Cytochemical Studies on the Embryonic Development of *Drosophila melanogaster*

II. Alkaline and Acid Phosphatases

BY

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With two Plates

The distribution of phosphatases during the embryonic development of *Drosophila melanogaster* Meig. was chosen as the next object of study for three main reasons: (1) a satisfactory technique for the demonstration of phosphatase (at least in the case of alkaline phosphatase) is available; (2) the possible significance of phosphatases during embryogenesis is indicated by the work of Moog (1944); and (3) the *Drosophila* embryo provides favourable material for a study of the relation between the phosphatases on one hand and growth and differentiation on the other, since a dissociation of these two morphogenetic processes is evident (Yao, 1950).

**MATERIAL AND METHODS**

The material and the procedures designed to obtain the requisite stages of the developing oocytes and embryos have been described in the first paper of this series.

For the demonstration of alkaline phosphatase, Danielli's (1946) method was generally adhered to. Certain minor modifications in timing were, however, found to be necessary for the present material, because of the relatively low content of the enzyme. Eighty per cent. alcohol was used as fixative. The duration of the steps between fixation and incubation was therefore deliberately shortened, especially that in the paraffin bath (m.-p. 52° C., 1–1½ hours at 56° C.). Sections were cut at 10 μ. Sodium glycerophosphate (B.D.H.), consisting of almost pure beta-salt, was used as substrate. The pH of the incubation mixture was 9·40. Since it was found that the alkaline phosphatase of *Drosophila* is also activated by Mg ion, magnesium chloride was added to the medium to give a final concentration of 0·01 M. The duration of incubation was 4 hours at 37·5° C. In several cases, the period was prolonged to 8, 12, and 24 hours.

Gomori's technique for the demonstration of acid phosphatase has recently been questioned on certain grounds (Bartelmez and Bensley, 1947, and others). The problem of non-specific adsorption was therefore studied, using *Drosophila* Quarterly Journal Microscopical Science, Vol. 91, part 1, March 1950.
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Drosophila ovaries, by superimposing active sections on inactivated (5–10 minutes in boiling water) sections and incubating them together for various lengths of time (8, 12, 24, 48 and 72 hours). It was found that after short incubation (8, 12 hours) the inactivated sections were practically unstained. Non-specific diffuse yellowish staining in these sections became evident in 24-hour series when the active sections gave a dark golden-brown reaction. Such contamination was more intense after 48- and 72-hour incubation. From this result, it appears that incubation requires very critical timing so as to give a maximal possible reaction but at the same time a minimum of non-specific complications. Moreover, lead phosphate was found to be rather stable, for alternate sections of ovaries show almost no difference in the reaction intensity whether they are immediately visualized after incubation or visualized after a preliminary soaking in distilled water for 24 hours subsequent to incubation. On the other hand, lead sulphide was found to be extractable by distilled water: soaking for a few hours can already produce a visible effect. Furthermore, the distribution of acid phosphatase in the Drosophila ovary has been checked by incubating fresh unfixed material: enzyme localization, identical to that found in the fixed sections, was obtained: this will be mentioned later.

Moog’s (1944) modified procedure for beta-glycerophosphate was used in this work. Chilled acetone was used as fixative throughout (80 per cent. alcohol destroyed nearly all the acid phosphatase activity in Drosophila material). The pH of the resulting incubation mixture as determined potentiometrically was 4.70. Incubation at pH 5.0 and 5.3 was also tried. However, owing to the heavy precipitation of beta-glycerophosphate under these conditions, detailed study was not possible. The duration of incubation was 16 hours at 37.5° C. Control sections were incubated either without substrate or in the presence of 0.01 M. fluoride.

The difficulties which other workers have experienced in the histochemical demonstration of acid phosphatase have also been encountered in the present material. Evidently this is mainly due to the fact that both fixation and paraffin embedding cause a far greater loss of activity for acid phosphatase than for alkaline phosphatase (Stafford and Atkinson, 1948). An effort was made to intensify the reaction by adding 1-ascorbic acid to the incubation mixture (Moog, 1944). About a fourfold increase of enzyme activity was obtained with a concentration of ascorbic acid as low as 0.005 M. The above value was estimated from the difference in the time of incubation required for attaining an approximately equal intensity of enzyme reaction in the presence and in the absence of ascorbic acid. However, the use of ascorbic acid was complicated by the formation of yellowish-white turbidity in the medium. The results to be described in this paper are based on reactions carried out without the addition of 1-ascorbic acid.

