A HISTOCHEMICAL STUDY OF THE DISTRIBUTIONS OF THIRTEEN ENZYMES IN THE EARLY CHICK BLASTODERM

> Thesis for the Degree of Ph. D. MICHIGAN STATE UNIVERSITY W. JOHN DALZELL, JR. 1967

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A HISTOCHEMICAL STUDY OF THE DISTRIBUTIONS OF THIRTEEN ENZYMES IN THE EARLY CHICK BLASTODERM

presented by

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## ABSTRACT

# A HISTOCHEMICAL STUDY OF THE DISTRIBUTIONS OF THIRTEEN ENZYMES IN THE EARLY CHICK BLASTODERM

by W. John Dalzell, Jr.

The objectives of this study were to localize with histochemical methods 13 enzymes in whole chick blastoderms at four stages of development: prestreak, definitive streak, head fold, and 7-somite. The enzymes were the following dehydrogenases: lactate, alcohol, isocitrate, succinate, glutamate, glucose-6-phosphate, and B-hydroxybutyrate; NAD diaphorase and NADP diaphorase; acid and alkaline phosphatase; carboxylic acid esterase; and phosphorylase.

The tests for the NAD and NADP linked dehydrogenases--lactate, alcohol, isocitrate, glutamate, glucose-6-phosphate and B-hydroxybutyrate--could not be controlled, so the patterns of these enzymes are invalid. The remaining seven enzymes--succinate dehydrogenase, NAD diaphorase, NADP diaphorase, acid and alkaline phosphatase, carboxylic acid esterase and phosphorylase--were satisfactorily localized. Their patterns are summarized below.

The activities of acid phosphatase and carboxylic acid esterase were uniform throughout the entire prestreak blastoderm except in the area opaca vitellina externa where



W. John Dalzell, Jr.

they were slightly stronger. Phosphorylase activity was uniform in the area pellucida but increased from medial to distal in the area opaca vitellina interna, becoming intense in the area opaca vitellina externa. The activities of the remaining enzymes were uniform throughout the entire blastoderm.

In the primitive streak blastoderm, the enzyme activities were slightly stronger in Hensen's node and the primitive ridges and much weaker in the primitive pit and groove than in the rest of the area pellucida. In the area opaca, phosphorylase activity increased from medial to distal in the vitellina interna, becoming intense in the vitellina externa. For the rest of the enzymes, the interna reacted uniformly with much stronger activity in the externa. Intense activity was present throughout the germ wall, except in the case of acid phosphatase, which was present only in randomly scattered patches. The activities of succinate dehydrogenase and the two diaphorases were stronger in the posterior one-third than in the anterior two-thirds of the germ wall. The formazan dye granules (succinate dehydrogenase and the two diaphorases) were much finer and the indigo dye granules (carboxylic acid esterase) were much coarser in the germ wall than elsewhere in the blastoderm.

In the head fold blastoderm, the enzyme activity in the embryonic area was generally uniform. The principle exceptions were the neural and primitive grooves in which



the activity was much weaker; the notochord in which the activities of succinate dehydrogenase, the two diaphorases, and carboxylic acid esterase were much stronger in the base than in the rest of the structure; and the primitive streak in which the activities of succinate dehydrogenase and the two diaphorases decreased from anterior to posterior. The rest of the area pellucida reacted more weakly than the embryonic area. In the area opaca, the activities of succinate dehydrogenase and the two diaphorases were uniform throughout the vasculosa and vitellina interna, whereas the activities of acid and alkaline phosphatase, carboxylic acid esterase, and phosphorylase were stronger in the vasculosa than in the vitellina interna. Phosphorylase activity in the interna increased from medial to distal. The activities of all enzymes were intense in the vitellina externa. The entire germ wall reacted strongly, with the activities of succinate dehydrogenase and the two diaphorases being strongest in the posterior one-third and the activity of alkaline phosphatase being strongest in the anterior one-half. The formazan dye granules (succinate dehydrogenase and the two diaphorases) were much finer and the azo dye granules (alkaline phosphatase) and indigo dye granules (carboxylic acid esterase) were much coarser in the germ wall than elsewhere in the blastoderm.

