 h at 4 °C with an initial voltage of 80 Volt; the initial current was 6 mA. At the end of the experiment the typical current value was 0.9 mA. The pH gradient along the gel was measured with an Ingold flat membrane combined electrode, at 4 °C. The gels were preincubed for three hours in 8 mM Tris-HCl buffer, pH 8.4, before staining by enzymatic activity (see below).

Starch-gel electrophoresis was carried out on layers prepared with 13% of partially hydrolyzed Connaught starch, in 30 mM borate buffer, pH 8.6, after Botte and Basile (Botte and Basile, 1973). In each slit were deposited 30 µl of the same supernatant as that utilized for isoelectric focusing, corresponding to 80–150 µg soluble proteins. The electrophoretic runs, in a vertical system, lasted 18 h at 4 °C.

The enzymatic activity was detected directly on the layers, using α-naphthyl acetate as the substrate, as in the method of Li *et al.* (1959).

The results of our experiments are reported in Pl. I. It is evident that non-specific esterases show some degree of multiplicity in the tadpole liver, as already observed in the adult of the same species (Botte and Basile, 1973).

The fractionation on starch-gel electrophoresis indicates only minor variations in the enzyme pattern during metamorphosis. The relative activity of two slowly moving bands, in fact, increases in the stages immediately preceding the climax (stage 31) and also in those tadpoles that have almots terminated metamorphosis (stage 33) (see the arrows in Pl. I).

The enzyme pattern in electrofocusing separation gradually changes during metamorphosis and the typical post-metamorphosis pattern (stage 33) seems to be reached immediately at the climax (stage 31).

The differences in enzymatic patterns displayed by the same samples when examined by starch-gel electrophoresis or electrofocusing, depend, in our opinion, upon the peculiar physico-chemical characteristics evidenced by the two methods. When the forms obtained on starch-gel are more numerous than those obtained on electrofocusing (see stage 25) it could be that several forms display a similar pH optimum and therefore focalize in very close positions.

These findings are another demonstration of the appearance of characteristics that will probably be useful during the terrestial life at a time when the tadpole is still living in the water. It seems to us particularly interesting that, for this type of investigation, the isoelectric focusing technique has shown some aspects of enzyme modifications that would have escaped detection using starch-gel electrophoretic analysis. At present it is not known whether the differences in the pattern during the various stages of metamorphosis depend on the synthesis of new proteins or are the expression of a modification in the physico-chemical properties of the enzymatic pool (i.e., shift in the pH optimum of some forms).

The functions of non-specific esterases, therefore, are not yet well established, but it is evident that the variations observed here should be somehow related to functions in the hepatocytes.

In adult frogs the activity of some forms of non-specific esterases extracted from the liver is under the control of ovarian steroids. These forms are probably linked to the synthesis in the hepatocytes of some yolk proteins (Botte and Basile, 1975). No observations are available for tadpoles, but it is well known that intestinal alkaline phosphates activity during metamorphosis is controlled by adrenal steroids (Chieffi and Carfagna, 1959; Botte and Buonanno, 1962; Botte, 1966).

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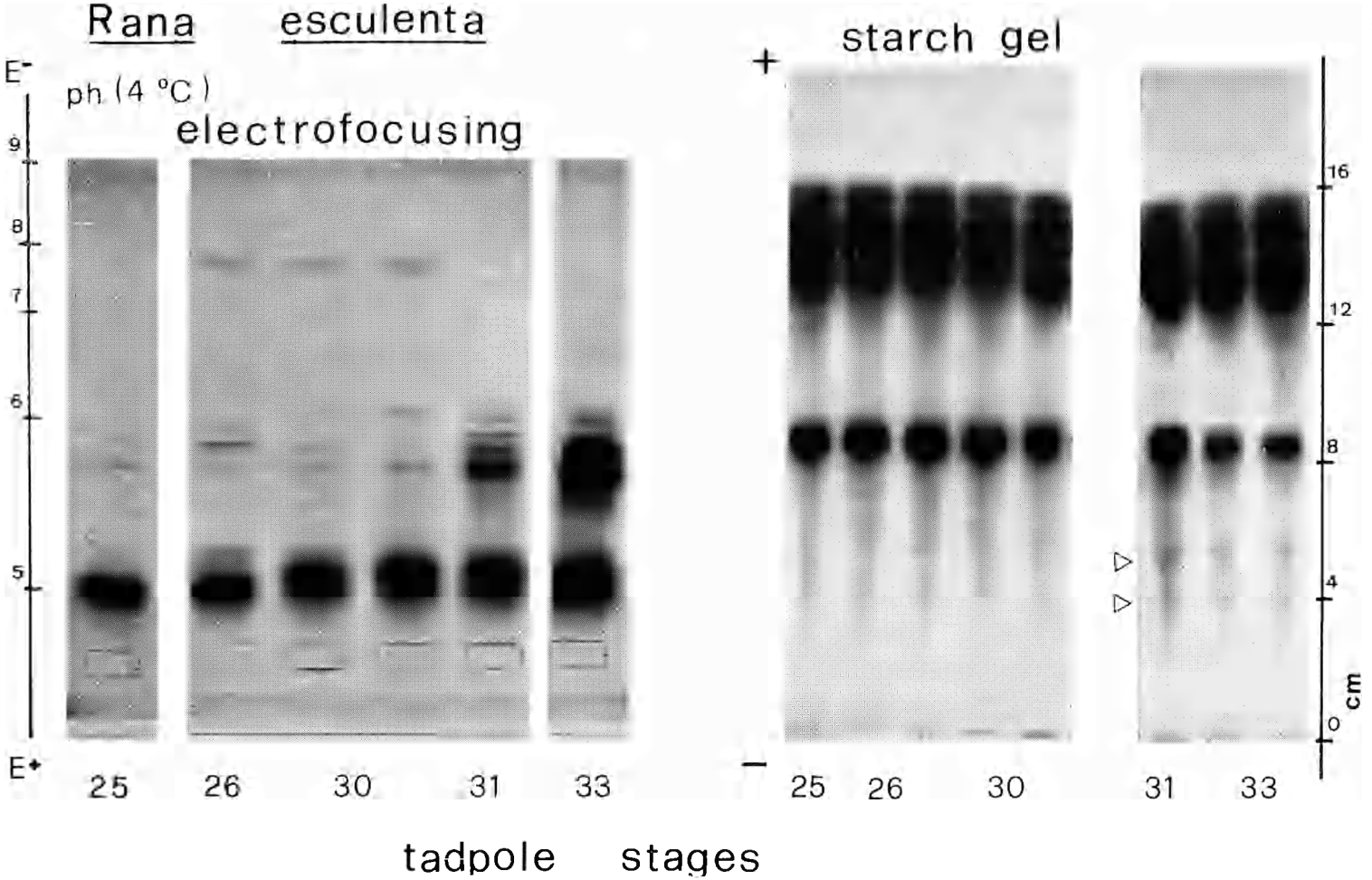

Thin layer isoeletric focusing (*left*) and starch-gel electrophoresis (*right*) of nonspecific liver esterases of *Rana esculenta* tadpoles during metamorphosis. (For tadpole stages see text).