The localization of phosphatases in Drosophila testes has also been studied: the result will be briefly dealt with.
RESULTS

I. Alkaline Phosphatase

(a) Oocyte development. Alkaline phosphatase is not demonstrable in Drosophila ovaries taken from 1-day-old females. Even when incubation was extended to 24 or 48 hours, only a very feeble reaction could be noticed in the nucleolar materials of the nurse cells and follicular epithelial cells. Both experimental and control sections appear greyish, possibly due to the presence of preformed phosphate. However, the epithelium of the oviducts gives a moderate to strong reaction.

Testes from adult males also show no histochemical trace of the enzyme.

(b) Embryonic development. Following on the negative reaction of mature oocyte and sperm, the enzyme is not detectable (even in the nuclei) in fertilized eggs nor in embryos up to a stage just prior to the contraction of the germ band. Alkaline phosphatase arises suddenly in the embryo during, or more likely immediately after, this morphogenetic stage. It invariably makes a first appearance in the ventral part of the embryo near the future thorax, although the exact centre of origin is rather difficult to locate. However, from a study of a great number of 8½- to 9-hour embryos, I got the impression that the centre lies probably in the latero-ventral ectoderm (opposite the ventral nerve cord) of the metathoracic and first two abdominal segments. Topographically, these segments occupy almost a central position along the antero-posterior axis of the embryo and their hypodermis always show a stronger alkaline phosphatase reaction than does the hypodermis of other segments in the period soon after the appearance of enzyme activity (Pl. 1, fig. 1). In most cases, only one such centre is found on the one side of the median axis. Occasionally two centres, one on either side of the median axis, are found.

From the centre (or centres) the enzyme activity spreads to the neighbouring hypodermal cells and to the interior organs in a quite characteristic manner, for definite gradients of alkaline phosphatase reaction are always observable in embryos fixed between the 9th and 12th hour after laying. At least two such gradients are noticeable:

1. Extero-interior gradient. This is indicated by (i) the hypodermis always showing more enzyme activity than internal organs; (ii) the ventral nerve cord being at first active in its ventral part only; later there is a distinct gradient with greater activity in the ventral region; (iii) the mesodermal components of the intestine invariably reacting earlier than the intestinal cells themselves to the wave of enzyme spread.

2. Antero-posterior gradient. The posterior part of the embryo is more reactive than the anterior part, a characteristic which can be very well seen in 10- to 11-hour embryos. This gradation also manifests itself within the hypodermis. In the case of the proctodaeum, the highest enzyme activity is found near the anal opening, gradually fading away towards the midgut. These two gradients of alkaline phosphatase reaction can both be seen in Pl. 1, fig. 2.
The possibility that gradient activities might be artifacts of some kind has been ruled out by the following experiment: a part of the enzyme activity of a number of fixed 11- to 12-hour embryos was destroyed individually by the local application of heat from a micro-cautery needle before embedding: the inactive areas in such embryos always gave a negative reaction irrespective of the positive reaction of the uninjured regions and of the duration of incubation. In other words, diffusion which might otherwise be the disturbing factor does not take place within the sections under experimental conditions. Consequently, the observed gradients must be attributed to the differential rates of spreading of enzyme activity.

Further spreading of enzyme activity takes place between the 12th and 14th hour, mainly in the anterior and central parts of the embryo. At this time the gradients of alkaline phosphatase reaction are no longer very distinct. In fact, the antero-posterior gradient is even replaced by a gradient in the reverse direction in 12- to 13-hour embryos. This is apparently due to the fact that organs in the anterior part of the embryo (such as the brain, stomodaeum, cephalopharyngeal apparatus, and head mesoderm) have reached their maximal enzyme activity, while the activity in the posterior part of the embryo is already in decline. Because the highest enzyme activity of a particular organ seems to be maintained for only a short period, it is difficult to say at which stage the Drosophila embryo, as a whole, has its maximal alkaline phosphatase content. It may be stated that all embryonic tissues show a positive reaction in 12- to 15-hour embryos (Pl. 1, fig. 3).