At the 7-somite stage, most of the enzymes had characteristic patterns of distribution in the axial

structures of the embryo. Succinate dehydrogenase and the two diaphorases were distributed along three gradients. Their activities decreased from anterior to posterior in the primitive ridges; from posterior to anterior in Hensen's node and the notochord, the unsegmented dorsal mesoderm and the somites, and the unclosed neural folds and the spinal cord; and from anterior to posterior in the Alkaline phosphatase activity increased sharply at brain. the level between the unsegmented dorsal mesoderm and the most recently formed pair of somites. Posterior to this level relatively weak activity was present in the spinal cord and unclosed neural folds, Hensen's node and the base of the notochord, and the unsegmented dorsal mesoderm; anterior to this level much stronger activity was present in the central nervous system, the notochord, and the somites. The activity of carboxylic acid esterase changed abruptly at the level of the anterior intestinal portal: the activity in the central nervous system was much stronger posterior to the portal than anterior to it, whereas the activity in the notochord was much stronger posterior to the portal than anterior to it. The unsegmented dorsal mesoderm and the two most recently formed pairs of somites reacted much more weakly than the older five pairs of somites. The patterns of acid phosphatase and phosphorylase were uniform in the axial structures with no indications of any gradients. In the area opaca, the activities of all the enzymes were



stronger in the vasculosa than in the vitellina interna. Furthermore, the dye granules in the vasculosa were distributed so as to give the region a mottled appearance. The patterns in the vitellina and germ wall were identical to those of the head fold blastoderm.

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# CHICK BLASTODERM

By ریکی W. John Dalzell, Jr.

# A THESIS

Submitted to Michigan State University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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#### INTRODUCTION

Very little is known about the distribution of enzymes in the chick embryo prior to the development of the organs, which, in the case of the heart and brain, is just getting underway at the 7-somite stage. Those most extensively studied have been the oxidases. Rulon (1935) stained whole blastoderms from the primitive streak stage to the late limb bud stage with Janus green, sealed them on slides, and then recorded the progressive anaerobic reduction of the stain. Lazarow and Cooperstein (1953) have since shown that Janus green is reduced by reduced flavin adenine dinucleotide (FADH2), its reduction therefore indicating the presence of this flavoprotein. Spratt (1958) observed the reduction of neotetrazolium chloride, potassium tellurite and methylene blue in head fold to 15-somite blastoderms that had been explanted onto albumin- or glucose-Ringer-agar medium containing one of these indicators. He also studied the effects of 15 specific substrates on the pattern of reduction by incubating head fold to 15-somite blastoderms, under aerobic and anaerobic conditions, on Ringer-agar medium containing specific substrates and neotetrazolium chloride. The reduction of neotetrazolium chloride and potassium

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tellurite indicates the presence of cytochrome oxidase, which is the principle reducing agent of these two compounds (Nachlas, <u>et al.</u>, 1960; Wachstein, 1949). The reduction of methylene blue indicates the presence of FADH<sub>2</sub> (White, <u>et al.</u>, 1964). The distribution of indophenol oxidase was investigated by Fraser (1956), who explanted prestreak, intermediate streak and definitive streak blastoderms onto media containing the Nadi reagent. This enzyme was localized in later stages by Moog (1943a), who immersed whole blastoderms of 16 to 96 hours in solutions containing the Nadi reagent. In both studies sodium azide was used to identify the indophenol oxidase activity that was due to cytochrome oxidase.

Considerable attention also has been given to the phosphatases. Using Gomori's (1941a & b) lead phosphate and calcium phosphate techniques, which are not very reliable, Moog (1943b, 1944) localized acid and alkaline phosphatases in sectioned blastoderms of 1 to 8 days. Acid phosphatase was localized also by Beck (1965) with naphthyl phosphate as a substrate and hexazonium pararosaniline as a coupler (Barka, 1960) in whole 10-somite blastoderms, some of which later were sectioned. His observations, however, were limited mainly to the area opaca. Perhaps the most meaningful study was that of Deuchar (1960a & b), who compared the adenosine triphosphatase activity in the unsegmented dorsal mesoderm with that in the somites of 13-somite blastoderms.



