3 F.J. Seil, A.L. Leiman, M.M. Herman and R.A. Fisk, Exp. Neurol. *55*, 390 (1977).
4 W. Rössner, Zbl. Vet. Med. A. *18*, 139 (1971).
5 G. Back, G. Seidel and W. Endell, Pharmacology *14*, 67 (1976).
6 T. Eriksson, S. Liljequist and A. Carlsson, J. Pharm. Pharmac. *31*, 636 (1979).
7 P.J. Basseches and G.J. DiGregorio, Drug Alcohol Depend. *5*, 305 (1980).
8 T. Tervo, J. Lehtosalo, V.-P. Lehto, M. Heino, J. Kantola and L.A. Laitinen, Aviat. Space Environm. Med. *51*, 137 (1980).
9 T. Tervo, F. Joo, A. Palkama and L. Salminen, Experientia *35*, 252 (1979).
10 K.O. Lindros, in: Research Advances in Alcohol and Drug Problems, vol.4, chapter 4. Ed. Y. Israel, F.B. Glaser, H. Kalant, R.E. Popham, W. Schmidt and R.G. Smart. Plenum Press, New York and London 1978.
11 K.O. Lindros and J.D. Sinclair, Drug Alcohol Depend. *4*, 95 (1979).
12 C.J.P. Eriksson, H.W. Sippel and O.A. Forsander, Analyt. Biochem. *80*, 116 (1977).
13 J.H. Chin, in: Biochemistry and Pharmacology of Ethanol, vol.2, chapter 7. Ed. E. Majchrowicz and E.P. Noble. Plenum Press, New York and London 1979.
14 T. Broman and A.M. Lindberg-Broman, Acta physiol. scand. *10*, 102 (1945).
15 S.I. Rapoport, Am. J. Physiol. *219*, 270 (1970).
16 S.I. Rapoport, M. Hori and I. Klatzo, Am. J. Physiol. *223*, 323 (1972).
17 H. Kalant, in: The Biology of Alcoholism, vol.1, p.1. Ed. B. Kissin and H. Begleiter. Plenum Press, New York 1971.
18 A. Stowell, M. Hillbom, M. Salaspuro and K.O. Lindros, Alcoholism: clin. exp. Res. *3*, 274 (1979).
19 L.D. MacLeod, Q. Jl Stud. Alcohol *11*, 385 (1950).
20 R. Hemmingsen, M.M. Hertz and T.G. Bolwig, Stroke *11*, 141 (1980).
21 H. Karwacka, Exp. Path. *18*, 118 (1980).

Some properties of *Bufo bufo* tyrosinase during development

M. Miranda[1], D. Botti, M. Di Cola and A.M. Ragnelli

Institute of General Biology, University of L'Aquila, via Assergi 6, I-67100 L'Aquila (Italy), 5 January 1981

Summary. Tyrosinase expression during *Bufo bufo* development has been investigated. Until stage 19, only 1 electrophoretic band is detectable, but at a later stage (25) 3 bands appear. The K_m for L-3,4-dihydroxyphenylalanine (L-dopa) was also determined.

Vertebrate tyrosinase (EC 1.14.18.1) is expressed in some neural crest derivatives; melanocytes and mosaic pigment cells such as erythrophores, melanophores and iridophores, which, according to Bagnara et al.[2], originate from a common stem cell. Transdetermination of some other neural crest derivatives, such as autonomic nervous system cells and chromaffin cells, may result in tyrosinase expression[3,4]. The compartmentation of this enzyme within melanosomes of the specialized melanogenic cells may assume a role in the regulation of L-tyrosine and L-dopa partition between melanogenesis and catecholaminosynthesis[2,5].

During amphibian development tyrosinase is expressed after neural induction, when a new synthesis of enzyme occurs[5]; information is available about enzyme properties and isozyme pattern in *Rana pipiens* skin[6,7]. The aim of the present work is to provide information about some kinetic properties of embryo tyrosinase, and the disc-gel electrophoresis pattern of the enzyme extracted from *Bufo bufo* embryo melanosomes during development.