Alkaline phosphatase is more concentrated in nuclei, presumably in the chromatin materials. In the cytoplasm, a diffuse and moderate reaction is demonstrable in every organ between the 12th and 15th hour. When a cell begins to acquire phosphatase activity, the nucleus seems to be the first region to become active. Activity in the cytoplasm comes later.

At about the 15th hour, enzyme activity starts to decrease in most tissues, especially in the cytoplasm. Cells of the tracheal tubes, hypodermis, and cephalopharyngeal apparatus still retain their full activity, with perhaps even more activity in their cytoplasm. It is noteworthy that these three organs have a common physiological function, i.e. the secretion of cuticle.

Except in the case of the salivary glands, the organs in the anterior part of the embryo only give some residual nuclear activity (after 24-hour incubation) at the time of hatching. These organs include the nervous system, cephalopharyngeal apparatus, fore-gut, proventriculus, gastric caeca, fat bodies, muscles, and hypodermis. On the other hand, the posterior part of the embryo is more active, due mainly to the presence of the reactive mid- and hind-guts (Pl. 1, fig. 4). A localization of alkaline phosphatase in the striated border of the intestinal cells and a gradient of activity between the border and the cell nucleus has been observed. The phosphatase reaction of the tracheae, fat bodies, muscles, and gonads in the posterior half of the embryo is also very weak. Posterior branches of the Malpighian tubes are, however, moderately active.
No alkaline phosphatase activity has been found in the yolk materials during most of the embryonic period. In older embryos (16- to 18-hour) the disintegrating yolk granules in certain sections of the mid-gut are reactive. Since in these cases the gut epithelia and its striated border always show a very high phosphatase content, the enzyme activity found in the yolk is probably not of intrinsic origin. Yolk nuclei show a moderate alkaline phosphatase reaction.

II. Acid Phosphatase

(a) Oocyte development. Quite contrary to alkaline phosphatase, acid phosphatase has a wide distribution in the Drosophila ovary. In ovarian follicles of all developmental stages, the oocyte, follicular epithelial cells and nurse cells are all very rich in acid phosphatase. The nuclear reaction is generally particularly strong and shows no variation throughout the development of the oocyte; on the other hand the cytoplasmic reaction is relatively weaker and varies between different developmental stages (especially in the nurse cells). Pl. 1, fig. 5, indicates the general features of acid phosphatase reaction of some young egg follicles.

There seems to be very little change of acid phosphatase activity in the follicular epithelial cells as the oocyte grows. Their nuclei stain as heavily as those of the nurse cells or oocytes, but their cytoplasmic activity is slightly weaker.

In the very young follicles, nurse cells are undergoing endomitosis and the resulting multiple chromatids show a strong tendency to remain together in definite groups (Painter and Reindorp, 1939). These polytene chromosomes give a very intense acid phosphatase reaction. Cytoplasmic activity of the nurse cells in these very young follicles is comparatively low. It increases substantially in the older follicles (stages 4 and 5), especially around the intracellular vacuoles and cell border. The activity falls off during stage 6, but the pyknotic nuclei remain very reactive.

As is evident from Pl. 1, fig. 5, the oocyte is very rich in acid phosphatase, both in the nucleus and cytoplasm. In the cytoplasm, the concentration of enzyme near the peripheral part of the egg is a distinct feature in the follicles of stages 4–6. The yolk granules as they are formed in this region are likewise endowed with high acid phosphatase activity. A similar concentration of enzyme is also noticeable around the vacuoles present in the ooplasm in stage 6. In mature oocytes cortical localization of acid phosphatase becomes less evident.