Only three studies have been made of esterase activity in the early chick blastoderm. Zacks (1954) used a simultaneous coupling azo dye method to demonstrate carboxylic acid esterase in whole, unincubated to 8-somite blastoderms. His results, however, may not be reliable because, as Zacks himself admits, at least one component of the incubating medium may not have penetrated to the enzyme sites rapidly enough to ensure a zero order reaction. The distribution of trimetaphosphatase in sectioned blastoderms of 24 hours to 8 days was studied by Berg and Szekerczes (1962) using a technique devised by Berg (1955). And Buno and Marino (1952), using Tween 60 as a substrate, localized lipase in sectioned blastoderms up to 3 days of age. Unfortunately, they were unable to distinguish between lipase and other esterases.

The only other enzyme whose distribution has been studied in the early chick blastoderm is B-glucuronidase, which was localized by Billett and Mulherkar (1958) in an unsuccessful attempt to find a correlation between the activity of this enzyme and the rate of cell proliferation. Using the ferric hydroxyquinoline method of Friedenwald and Becker (1948), they localized the enzyme in primitive streak to 12-somite blastoderms.

In view of the importance of enzymes in cell metabolism, the need for additional studies of enzyme distribution in the early chick blastoderm is apparent.



Furthermore, some of the studies mentioned above obviously need to be repeated using more reliable techniques. Such studies not only should add to our understanding of the development of the early chick embryo but should provide a good foundation upon which additional investigations can be made. For these reasons the present study was undertaken.

The objectives of this study were to map the locations of 13 enzymes in the early chick blastoderm using the most reliable techniques available. The enzymes were the following dehydrogenases: lactate, alcohol, isocitrate, succinate, glutamate, glucose-6-phosphate and B-hydroxybutyrate; nicotinamide adenine dinucleotide (NAD) diaphorase and nicotinamide adenine dinucleotide phosphate (NADP) diaphorase; acid and alkaline phosphatase; carboxylic acid esterase; and phosphorylase. All of the dehydrogenases were regarded as NAD linked, except glucose-6-phosphate dehydrogenase, a NADP linked enzyme. Each enzyme was localized in whole chick blastoderms at four stages of development, according to the Hamburger and Hamilton (1951) stage series: prestreak (6 hours), definitive streak (18 hours), head fold (24 hours) and 7-somite (33 hours).



### MATERIALS AND METHODS

White Leghorn chicken eggs were incubated in a forced-draft incubator in which the dry-bulb thermometer reading averaged 99 3/4°F., and the wet-bulb thermometer reading varied between 84 and 86°F. After incubating the eggs for appropriate lengths of time, the blastoderms were removed by a technique similar to that used by Spratt (1947). A large hole was broken in the large end of the egg shell, and the contents were poured into a small fingerbowl about one-half full of warm (37°C.) chick Ringer's solution. With a pair of fine scissors, the vitelline membrane was cut around the blastoderm, keeping about 1/8 inch away from the edge of the blastoderm. The vitelline membrane, with the blastoderm adhering to its ventral surface, was then carefully pulled off the surface of the yolk, floated into a small watchglass, and transferred to a Petri dish containing warm (37°C.) Ringer's solution. Here the loose yolk was carefully scraped from the blastoderm with a glass needle, so the incubating medium could penetrate more easily to the ventral surface of the area opaca. Finally, the blastoderm was separated from the vitelline membrane. This was done by carefully tearing the vitelline membrane up to and slightly beyond

the edge of the blastoderm, inserting the tip of a glass needle into the space between the blastoderm and the vitelline membrane, and then working the needle around the blastoderm so as to separate its edge from the vitelline membrane. The freed, and relatively yolk-free, blastoderm then was floated into a small watchglass where the histochemical test was performed after the Ringer's solution had been removed with a small bore evedropper.

Some of the blastoderms were quickly frozen and thawed before the histochemical test was performed. This was done according to the method of Gonzales and Luyet (1954). After the blastoderm and vitelline membrane had been separated, the blastoderm was floated onto a small square of aluminum foil; the Ringer's solution was soaked off with a piece of filter paper: and the blastoderm was partially dehydrated by covering it with several drops of 60 per cent ethylene glycol for one minute. The ethylene glycol then was removed with a piece of filter paper, and the blastoderm was frozen quickly by placing the piece of aluminum foil on the end of a metal rod that dipped into a mixture of acetone and solid carbon dioxide. After the blastoderm was frozen, it (along with the aluminum foil) was placed in a watchglass, and the incubating medium. warmed to 37°C.. quickly was pipetted onto it.

