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β -Glucuronidase Activity During the Early Development of *Xenopus laevis* Embryos

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β -Glucuronidase has been associated with the proliferation of cells in normal and neoplastic tissue (Levy, Kerr & Campbell, 1948; Fishman & Anylan, 1947). Rapid cell divisions are characteristic of certain phases of embryonic development; it is therefore of some interest to study the behaviour of β -glucuronidase during the early development of embryos. The possible implication of steroids in the primary differentiation of the amphibian embryo (Waddington, Needham, Nowinski, Needham & Lemberg, 1934) adds interest to such a study, particularly in view of the suggestion of Fishman (1947) that β -glucuronidase may be connected with the metabolism of steroids in mammalian tissues. Apart from these special considerations, there is clearly a general need for more information on the behaviour of enzymes during early embryonic development.

The South African clawed toad (*Xenopus laevis*) was chosen for this investigation because large numbers of fertile eggs can be obtained from these animals, at any time of the year, by injections of chorionic gonadotrophin. Initially a number of determinations showed that a small amount of β -glucuronidase activity was present in extracts of embryos, ranging from two-cell to newly hatched larvae. In this paper the results of a large number of determinations show that small, real variations of activity occur during the early stages of development. A preliminary account of this work has been given elsewhere (Billett, 1956).

EXPERIMENTAL

Fertilized eggs were obtained from the toads by injection of chorionic gonadotrophin. Egg capsules were removed by papain and thioglycollate (Spiegel, 1951). Approximately 300 embryos of about the same stage were selected and divided into groups of about 60. After several washes in Holtfreter solution diluted ten times with water (Hamburger, 1947) the embryos were allowed to develop, in the solution, at 18°. The development of the embryos was stopped at a known time by placing them in a deep-freeze at -20°. Before freezing the embryos were grouped in stages according to the second column of Table 1. This staging is based on a description of the external appearance of the embryos at intervals of approx. 2 hr. at 18°. The stages chosen correspond fairly well with those of Nieuwkoop & Faber (1956).

After 2-14 days at -20°, each sample was homogenized at about 5° in a small quantity of water and made up to 1 or 2 ml. An all-glass homogenizer of the Potter-Elvehjem type was used, operated by hand. The material was readily broken down during the course of 1 min. This operation was carried out at about 5°. Homogenates were stored in a refrigerator overnight and then centrifuged at about 3000 g. The centrifuging resulted in a fractionation similar to that described by Panijel (1950). Residues were washed once by centrifuging in the original volume of water. The original supernatant, together with the washing from the residue, constituted the enzyme extract. Microscopic examination of the residue showed it to consist largely of intact yolk platelets.

The β -glucuronidase activity of the extracts was determined by a method previously described (Billett, 1954). This method is based on the colorimetric determination of 8-hydroxyquinoline formed by the hydrolysis of its

glucosiduronic acid. Briefly, the assay was carried out as follows. Enzyme extract (0.5 ml., corresponding to six to eight embryos), 0.5 ml. of 0.005 M-substrate in M-acetate buffer, pH 4.5, and 0.5 ml. of water (test) or 0.5 ml. of 0.001 M-potassium hydrogen saccharate (control) were incubated together for 2 hr. at 25°. The reaction was stopped by cooling in ice and adding trichloroacetic acid. The quinoline was determined by means of Ehrlich's diazo reagent. Assays were in duplicate. The optimum pH for enzyme activity in acetate buffer was found to be in the region of 4.5 for all stages. The activities of the extracts were expressed as $\mu\text{g.}$

Fig. 1. β -Glucuronidase activity of extract of mid-cleavage embryos. O, with water (test); ●, with 0.001 M-potassium hydrogen saccharate (control).

of 8-hydroxyquinoline liberated/hr./g. dry wt. of the original material. The dry weight of the embryos was determined in two ways. Initially, a small amount (0.2–0.5 ml.) of the original homogenate from each embryonic stage from a single ovulation was dried to constant weight in a vacuum desiccator over P_2O_5 . These determinations showed that no appreciable gain or loss in weight of the embryos occurred during the developmental period under investigation. In later experiments a batch of about thirty embryos of a known stage was taken as representative of all the embryos from a single ovulation. The dry weight of these was determined as before. The results of the determinations of dry weight are given in Table 1. The number of determinations for each stage is given in parenthesis after the average value (in $\mu\text{g.}$) for the dry weight calculated for a single embryo. The two methods gave similar values.