The distribution of acid phosphatase in the Drosophila ovary has also been studied by incubating fresh ovaries directly in lead glycerophosphate reagent. After half an hour of incubation, the nuclear reaction is already evident in the peripheral follicles of the ovaries, chiefly in the follicular epithelial cells and nurse cells. The slower reaction of the oocyte is perhaps due to the limitation set by the rate of penetration of the reagents. The reaction becomes more intense and definite as the duration of incubation increases. Figs. 6 and 7 on
Pl. 2 present the differences found between a group of experimental and control follicles after 2-hour incubation (sectioned materials after the same period of incubation show practically no measurable activity). If one compares Pl. 2, fig. 6, with Pl. 1, fig. 5, it is evident that the differential concentration of acid phosphatase between nucleus and cytoplasm as well as that between the cytoplasm of the nurse cells of different developmental stages are precisely the same in both cases.

Acid phosphatase is also demonstrable both in the nucleus and cytoplasm of the oviduct epithelium.

Like the ovary, the adult testis is rich in acid phosphatase. Spermatogonia were found to contain less acid phosphatase than spermatocytes which, in turn, are less reactive than spermatids and sperm. As regards mature sperm, both the heads and the whole matrix in which the sperm are embedded seem to contain the enzyme.

From this outlined description it is clear that the cytoplasmic localization of acid phosphatase during *Drosophila* oogenesis shows, on the whole, a close correlation with the distribution of ribonucleic acid (Yao, 1950). Such a correlation, however, does not exist in the case of spermatogenesis.

*(b) Embryonic development.* Acid phosphatase can be demonstrated in the developing embryos from cleavage up to the hatching of the larva. No apparent change in enzyme activity seems to occur during the whole embryonic development, although irregular results have often been encountered due to the lability of this enzyme. Generally speaking, the most active site is the yolk which stains dark brown to black. Nuclei of embryo cells are the next most active structures, giving a golden-brown appearance. Cytoplasm is the site of weakest activity, showing a light yellow to yellowish-brown colour. Alternate sections as controls are always colourless.

In cleavage stages, acid phosphatase is mostly concentrated in the subcortical plasma and in the yolk; whereas the egg cortex appears transparent with a slight yellowish tint and is possibly free from the enzyme. In dividing nuclei, chromosomes and spindle area are found to be moderately active.

In the single blastoderm stage, acid phosphatase is localized in the nuclei of the blastoderm cells and in the yolk (Pl. 2, fig. 8). In this same figure a stronger reaction in the ‘innere Blastema’ which corresponds more or less to the subcortical plasma in the cleavage stage is clearly visible. Throughout the gastrulation stage, the situation remains the same: a stronger reaction is found in the yolk and in the cell nuclei (Pl. 2, fig. 9). No difference has been found between the cells of the three germ layers, nor is there any differential intensity of reaction in the different parts of an embryo at this or any other later stages.

Pl. 2, fig. 10, is taken from a 12-hour embryo. As can be seen in the figure, the nuclear reaction in the nervous system, proventriculus, cephalopharyngeal apparatus and hind-gut is still comparable to that of the early embryonic cells. The mid-gut with its enclosed yolk forms the most active centre of acid phosphatase activity. Comparing this distribution with the alkaline phos-
phatase reaction of an embryo of the same age, a most noticeable difference is that the yolk and nerve-fibres give a moderate to strong acid phosphatase reaction but no trace of (yolk) or very weak (nerve-fibres) alkaline phosphatase activity.

Further difference between the behaviour of the two kinds of phosphatase during Drosophila embryogenesis is found in embryos prior to hatching. No decline of acid phosphatase activity has been noticed (Pl. 2, fig. 11). Both nuclear and cytoplasmic reactions are still demonstrable in every tissue. In the case of gut epithelia (including proventriculus and gastric caeca) and salivary glands, it is even possible that there is some increase in cytoplasmic activity.

Due to the extreme impermeability of the vitelline membrane, it was not possible to check the distribution of acid phosphatase in Drosophila embryo by incubating fresh unfixed material.