The enzymes were localized with the procedures described in the following paragraphs. The exact



compositions of the incubating media are given in Appendix A, and the numbers of experimental and control blastoderms in which each enzyme was localized are shown in Tables 1 through 10. Once the technique for removing the blastoderms had been refined and the procedures for localizing the enzymes had been perfected, it was possible consistently to obtain reproducible patterns (with one exception) at any given stage of development with scarcely any failures. These numbers, therefore, include only successful localizations.

#### Localization of Dehydrogenases and Diaphorases

The oxidative enzymes were localized with modifications of the method described by Thomas and Pearse (1961). Two tetrazolium salts were used: 2,2'di-p-nitro-phenyl-5,5'diphenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride and 3-(4,5-dimethyl-thiazolyl-2)-2,5diphenyl tetrazolium bromide, commonly known as Nitro-BT and MTT respectively. At each of the four stages of chick development, each enzyme was localized in (a) unfrozen blastoderms with Nitro-BT, (b) unfrozen blastoderms with MTT, (c) frozen blastoderms with Nitro-BT, and (d) frozen blastoderms with MTT. The general procedure was as follows. Each blastoderm was immersed in 1.0 ml. of a buffered medium containing the appropriate substrate, a co-enzyme (if required), a tetrazolium salt, sodium azide or sodium

cyanide, magnesium chloride, cobaltous chloride (if required), and polyvinylpyrrolidone (molecular weight = 10,000). The blastoderms were incubated in this solution, maintained at  $37^{\circ}C.$ , for different lengths of time. With Nitro-BT, unfrozen blastoderms were incubated for 2 1/2 hours and frozen blastoderms for 30 minutes; with MTT, both unfrozen and frozen blastoderms were incubated for 45 minutes. At the end of the incubation period, the blastoderms were fixed for 30 minutes in calcium formol (150 ml. of 40% formaldehyde and 850 ml. of 1.3% calcium chloride) and then washed several times with Ringer's solution. These tests were controlled by incubating blastoderms in media lacking substrates.

#### Localization of Acid and Alkaline Phosphatase

Acid phosphatase was localized with a simultaneous coupling azo dye method using a substituted naphthol (Burstone, 1961). Unfrozen blastoderms were fixed in pure acetone for 30 minutes at 4°C. After fixation, each blastoderm was incubated for 55 minutes in 1.0 ml. of histochemical medium maintained at 37°C. This solution, buffered at pH 5.2, contained naphthol AS-BI (2-hydroxy-3-naphtho-<u>o</u>-anisidide) phosphoric acid as a substrate and fast red violet salt LB (diazotized 5-benzamido-4-chloro-2-toluidine) as a coupler. Following incubation, the blastoderms were washed several times with Ringer's solution.



Alkaline phosphatase also was localized with a simultaneous coupling azo dye method using a substituted naphthol (Burstone, 1961). Unfrozen blastoderms were fixed for 30 minutes at  $4^{\circ}$ C. in a sucrose formaldehyde solution (0.88 M sucrose in 10% formalin). Following fixation, each blastoderm was immersed in 1.0 ml. of incubating medium, buffered at pH 8.3 and containing naphthol AS-MX (4'-methyl-2-hydroxy-3-naphtho-o-toluidide) phosphoric acid as a substrate and fast red violet salt LB (diazotized 5-benzamido-4-chloro-2-toluidine) as a coupler. The blastoderms were incubated in this solution for 50 minutes at  $37^{\circ}$ C. and then washed several times with Ringer's solution.

The histochemical tests for the phosphatases were controlled in two ways. Some blastoderms were fixed and then incubated in a medium lacking a substrate. Others, after being fixed, were immersed for 10 minutes in distilled water at  $90^{\circ}$ C. in order to denature the enzyme, and then were incubated in the complete medium.

