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[5] The basal solution contained per liter 50 g. dextrose, 1.5 g. KH_2PO_4, 0.5 g. $MgSO_4 \cdot 7H_2O$ and 2 g. asparagine. To this solution the following trace elements were added in p. p. m.: 0.005 B, 0.02 Cu, 0.1 Fe, 0.01 Ga, 0.01 Mn, 0.01 Mo and 0.09 Zn. To each flask there were added also 0.1 μg. biotin methyl ester and 10 mμ moles thiamine. For the experiment summarized in part in table 1 the reaction of the basal solution was adjusted with KOH to pH 5.2 and for the one presented in table 2 to pH 5.8. Solutions were autoclaved at 15 lbs. pressure for 30 minutes.

[6] We used S. M. A. vitamin-free casein hydrolysate which had been treated with excess $CaCO_3$ to neutralize the acidity.

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*THE DISTRIBUTION OF PHOSPHATASE IN THE SPINAL CORD OF CHICK EMBRYOS OF ONE TO EIGHT DAYS' INCUBATION**

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Despite the enormous interest that has grown around the phosphatases since their discovery 36 years ago, our knowledge of the rôles they play in the economy of living organisms, in which they are almost ubiquitous, is but slight. In part the reason for this lack is, as Kay (1932) pointed out in another connection, the fact that the method of studying phosphatase activity in tissue extracts precludes any chance of discovering the intimate distribution of the enzyme within the tissues. In 1939, however, Gomori, and also Takamatsu, offered a histochemical method whereby the localization of alkaline phosphatase in cells and tissues may be determined with great exactness, and more recently Gomori (1941*b*) has extended this method to acid phosphatase.

With the thought that a histochemical study of the embryogenesis of these enzymes might produce clues concerning their biological significance, the writer undertook a comprehensive survey of the development and distribution of phosphatases in the embryo of the chick. Among the striking results to emerge from this study is the fact that both these enzymes appear in a remarkable pattern in the embryonic spinal cord.

Method.—The techniques outlined by Gomori (1941*a*, *b*) for the histological demonstration of alkaline and acid phosphatases were followed.

They were used on White Leghorn embryos of one to eight days' incubation, which were fixed in ice-cold acetone for 24 hours, embedded in a paraffin-beeswax-bayberry wax mixture by routine methods, and sectioned serially at 15 micra. Chilled acetone proved an excellent fixative for the gross histological examination of objects even as large as eight-day chick embryos, but the cell structures were somewhat distorted by shrinkage.

The sodium glycerophosphate used contained equal parts of the alpha and beta salts; pure alpha glycerophosphate was not available. The incubating solution for alkaline phosphatase was made according to Gomori's (1941*a*) instructions, at pH 9.3. Incubation was continued for three hours at 38 degrees. After treatment with cobalt nitrate and ammonium sulfide, the sections were counter-stained lightly with erythrosin. To compensate for the precipitation of lead beta-glycerophosphate, the formula (Gomori, 1941*b*) for the incubating solution for acid phosphatase, was altered as follows:

Molar acetate buffer at pH 4.8	$2^1/_2$ parts
5% $PbNO_3$	2 parts
Distilled water	30 parts
Na glycerophosphate	5 parts

The solution was made up fresh for each test, and the precipitate removed by filtration. The pH is 5.1. Incubation was continued for six hours at 38 degrees. The sections were then treated with sulfide and counter-stained as before. Collodion protection was not found useful in either case.

The alkaline phosphatase showed remarkable stability in the course of handling. Keeping the specimens in acetone in the refrigerator for a week did not cause any variation in the excellently uniform pictures, nor did allowing the paraffin blocks or even the unstained mounted sections to stand for a month have any deleterious effect. The acid phosphatase, on the other hand, proved extremely labile, even on standing at icebox temperature, so that the achievement of comparable results required that the embryos be processed as rapidly as possible; three to four days were generally allowed from killing to staining. The embedding heat also had to be controlled carefully for, unlike the enzyme reported by Gomori (1941*b*) for adult mammalian tissues, this phosphatase was destroyed by temperatures above 60 degrees. Yet, in spite of these precautions, individual embryos showed considerable variation in the intensity of acid phosphatase activity. The pattern of distribution in the various tissues, however, was constant.