The high acid phosphatase activity during Drosophila embryogenesis and a similar high concentration of ribonucleic acid in Drosophila embryo cells (Yao, 1950) naturally suggests a functional correlation between these substances. Particularly illustrative are organs such as gut epithelia and salivary glands which show a parallel increase in cytoplasmic basiphily and acid phosphatase activity in later embryonic period. However, it should be pointed out again that such a correlation is not an absolute one, since both the yolk and nerve-fibre region are enzymatically active, but are void of ribonucleic acid.

**DISCUSSION**

Perhaps the most interesting and significant fact emerging from the present study is the demonstration of the presence of two different patterns of phosphatase activity in Drosophila embryogenesis: acid phosphatase shows no change in activity, at least histochemically, throughout embryonic life; whereas alkaline phosphatase arises at a certain stage, increases in activity and then recedes (in most tissues). Quite recently, some preliminary data concerning the phosphatase activity in the early development of Arbacia has become available (Mazia et al., 1948). They found that in the unfertilized eggs acid phosphatase predominates and its activity remains constant during early development. On the other hand, alkaline phosphatase activity remains constant until gastrulation, but increases very sharply after this event. The general pattern of phosphatase activity in the early development of Drosophila and of Arbacia is evidently much the same. In the case of the chick embryogenesis, the situation is somewhat different because the concentration of alkaline phosphatase is much greater than that of acid phosphatase from the very beginning (Moog, 1944, 1946). Nevertheless, the trend of the change of acid phosphatase activity between 2- and 12-day-old chick embryos is far less marked than that of alkaline phosphatase in the same period (Moog, 1946). From the above facts, it seems quite safe to say that acid and alkaline phosphatase are two different enzymes and play quite different physiological roles, even though they may be present simultaneously within the same cell.
The particular richness of acid phosphatase in the *Drosophila* embryo can be traced back to the development of the germ cells. In fact, I have found that high acid phosphatase activity is a characteristic of *Drosophila* gonads throughout the whole life-cycle of the fly. The physiological function of acid phosphatase is difficult to understand at present. In view of its constant activity during embryonic development, the enzyme probably has a more general relation to cell function than its suggested specific relation to proliferation and chemo-differentiation (Moog, 1944).

Whether or not alkaline phosphatase is present in small amounts in *Drosophila* ovaries and early embryos is not known. Quantitative determination of phosphatase activity, now contemplated, could provide an answer. At any rate, the histochemical evidence indicates that the concentration of the enzyme, if present, would be very low.

The factors which determine the origin (or activation?) of alkaline phosphatase in the ventral ectoderm after the contraction of the germ band as well as the differential rates of spreading after its first appearance are not clear. The fact that the spreading of enzyme activity is a gradual process seems to suggest that the primary factor involved in such spreading is actually direct contact between the active and inactive cells (in fact it reminds one of the process of infective colour transformation of guinea-pig skin (Medawar, 1947)). If this is true, the observed extero-interior gradient of alkaline phosphatase activity can be partially accounted for. Thus, the closer contact between hypodermal cells themselves than between a hypodermal cell and, for example, a nerve-cell would explain a quicker spreading within the hypodermis than spreading from the hypodermis to interior parts of an embryo. In this connexion, it is worthy of mention that the salivary glands which are still directly connected with the ventro-lateral ectoderm (shortly after the germ-band contraction stage) show a strong alkaline phosphatase reaction earlier than do other internal organs.

That contact is not the sole factor is indicated by the presence of an antero-posterior gradient which is also observable within the hypodermis itself. Evidently, there must exist within the embryo some physiological differences between the anterior and posterior parts and such differences control, in turn, the differential rate of enzyme spreading along the antero-posterior axis. The presence in the *Drosophila* embryo of a physiological gradient of some kind is also shown by Geigy's (1931) irradiation experiments. He discovered that the sensitization and de-sensitization of ventral ectoderm towards ultra-violet light exhibit a quite distinct 'thoraco-abdominal' gradation.

For various reasons it has been suggested in the first paper of this series that the stage of the contraction of the germ band divides, more or less, the growth phase of embryonic development of *Drosophila* from the phase of histological differentiation. The appearance of alkaline phosphatase during or immediately after the contraction of the germ band and its disappearance before hatching thus indicate that this enzyme is principally concerned with histo-differentiation. It is likely that the contraction of the germ band
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represents a very important morphogenetic event and further investigation might be expected to reveal many biochemical differences between embryos before and after this stage.