### Localization of Carboxylic Acid Esterase

Carboxylic acid esterase was localized with the indoxyl acetate method of Holt and Withers (1952). Unfrozen blastoderms were fixed for 30 minutes at  $4^{\circ}$ C. in calcium formol (1% calcium chloride in 10% formalin). Each blastoderm then was incubated for 18 1/2 hours at  $37^{\circ}$ C. in


2.0 ml. of incubating medium, a buffered (pH 6.1) solution containing 5-bromoindoxyl acetate as a substrate and a potassium ferri-ferrocyanide redox system. Because of the long incubation period, the incubating medium was replenished every three hours. After incubation, the medium was removed and the blastoderms were washed several times with Ringer's solution. To control this test, blastoderms were treated exactly as described above, with one exception. Following fixation, the esterases were denatured by immersing the blastoderms in distilled water at 90°C. for 10 minutes. The blastoderms were then incubated in the complete histochemical medium.

## Localization of Phosphorylase

The method Eranko and Palkama (1961), after slight modification, was used to localize phosphorylase. The blastoderms were frozen quickly, and then each was immersed for 40 minutes in 2.0 ml. of incubating medium at a temperature of  $37^{\circ}$ C. This solution, buffered at pH 5.9, contained glucose-1-phosphate, glycogen, adenylic acid, insulin, mercuric chloride and polyvinylpyrrolidone (molecular weight = 10,000). Following incubation, the blastoderms were fixed for 4 minutes in 40 per cent ethyl alcohol and then stained for 10 minutes in full strength Gram's iodine solution made in 0.32 M sucrose. Finally, they were washed with a Gram's iodine-sucrose solution



that had been diluted five times with 0.32 M sucrose. The control blastoderms were treated exactly as described above except that glucose-l-phosphate was omitted from the incubating medium.

Upon completion of each of the tests described above, the blastoderms were immediately studied under a dissecting microscope and the results recorded before the dyes could fade. Rather than mount the blastoderms on glass slides, each was immersed in Ringer's solution in a small watchglass where it could be moved about and studied from both dorsal and ventral surfaces (for phosphorylase, the blastoderms were immersed in a Gram's iodine-sucrose solution of the same concentration as that used to wash them). Since all structures could be seen clearly, the blastoderms were not counterstained. However, the endoderm of the 7-somite blastoderms was frequently stripped off, so the internal structures could be seen more clearly.



### RESULTS

Before presenting the results it would be helpful to review briefly the anatomy of the area opaca and to define some terminology. This part of the blastoderm. which rests upon the yolk, can be divided into several concentric regions (Lillie, 1952). The distal region. known as the margin of overgrowth, is a thin shelf of tissue that lies upon the surface of the yolk but to which the yolk does not adhere. Inside the margin of overgrowth lies the zone of junction to which yolk firmly adheres when the blastoderm is removed. These two regions together constitute the area opaca vitellina externa. The medial region of the prestreak and definitive streak blastoderms is the area opaca vitellina interna. Although yolk does adhere to the ventral surface of this region, it can be scraped off easily. The externa and the interna constitute the area opaca vitellina. In the head fold and 7-somite blastoderms, there is an additional region, the area opaca vasculosa, between the area opaca vitellina and the area pellucida. In contrast to the vitellina, the vasculosa contains a layer of mesoderm, the cells of which aggregate into clumps and short cords which give this region a mottled appearance. The inner edge of the area opaca. which borders the area pellucida, is known as the germ wall,

### Distribution of Dehydrogenases and Diaphorases

The results of the histochemical tests for the dehydrogenases and diaphorases depended upon which tetrazolium salt was used as an electron acceptor and whether the histochemical test was performed on unfrozen or frozen blastoderms. When these enzymes were localized in unfrozen blastoderms with Nitro-BT, the dye granules were distributed in unsymmetrical. non-reproducible patterns. Furthermore, the blastoderms had to be incubated for a minimum of 2 1/2 hours before an appreciable reduction of the tetrazolium salt occurred. However, when the blastoderms were quickly frozen and thawed before the histochemical tests, symmetrical, reproducible patterns appeared after 15 minutes of incubation. These same patterns were also produced by the reduction of MTT in both unfrozen and frozen blastoderms after 45 minutes of incubation. The diformazan granules produced by the reduction of Nitro-BT were bluish-black, whereas the monoformazan granules formed by the reduction of MTT were first violet, but turned bluish-black if the fixed blastoderms remained overnight in Ringer's solution. Furthermore, the diformazan granules were much coarser than the monoformazan granules.