The activity of the extracts varied linearly over a concentration range corresponding to 4–20 embryos/ml. of homogenate. The amount of 8-hydroxyquinoline liberated was directly proportional to time over an incubation period of 4 hr. (Fig. 1).

Estimations were made on embryos from twenty different ovulations, obtained from about twelve different pairs of toads. A large number of determinations were necessary because of the large variation in enzyme activity between embryos of the same stage from different ovulations. It was not possible to cover the whole range of early development with eggs from a single ovulation. Usually a maximum of six consecutive stages out of a desired seventeen could be obtained from a single ovulation.

RESULTS

The results are summarized in Table 1, in which the mean values of several determinations on embryos of the same stage from different ovulations are given. The individual values for each stage cover a wide

Table 1. β -Glucuronidase activity of *Xenopus* embryos

The number of determinations is indicated in parentheses.

Embryonic process	Description of stage	Age (hr. at 18°)	Average dry wt./embryo ($\mu\text{g.}$)	Activity ($\mu\text{g.}$ of 8-hydroxyquinoline/g./hr.)		Percentage of total activity in homogenate residues
				Mean value	Range	
Cleavage	Unfertilized	0	555 (1)	531 (4)	450–670	—
	2–4 cells	2–3	560 (1)	482 (3)	340–555	—
	8–16 cells	4–6	578 (1)	407 (3)	262–540	—
	Mid-cleavage	7–8	460 (2)	448 (3)	313–670	—
	Late cleavage	9–10	537 (1)	586 (4)	498–630	3
	Blastula	12	598 (2)	441 (3)	385–550	—
Gastrulation	Dorsal lip a small crescent	13.5	571 (3)	438 (10)	330–575	<1
	Dorsal lip complete and $\frac{1}{2}$ diam. of embryo	17	626 (2)	421 (5)	330–600	—
	Dorsal lip $\frac{1}{2}$ diam. of embryo	19	613 (2)	437 (7)	305–603	—
	Dorsal lip $\frac{1}{2}$ diam. of embryo	21	—	441 (6)	350–550	—
	Yolk plug	24	552 (4)	407 (7)	232–600	<1
	Beginning of neural plate	26	—	351 (11)	210–465	<1
Neurulation	Neural plate outlined	28	553 (5)	342 (4)	240–510	—
	Neural folds forming	30	—	391 (6)	300–570	—
	Neural folds fusing in mid-dorsal line	32	564 (2)	408 (5)	300–470	10
	Neural folds completely fused	36	—	380 (2)	340–420	—
	Hatching	72	506 (1)	393 (3)	220–490	4

range. The large variation within a stage appears to be the result of two factors. In the first place there is an inherent variation in the biological material; embryos of the same age from different ovulations have different activities. The second contribution to the variation will come from the error involved in the determinations themselves.

The variation inherent in the material is illustrated in Fig. 2. In this figure mean values for stages corresponding to cleavage, gastrulation, neural-plate formation and neural-tube formation of embryos from the same ovulation are joined by straight lines. Data from seven ovulations are given. The variation of activity of embryos from different ovulations is obviously large; it appears to be of the same order of magnitude as that described by Cohen (1954) for the glycolytic activity of embryonic extracts of *Rana pipiens*. An estimate of the experimental error was made by analysing the results of replicate determinations. The analyses showed that the standard deviation for a single determination was about 100 units, corresponding to an error of 70 units for a duplicate determination, or to a coefficient of variation of about 15%. Although this error is large, it is considerably smaller than the inherent variation of the material.

Analysis of the variation between stages does not reveal any significant change of enzyme activity during cleavage and gastrulation. The activity of the unfertilized egg appears to be more or less maintained during these early stages. The values obtained for the neural plates are, however, significantly lower than those for the preceding and the subsequent stages. The probability that this difference is due to chance is less than 1% (*t* test).

Fig. 2. Comparison of β -glucuronidase activity of embryos from seven different ovulations. (Values for individual ovulations are joined by lines.)