Since Seidal’s classical work on Platycnemis, it is generally admitted in experimental embryology that the organized development of insects involves the operation of two centres of organization, namely the activation centre and the differentiation centre. The presence of these centres in Drosophila has hitherto not been demonstrated though the work of Geigy (1931) points to their existence. Whether or not the centre of origin of alkaline phosphatase activity in the ventral ectoderm near to the future thorax represents the actual differentiation centre in Drosophila cannot yet be fully ascertained; but that it is so appears very likely.

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Summary

1. Drosophila ovary and testis are very rich in acid phosphatase, but contain no histochemical trace of alkaline phosphatase. Thus the mature oocyte shows a strong acid phosphatase reaction both in the nucleus and cytoplasm. Sperm heads are equally reactive.

2. Acid phosphatase is demonstrable in Drosophila embryos from early cleavage up to the hatched larva. No striking change in enzyme activity has been observed during this period.

3. Alkaline phosphatase is not detectable in the first half of embryonic life. It suddenly appears in the ventral ectoderm near the future thorax during or shortly after the contraction of the germ band. The enzyme activity then spreads to the other parts of the embryo following definite patterns, until finally the whole embryo becomes active. The possible mechanism of the spreading of enzyme activity is discussed.

4. Alkaline phosphatase disappears in most tissues before hatching, but is retained in the gut epithelia, salivary glands, and Malpighian tubes. The relationship of this enzyme to histo-differentiation is suggested.

5. The centre of origin of alkaline phosphatase activity is considered as the ‘differentiation centre’ of the Drosophila embryo.

6. The high cytoplasmic acid phosphatase activity of the oocyte and nurse cells and a similar activity of the yolk in the developing embryos indicate that the enzyme plays some role both in the synthesis and in the degradation of yolk.
REFERENCES

Yao, T., 1950. (In the press.)

EXPLANATION OF PLATES 1 AND 2

$G$, cerebral ganglion or brain
$hg$, hind-gut
$mg$, mid-gut
$P$, proventriculus
$ov$, oviduct
$s$, salivary gland
$Vg$, ventral nerve cord

PLATE 1

Fig. 1. Oblique sagittal section of a 9-hour embryo showing the appearance of alkaline phosphatase in ventral hypodermis. The arrow-head indicates approximately the centre of origin of enzyme activity.  $\times$ 120.

Fig. 2. Sagittal section of a 10–10½-hour embryo showing the spreading of alkaline phosphatase activity towards the interior and posterior parts of the embryo.  $\times$ 120.

Fig. 3. Longitudinal section of a 14-hour embryo showing the distribution of alkaline phosphatase activity.  $\times$ 120.

Fig. 4. Sagittal section of an embryo just before hatching. Note the alkaline phosphatase activity of the salivary gland and gut.  $\times$ 120.

Fig. 5. Part of a section of a 24-hour ovary. Note the intense acid phosphatase activity of the nuclei of the nurse cells and oocytes.  $\times$ 95.

PLATE 2

For list of abbreviations, see Plate 1.

Fig. 6. Acid phosphatase reaction in fresh ovarian follicles incubated for 2 hours in lead glycerophosphate mixture. Compare with Pl. 1, fig. 5.  $\times$ 82.

Fig. 7. Same as fig. 6, incubated in the presence of 0.01 M. fluoride.  $\times$ 82.

Fig. 8. Longitudinal section of an embryo in the single blastoderm stage showing acid phosphatase distribution.  $\times$ 120.

Fig. 9. Sagittal section of a 4½-hour embryo in an advanced stage of gastrulation showing acid phosphatase distribution.  $\times$ 120.

Fig. 10. Sagittal section of a 12½-hour embryo showing acid phosphatase distribution.  $\times$ 120.

Fig. 11. Sagittal section of a 17½-hour embryo showing acid phosphatase distribution. The arrow-head indicates the point of puncture during fixation.  $\times$ 120.