Experimental. 50 ml of *Bufo bufo* embryos, from the same ovoposition which occurred in the laboratory, at the stages 8 and 25 of development[8], were washed thrice with 0.5 M sucrose, resuspended with 30 ml of the same medium and homogenized by hand with a Potter homogenizer by 4 pestle strokes. The homogenate was centrifuged for 5 min at 650 × g, and the supernatant was collected and recentrifuged as above; 2 volumes of this supernatant were layered on a sucrose discontinuous density gradient consisting of 1.8 M sucrose (1 vol.) and 1.4 M sucrose (1 vol.), and centrifuged for 120 min at 73,000 × g. Premelanosomes were recovered at the bottom of the tube, as ascertained by electron microscopy and tyrosinase detection (figs 1A and

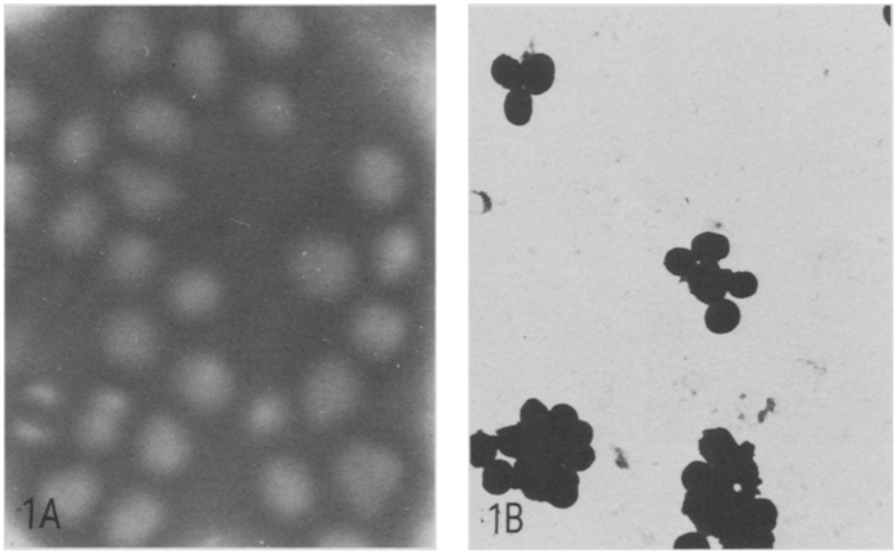

Figure 1. Electron micrographs of negatively stained *Bufo bufo* embryo melanosomes. *A* Premelanosomes from stage 8. × 20,000. *B* Late melanosomes from stage 14. × 12,750.

2A). Different methods[9] were employed to prepare melanized 'late' melanosomes (fig. 1B) from the stages 3, 6, 9, 12, 14 and 19. The negatively stained melanosome preparations for the electron microscopy were prepared as previously described[9]. Tyrosinase was extracted from melanosomes by resuspending the melanosomal pellet with 0.10 M sodium phosphate buffer, pH 7.0, containing 1% of sodium deoxycholate, and stirring for 60 min at 4 °C. The solubilized melanosome suspension was centrifuged for 60 min at 110,000 × g with a Spinco-Beckman L2-65B centrifuge. The supernatant was used for tyrosinase investigation. Tyrosinase activity was spectrophotometrically measured according to Pomerantz and Peh-Chen Li[10]; under our conditions of assay the detection limit for tyrosinase activity was 2.7 μU.

Tyrosinase polyacrylamide disc-gel electrophoresis detection was carried out as previously described[9,11]. Controls for tyrosinase activity were performed by the addition of phenylthiourea (PTU) to the reaction mixtures (0.1 M final concentration). Proteins were determined by the biuret reaction according to King[12]. Reagents were purchased from Sigma and Boehringer.

Results and discussion. Both electron microscope (fig. 1) and disc-gel electrophoresis (fig. 2A) showed that our preparations consisted mainly of melanosomes, since tyrosinase was extracted from these organelles. As judged by the intensity of pigmentation, the preparation in figure 1A (from stages 8 and 25), is enriched with premelanosomes. For comparison, the preparation in figure 1B obtained by a different method[9], is enriched with late melanosomes.