The activity of the extracts of neural plate appears to be some 30% lower than that of extracts from the other stages.

A small number of determinations were made on the residues obtained by centrifuging the homogenates. These determinations indicated an activity of less than 5% of the total in most cases (final column of Table 1).

DISCUSSION

Investigations of regenerating liver and nerve (Levy *et al.* 1948; Hollinger & Rossiter, 1952) suggest that β -glucuronidase is in some way related to the metabolism of proliferating cells. This idea is supported by data which indicate that in some cancerous tissue the β -glucuronidase level is raised compared with the normal homologous tissue (Fishman, 1950). More recently, however, it has been shown that β -glucuronidase activity increases during atrophy of lymphatic tissue (Pellegrino & Villani, 1956). Thus for vertebrate tissues enhanced β -glucuronidase activity cannot be specifically associated with cell proliferations, particularly when it is remembered that other enzymes show enhanced activity under similar conditions (Greenstein, 1947; Needham, 1952). The presence of large amounts of β -glucuronidase in the digestive glands and juices of certain molluscs and insects is also incompatible with any generalized hypothesis connecting β -glucuronidase with cell proliferation.

The work which has been presented in this paper does not suggest any connexion between β -glucuronidase activity and rapidly dividing cells. During the early development of *Xenopus* embryos no dramatic increase in the activity of this enzyme appears to occur, although rapid cell cleavage is manifestly a feature of this development. The only marked change to occur is an undeniable fall in activity when the neural plate is formed. This fall in activity corresponds to a period which marks the onset of differentiation in the amphibian embryo. At this time various physiological changes are known or suspected to occur. Glycogenase activity appears to decrease in certain amphibian embryos at about this time (Barnes, 1939). It appears to be well established that the glycogen content of such embryos decreases during gastrulation (Jaeger, 1945). It has also been suggested that tissues which become invaginated at about this time will necessarily tend to have an anaerobic metabolism (Barth, 1942). It is difficult to imagine how such changes could be related to a fall in β -glucuronidase activity or to variations in the activity of nucleases shown to occur during the development of *Rana pipiens* (Finnamore, 1955).

The role of β -glucuronidase in the embryo is the subject of speculation. Waddington & Needham

(cf. Needham, 1942) suggested that the primary evocator, upon which the differentiation of the embryo depends, is a steroid substance of some kind. They also proposed that this substance was present in a masked condition in the tissues, the active steroid presumably being liberated at the right time and place to cause differentiation. It is tempting to suggest that β -glucuronidase acting on a steroid glucuronide could provide the actual mechanism of this change. It is, of course, dangerous to assume that the measured activity of the enzyme in the crude extracts bears any relation to the activity in the intact embryos. Possibly, β -glucuronidase is best considered as analogous to enzymes such as cathepsin. Such enzymes, showing high activity in tissue extracts, may not be very active in the living tissues. In this connexion it may be noted that de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) have demonstrated that in certain tissues cathepsin and β -glucuronidase are associated in one type of cytoplasmic particle, which releases active enzymes after the tissues are homogenized.

SUMMARY

1. The β -glucuronidase activity of extracts from various stages of *Xenopus laevis* embryos has been measured.

2. The only significant change detected was a fall in the activity of the enzyme in the extracts of the neural plates.

3. These results do not support the idea that β -glucuronidase is associated with the proliferation of cells.

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The Phosphoesterase of Pea Plants (*Pisum sativum* L.)

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A number of polyphosphatase activities present in extracts of pea plants (*Pisum sativum* L.) are associated with an unspecified phosphoesterase (Pierpoint, 1957). This enzyme liberates orthophosphate from trimetaphosphate, tripolyphosphate, commercial 'hexametaphosphate' and a number of organic phosphates, although not from tetrametaphosphate. Chromatography of the enzyme on an ion-exchange resin resolves it into two fractions which have similar enzymic activities.

Two problems arising out of this work were the heterogeneity of the enzyme, and the association of the phosphomonoesterase activity with the diesterase activity against trimetaphosphate. The present paper presents evidence that the heterogeneity of the enzyme is not due to the 'purification' procedure to which it is subjected, and confirms the association of the mono- and di-esterase activities. It also provides additional information on the specificity of the enzyme, and the path by which it breaks down polyphosphates.