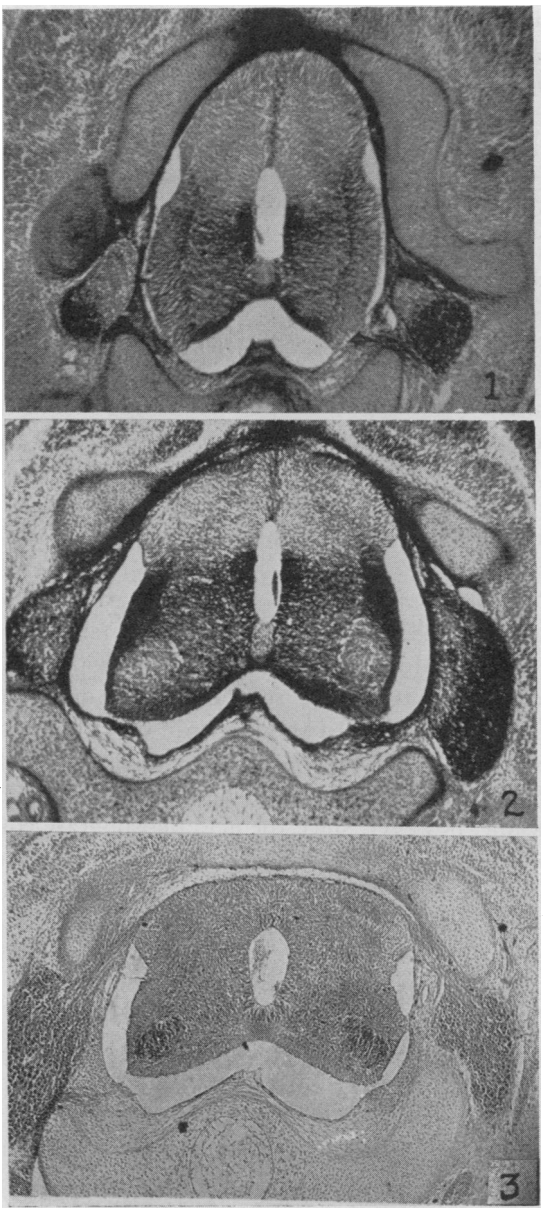
Results.—(1) *Alkaline Phosphatase.* The activity of alkaline phosphatase, as judged by the amount of phosphate deposited within a given time,

is generally strong in the embryo, for heavy black deposits appear in many tissues even if incubation is continued for only one hour. A high concentration of alkaline phosphatase is found in the neural tissue at the end of the first day, the earliest stage examined. Through the second day the neural tube is uniformly active at anterior levels, but near the end of the region of differentiated somites the reactivity wanes and almost disappears. Anteriorly, on the third day, the dorsal half of the cord and the neural crests tend to react more strongly than the ventral half, but this difference fades into uniformity at posterior levels. On the next two days small portions of the ventral region of the cord continue to lose their alkaline phosphatase content, and it soon becomes clear that these portions represent the future motor horns of the limb plexus. The white matter differentiating outside of the horn area is strongly positive. In the tail region, on the fourth day, the distribution is still fairly uniform.

From the end of the fourth day on, the enzyme disappears according to an antero-posterior gradient from the dorsal half of the gray matter, while it becomes more concentrated in the ventral half, so that by the seventh day a definite and apparently persistent pattern is established. The striking feature of this pattern is a heavy black crossband that virtually bisects the cord; the band reaches from the lumen to the outer edge, thus involving ependymal, marginal and mantle layers. Dorsal to the band the ependyma and gray matter are almost negative, except for a small positive area just above the lumen, at the base of the dorsal funiculus; but ventrally both layers show a marked reaction, with the exception of a limited portion of the ependyma immediately under the lumen, and of the large lateral motor groups which, by the sixth day, are established in the pelvic as well as the pectoral region. The mesial motor groups are also less active than the surrounding gray matter, but the demarcation is not as sharp as in the other cases cited. The white matter contains a high concentration of alkaline phosphatase throughout, though the activity is greater ventral to the crossband than dorsal to it; the ventral commissure is extremely active.

This pattern does not change through the eighth day. Its appearance in the cervical and fore-limb regions is shown in figures 1 and 2.

The absence of alkaline phosphatase described refers only to the cytoplasm of the cell bodies and processes in question. Within such cells the nuclear membranes are clearly outlined by black deposits, and the nucleoli are also stained black. In the cells which show phosphatase in their cytoplasm, when the deposits are light enough to permit examination of the interior of the cell, it can be seen that the nuclear membranes and nucleoli are also strongly reactive. There are no cytoplasmic inclusions seen in either case.