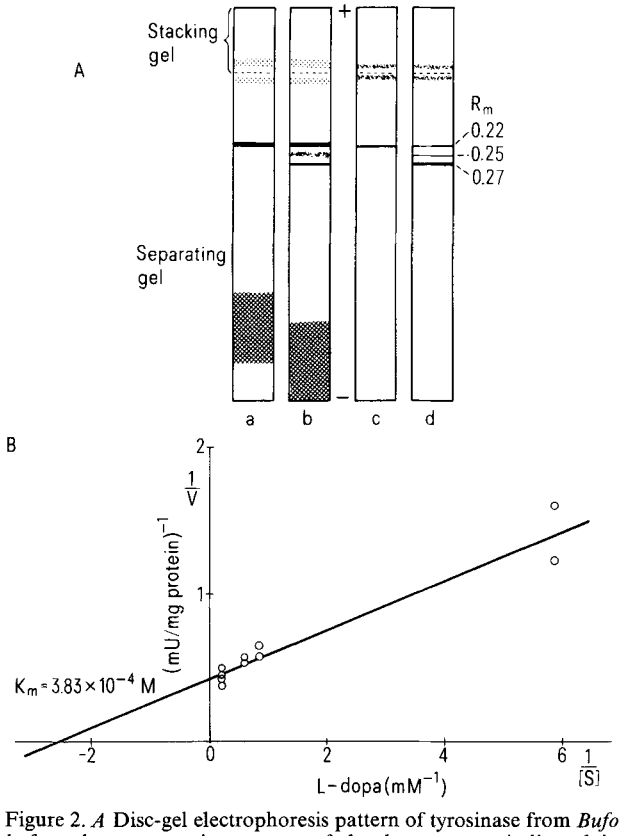

Figure 2. *A* Disc-gel electrophoresis pattern of tyrosinase from *Bufo bufo* embryos at various stages of development, as indicated in Experimental: *a* (stages 3–19) and *b* (stage 25) Coomassie staining; *c* (stages 3–19) and *d* (stage 25) L-dopa staining. *B* Double reciprocal plot of whole tyrosinase activity from the stage 25 premelanosomes. The K_m-value is indicated. The curve was calculated by the least-squares method.

The melanosomes prepared by either method express the same electrophoretic pattern of tyrosinase at each stage tested (fig. 2A), except at stage 25, when 3 bands appear. Spectrophotometrically, tyrosinase activity was detectable only starting from stage 19. This agrees with the findings in *Rana pipiens*[5] where new tyrosinase is synthesized at the tail bud stage; however, in our experiments tyrosinase was electrophoretically detectable at stages of development previous to stage 19. This may be due to the extraction from concentrated melanosomes.

The absence of a spectrophotometrically detectable tyrosinase activity in *Bufo bufo* embryos prior to stage 19, when the existence of the protein can be shown by electrophoresis, shows that the protein, possibly of maternal origin, is either inactivated or must be processed by a post-translational control system. The findings of Benson and Triplett[5] support the view that before neurulation no enzyme activity is detectable and no new enzyme is synthesized. Our findings also are in agreement with this point of view, because as the tyrosinase reaction proceeds the enzyme loses its activity due to the binding with reaction products[10,11] and no difference in enzyme activity appears to exist between melanosome populations (pre- and late). In fact, premelanosomes from the stage 8 and late melanosomes from the stages 3, 6, 9, 12, 14 show no spectrophotometrically detectable activity but the same electrophoretic tyrosinase band; starting from stage 19 enzyme activity from late melanosomes dramatically increases but the electrophoretic band of tyrosinase is the same as in previous stages.

On the other hand, the appearance of novel tyrosinase bands occurring at stage 25 may be due either to the expression of a different tyrosinase gene or genes, or to the processing of the protein expressed at stage 19.

In figure 2A the R_m-values of the tyrosinase bands are indicated. Some melanized material does not enter the gels; this material may be inactivated tyrosinase, according to previous findings with mushroom tyrosinase[11].

The K_m of whole tyrosinase from stage 25 is 3.83×10^{-4} M, as calculated by the double reciprocal plot (figure 2B); this value is in the range of vertebrate tyrosinases[10].

In conclusion, during *Bufo bufo* development tyrosinase is electrophoretically detectable from stage 3 to stage 25, but only after stage 14 is it detectable using the spectrophotometer; at stage 25 new isozymes appear. Whether new tyrosinase genes are expressed and/or a post-translational control is responsible for the increase of activity and for the change of the isozyme pattern is a matter for future work.

1 To whom the reprint request should be addressed.
2 J.T. Bagnara, J. Matsumoto, W. Ferris, S.K. Frost, W.A. Turner, Jr, T.T. Chen and J.D. Taylor, Science *203,* 410 (1979).
3 Y. Itoh, T.S. Okada, H. Ide and G. Eguchi, Devl Growth Different. *17,* 39 (1975).
4 D.H. Nichols and J.A. Weston, Devl Biol. *60,* 217 (1977).
5 S.C. Benson and E.L. Triplett, Devl Biol. *40,* 270 (1974).
6 R.B. Mikkelsen and E.L. Triplett, J. biol. Chem. *250,* 638 (1975).
7 B.G. Barisas and J.S. McGuire, J. biol. Chem. *249,* 3151 (1974).
8 R. Rugh, in: Experimental Embryology, p. 56. Burgess Publishing Company, Minneapolis 1962.
9 M. Miranda, D. Botti, C. Pantani, P. Conti and L. Nicoli, Devl Growth Different. *21,* 465 (1979).
10 S.H. Pomerantz and J. Peh-Chen Li, in: Methods in Enzymology, vol. XVII A, p. 620. Ed. S.P. Colowick and N.O. Kaplan. Academic Press, New York 1970.
11 M. Miranda, G. Urbani, L. Di Vito and D. Botti, Biochim. biophys. Acta *585,* 398 (1979).
12 T.E. King, in: Methods in Enzymology, vol. X, p. 202. Ed. S.P. Colowick and N.O. Kaplan. Academic Press, New York 1967.