

above effect of ovariectomy is interfered with by the interaction of acid extract of cattle anterior pituitary, which has the tendency to exert its specific growth-promoting and calcifying influence on the cartilage. If the two factors act simultaneously, a competition between both principles takes place and a combination effect results, the outcome of which determines the course of the endochondral ossification.

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Enzymes in Ontogenesis (Orthoptera). III. Activation of Naturally Occurring Enzymes (Tyrosinase).*

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Interest in the enzyme tyrosinase seems largely to have centered around its distribution as well as the nature of its action on various natural and artificial substrates. Its rather wide occurrence in plants seems well established while for animals data are available which would lead one to assume a rather limited or restricted distribution (Graubard and Nelson,¹ v. Euler,² Graubard,³ Raper⁴). Insects appear to be rich in the enzyme (Bodine and Boell⁵).

The present paper treats of results of a study on the growth, activation and substrate relations of tyrosinase as found in the developing egg of the grasshopper, *Melanoplus differentialis*. The advantages in using such biological material have been previously indicated and mention will only be made where departures from usual procedures occur.⁵ Enzyme preparations were obtained from centrifuged fractions of egg brei after the following manner. For each set of experiments, 200 eggs of known age were removed from pods and carefully selected for morphological stage of development. They were thoroughly washed in tap water, then sterilized in 70% alcohol, washed three times with sterile distilled water and finally

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¹ Graubard, Mark, and Nelson, J. M., *J. Biol. Chem.*, 1935, **112**, 135.

² v. Euler, H., 1934, *Chemie der Enzyme*, J. F. Bergmann, München.

³ Graubard, Mark, *J. Genetics*, 1933, **27**, 199.

⁴ Raper, H. S., *Physiol. Reviews*, 1928, **8**, 245.

⁵ Bodine, J. H., and Boell, E. J., *J. Cell. and Comp. Physiol.*, 1935, **6**, 263.

twice with sterile buffered (phosphate pH 6.8) 0.9% sodium chloride. Excess fluid was allowed to drain from the eggs which were then transferred to a sterile glass mortar and thoroughly ground. Two cc. of buffered 0.9% sodium chloride were added and mixed with the brei. The mixture was then poured into a sterile graduated centrifuge tube. The washing of the mortar with buffered 0.9% sodium chloride was repeated 3 times and the contents of the centrifuge tube brought to volume, 10.5 cc., with buffered 0.9% sodium chloride. This diluted egg brei was then centrifuged for 10 minutes in an International Clinical centrifuge at a speed of 1060 r.p.m. At the end of this period the contents were stratified into 3 definite and clear-cut layers: a thin topmost layer, *A*, of lipoidal and yellow pigment material; a middle, slightly turbid layer, *B*, making up by far the greatest of the three; a dark layer, *C*, about 0.5 cc. in volume and made up of the remains of the membranes, etc., of the eggs. The yellow lipoidal layer was carefully removed by means of filter paper and a capillary pipette attached to an aspirator. The layer *B* was poured into a sterile centrifuge tube leaving *C* behind. Fraction *B*, which was the one employed in the following experiments was then ready for use and as prepared was equivalent to 20 eggs per cc. of solution. All determinations of enzyme concentration were carried out in standard Warburg manometers at 25°C. using tyramine hydrochloride as substrate, essentially as indicated previously.⁵

In preliminary experiments, it was easily demonstrated that the enzyme tyrosinase was largely if not solely confined to the *B* fraction of the above mixture. However, if the *B* fraction is added to the substrate, tyramine HCl, very little activity occurs over long periods of time, indicating the possible existence of an inhibitor or the lack of a naturally occurring activator. By checking back it was soon discovered that the addition of the fraction *A* caused an almost immediate response on the part of the enzyme in the *B* fraction. A detailed study was next made on the question of other possible activators for the enzyme. A large list of compounds was found to work rather favorably in this respect. The list of activators includes the following: chloroform, acetone, ethyl urethane, sodium taurocholate, ether, heat (60°C. for 10 min.), sodium oleate, toluol, thymol, olive oil, etc. Results are presented using only sodium oleate as activator since this compound has been found to function in this respect especially well. In a typical experiment the various constituents of the manometer flasks were made up as follows: 1 cc. of *B*; 0.1 cc. of 5% sodium oleate; 0.1 cc. of 10% potassium hydroxide (center well); 1.6 cc. of buffered (pH 6.8) 0.9% sodium chloride; 0.3 cc. of 0.4% tyramine HCl (in side bulb).