FIGURES 1-3

All sections are from one eight-day embryo. Magnification 50 ×. (1) Alkaline phosphatase, neck region. The heavily stained distal portions of the ganglia are made up of the large cells. (2) Alkaline phosphatase, fore-limb region. (3) Acid phosphatase, hind-limb region. Note the darkly stained masses in the large cells of the ganglia.

In the sensory ganglia, which are made up of cells of two sizes, a clear-cut disparity exists in the alkaline phosphatase content of the cytoplasm of the large and the small cells: the former reacts strongly, whereas the latter seems devoid of the enzyme. Thus the clustering of the large cells distal to the nerve cord gives the phosphatase-stained ganglion the appearance of being divided into two parts (see Fig. 1). As in the cells of the cord, the nucleoli and nuclear membranes of the small cells stain deeply, and in addition a faint darkened network, probably of chromatin, may occasionally be seen inside the nucleus. The interior of the large cells is obscured by the heavy sulfide deposits.

The dorsal and ventral roots are strongly positive, as are the nerve trunks peripheral to the ganglia.

Tests run on six-day embryos at pH 8.5, 8.0 and 7.5 showed that the activity is markedly less at pH 8.5 than at 9.3, and it is abolished at pH 8.0 or lower. Other tests, run for one hour, in which the incubating solution was made 0.001 *M* and 0.01 *M* with respect to $MgCl_2$, revealed some activation at the lower concentration and very marked activation at the higher.

(2) *Acid Phosphatase.* Like its alkaline counterpart, acid phosphatase also appears in the neural tissue at the end of the first day, but it does not seem to be as concentrated, or as active, as the alkaline enzyme. Slides treated by the alkaline technique regularly show dense black deposits, whereas after the acid treatment the deposits in the nerve tissue never have more than a deep golden brown color, even when the incubation is continued for 12 hours. Strict comparison between the two enzymes on this basis is not possible, however, since the acid phosphatase could operate on alpha-glycerophosphate only; and the beta salt is the preferred substrate of the phosphomonoesterases of both Class AI and Class AII (Folley and Kay, 1936). But it is noteworthy that Fleischhacker (1938) found a similarly great disparity between the activity of the two enzymes in mammalian brain tissue, even when beta-glycerophosphate was the substrate for both.

As the cell layers of the cord differentiate, acid phosphatase becomes concentrated chiefly in the ependyma, while the surrounding tissue shows lessened activity. Between the fifth and the seventh day acid phosphatase largely disappears from all three layers of the dorsal half of the cord, except for a few cells just dorsal to the lumen—the same spot that also shows alkaline phosphatase activity. In the ventral half the enzyme occurs in reactive cells scattered through a negative ground substance; but heavier uniform concentrations are found in the lateral and mesial motor groups. These deposits appear in posterior progression, demarcating the lateral horn in the fore-limb region on the fifth day, and in the hind-limb region less than a day later. The ventral commissure also stains more heavily

than its surroundings, and so do an extensive group of ependymal cells below the lumen.

Thus a definite pattern, as shown in figure 3, is laid down by the end of the sixth day. Aside from more complete withdrawal of the enzyme from the dorsal half of the cord, the pattern does not change essentially through the eighth day.

Unlike alkaline phosphatase, the acid enzyme is apparently absent from the nuclei of cells which have unreactive cytoplasm. In the reactive cells the nuclei stain more lightly than the cytoplasm and show no inclusions; but there is in each cell a small, intensely stained body outside the nucleus but closely applied to it. The nature of these bodies is not evident.

The small cells of the ganglia appear to contain only a little acid phosphatase in their nuclei and cytoplasm; they do, however, show the same reactive body on the surface of the nuclei as was found in the active cells of the cord. The large cells contain a very large, intensely reactive mass which is bigger than the nucleus and apparently includes all the cytoplasm of the cell body except for a negative rim continuous with the processes. The clump has a granulated appearance which seems to be caused by darkly staining inclusions. Possibly it represents the tigroid substance shrunken into a rounded lump (see Fig. 3).

The dorsal and ventral roots, and the nerve trunks leading from the ganglia, are lightly positive.