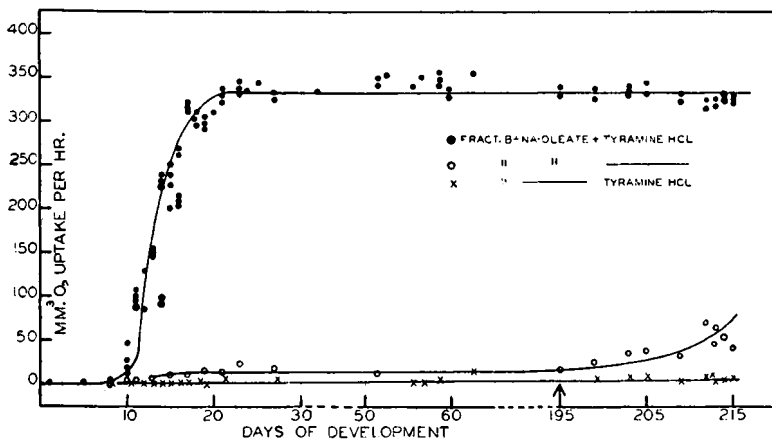


FIG. 1.

Figure shows amount of enzyme (tyrosinase), its relative activity, as well as naturally occurring substrate in the developing egg of the grasshopper (*M. differentialis*) from laying to hatching. Abscissa, indicates days of development at 25°C. Arrow indicates beginning of post-diapause. Ordinate, cubic millimeters of oxygen consumed per hour by enzyme extract B (equivalent to 20 eggs per cc. of extract). Points indicated for individual experiment in most cases, otherwise represent averages.

Results of many experiments have been graphically shown in the accompanying figure. From an inspection of this figure it will be noted that the enzyme in fraction B without the addition of sodium oleate shows practically no activity during the entire course of the development of the egg. Upon addition of sodium oleate, however, marked activity occurs. As a matter of fact the theoretical oxygen uptake in many cases is approximated at the end of an hour. That the total enzyme present has not been used up can be clearly demonstrated upon the addition of more substrate, either tyramine HCl, p-cresol, or tyrosine. It would thus suggest that in the fraction B tyrosinase is normally inhibited or non-activated even though its amount naturally may be increasingly great. Activation of B can also be accomplished by the addition of A, the 'naturally occurring activator(?)'. The growth of the enzyme during ontogeny is indicated in the figure and agrees essentially with that previously pointed out.⁵ It is of some interest to again recall that the maximum amount of the enzyme is produced early in ontogeny, *i. e.*, during the first 21 days, and stays relatively constant thereafter. The question of naturally occurring substrate is of interest since these embryos apparently are not pigmented until after or upon hatching. From an inspection of the middle curve (substrate curve) in the figure it will be seen that substrate occurs naturally only late in embryonic development. It would thus seem that the embryo's content of tyro-

sinase is produced long before the substrate upon which it normally reacts is formed.

The question of the activation of the enzyme tyrosinase is of some further interest in view of the fact that in many cases its presence in tissues has been often questioned. It would seem from the present results that the enzyme may be present, but lacking an activator or substrate, no indications of its activity are evident.

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Deposition of Amyloid in Kidneys with Restricted Circulation.

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In an earlier report,¹ one of us described a method for production of renal insufficiency in rabbits, by reducing the blood flow through the renal artery of one of the kidneys by means of a loop of thread of a definite diameter. The loop is put on when the animals are small, the artery grows up to the size of the loop, and thereafter growth of the kidney stops. The other kidney continues to grow, and it is removed at some time later with the resultant production of renal insufficiency.

It has been our experience that the rabbits will survive if the restricted kidney represents 30% or more of the total previous kidney mass; in other words, if the restricted kidney is about half or more of the mass of the one removed. A group of 6 such animals survived for periods varying from 1½ to 10 months. The remaining kidney in each of these animals was swollen and pale, and microscopic examination revealed the presence of amyloid in the glomerular tufts and about the intertubular capillaries. This infiltrating substance was identified as amyloid by the methyl violet, congo red, and iodine staining methods. No amyloid was found in other organs.

No amyloid was found in the kidneys of a group of 10 rabbits surviving, one month or less, the removal of the normal kidney. Furthermore, no amyloid was found in the kidneys with restricted blood supply where the normal kidney was not removed.

¹ Drury, D. R., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 856.