Discussion.—In a consideration of the nature of these enzymes, the thought arises that the alkaline phosphatase, at least, is a phosphomonoesterase component of a lecithinase, a substance which might reasonably be expected to occur in nerve tissue. Comparison of the pH tests reported here with those of King (1931), however, enable the dismissal of this possibility; for King showed that lecithinase has a pH optimun of 7.0–7.4, whereas the alkaline phosphatase of this study did not act at all at pH 8.0 or lower. Similarly it can be seen that neither the acid nor the alkaline phosphatase resembles the 5-nucleotidase found widespread in nerve tissue by Reis (1937). The Reis enzyme is only slightly active against glycerophosphate, but these enzymes are as effective on this substrate as are most other phosphatase-containing tissues of the embryo. Although the 5-nucleotidase is virtually inactive at pH 5, it retained considerable activity at pH 9, but again it fails to correspond to the alkaline enzyme of this study in being unaffected by the presence of magnesium ions.

The enzymes found in the embryonic chick cord do, however, correspond well with the brain phosphatases delineated by Fleischhacker (1938) and Kotkova (1939). The latter demonstrated in the brain of the sparrow and pigeon, as well as of members of other classes, phosphomonoesterases with pH optima of 4.5–5.6, and 8.9–9.6; the second was activated by magnesium, and the first inhibited by fluoride. Fleischhacker obtained somewhat

similar results, except that he claimed the acid phosphatase to be activated by magnesium; but the acid activities he reported were in all cases so low that the differences may be unreliable. Probably all these nerve phosphatases can be classified, according to Folley and Kay's (1936) scheme, as phosphomonoesterases of Classes AI and AII. They may be concerned in the metabolism of the carbohydrate substrates of nerve tissue.

The change in the distribution of the embryonic cord enzymes with time indicates that they may have more than one function. The uniform distribution of the enzymes in the neural tissue of the earliest stages, and their later disappearance from certain regions, and concentration in others, are phenomena shared by practically every organ of the embryo (unpublished results). Thus the observer is led to feel that the phosphatases are essential in the initiation, at least, of differentiation; for every indifferent cell contains phosphatase in its cytoplasm. As differentiation proceeds, the enzymes may disappear, or they may become more concentrated. In the latter case the accumulation of the enzymes in local regions may be regarded as a consequence of differentiation in itself, or in other words a chemical differentiation paralleling the histological differentiation; presumably the enzymes are thereafter concerned in the functioning of the specific cells in which they are localized. In this connection it is suggestive that the establishment of the pattern described, and especially the accumulation of acid phosphatase in the motor groups, precedes by about 24 hours the appearance (at $6^1/_2$ to 7 days) of the first local reflexes (Orr and Windle, 1934). In any case the results suggest for phosphatase a hitherto unsuspected rôle in the actual functioning of nerve elements.

That the localization of acid phosphatase at least is not a transitory embryonic phenomenon is shown by the fact that Fleischhacker (1938), using a crude histochemical method adapted directly from the colorimetric method of Kuttner and Lichtenstein, found similar concentrations in the anterior horn cells of adult rats; he did not mention the pH. Gomori's (1941*a*) failure to find any alkaline phosphatase in mammalian nerve tissue, however, remains to be explained.

Summary.—1. The presence of acid and alkaline phosphatases in the spinal cord of the embryonic chick has been demonstrated by the histochemical techniques developed by Gomori.

2. At all stages examined, alkaline phosphatase reacts much more strongly than acid phosphatase.

3. As early as the end of the first day of incubation, both acid and alkaline phosphatase are found in fairly high concentration in the neural tissue.

4. As development proceeds, the alkaline phosphatase becomes localized in the white matter throughout, and in the gray matter and ependyma of the ventral half of the cord, with the exception of the cells of the motor

groups. It is absent from a small part of the ependyma just below the central canal, and is concentrated in a similar small area just above the canal.

5. The acid phosphatase becomes largely restricted to the ventral half of the cord, and is especially concentrated in the motor groups. It also occurs in small regions just dorsal and ventral to the lumen.

6. The pattern described appears in antero-posterior progression on the fourth, fifth and sixth days.

7. The large cells of the spinal ganglia contain large amounts of both acid and alkaline phosphatase, the small cells very little of either.

8. As far as can be detected, all nucleoli and nuclear membranes are positive for alkaline phosphatase.

9. Acid phosphatase is not found in nuclei, but even where the cytoplasm is not reactive the enzyme is generally present in a small body, of unidentified nature, which is closely applied to the nucleus. In the large ganglionic cells, the cytoplasm is not uniformly reactive, but contains a very large rounded clump which is strongly positive.

10. Evidence is presented which indicates that these enzymes are phosphomonoesterases of Classes AI and AII.

11. It is suggested that both these phosphatases first play a part in early differentiation, and later are involved in the specific functions of the cells in which they become concentrated.

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