The seven dehydrogenases and two diaphorases all had identical patterns of distribution, which are described in the following paragraphs. These results were obtained with MTT on unfrozen blastoderms.



Dye granules were uniformly distributed throughout the prestreak blastoderm. The area pellucida of many blastoderms was wrinkled, and the ridges of the wrinkles appeared to react more intensely than the furrows. But when the wrinkles were flattened with a blunt probe, the apparent difference in activity disappeared. This same difficulty was encountered with prestreak blastoderms in all of the other histochemical tests. Also, small clumps of firm, white yolk globules adhered to the ventral surface of the area opaca of some blastoderms. In each of these blastoderms the area opaca appeared blotched, because the tissue overlying each clump of yolk reacted less intensely than the surrounding tissue, which was relatively free of yolk.

In the definitive streak blastoderm, dye granules were uniformly distributed in Hensen's node and along the primitive ridges. Much less reactive were the primitive pit and primitive groove. The notochord had begun to develop in five blastoderms and accompanying its appearance was a distinct increase of activity in Hensen's node and in the anterior ends of the primitive ridges immediately adjacent to the node; the notochord itself reacted slightly weaker than the posterior portion of the primitive ridges (Figure 1). Proceeding from the node and ridges to the periphery of the area pellucida, the activity gradually diminished and then continued without further decrease



through the area opaca. Upon reaching the area opaca vitellina externa, the intensity of the reaction increased greatly, so this region appeared as a dark, violet-colored band encircling the blastoderm. The germ wall, in contrast to the rest of the blastoderm, was tinged with violet but contained no discrete dye granules; the coloring was deepest along its posterior one-third.

By the time the head fold appeared, there was a distinct gradient of activity in the primitive ridges: from the posterior ends of the ridges, the activity gradually increased to reach a maximum in the anterior ends of the ridges and in Hensen's node. The primitive pit and primitive groove reacted weakly and displayed no gradient. The neural folds reacted uniformly with the same intensity as Hensen's node. At their anterior ends the activity decreased slightly as it continued into the head fold. The neural groove reacted weakly. The high activity of Hensen's node extended anteriorly into the base of the notochord and then abruptly declined to reach a minimum in the rest of the notochord. The remainder of the area pellucida, including the foregut, reacted like the posterior ends of the primitive ridges with a slight decrease in activity from the axial structures to the periphery of the area pellucida. The area opaca reacted exactly as it did in the definitive streak blastoderm with one exception: scattered through the area opaca vitellina interna of



several blastoderms were patches of tissue to which clumps of yolk adhered and which reacted more weakly than the surrounding relatively yolk-free tissue.

At the 7-somite stage, the notochord, somites and spinal cord all displayed similar gradients of activity (Figure 2). Strong activity was present in the unclosed neural folds, which form the margins of the sinus rhomboidalis; in that portion of the notochord which lies directly under the sinus rhomboidalis (from a dosal view it appears to be enclosed within the sinus): and in the unsegmented dorsal mesoderm and most recently formed pair of somites, which lie on either side of the sinus rhomboidalis. From the anterior end of the sinus rhomboidalis to the posterior end of the rhombencephalon the enzyme activity in the spinal cord, the notochord, and the somites progressively decreased. The activity in any pair of somites was the same as the activity in the segment of notochord lying between them. At the anterior end of the spinal cord, the weak activity continued into the brain through which it progressively increased, reaching a peak in the edges of the anterior neuropore. Extending from the anterior neuropore to the anterior end of the sinus rhomboidalis. the line of closure of the neural folds was marked by a fine, dark line of dye granules. A slight gradient, decreasing from anterior to posterior, persisted

in the primitive ridges. The rest of the embryonic area, including the foregut and the heart with its associated vessels, reacted essentially the same as the brain. In the extraembryonic part of the blastoderm, the area opaca vitellina externa reacted strongly in contrast to the interna and the proamnion, which reacted much more weakly. Slightly less reactive than the interna and having a mottled appearance was that part of the blastoderm in which the blood islands were developing. As in the two previous stages, the germ wall contained no discrete dye granules, but nevertheless was tinged with violet, especially in its posterior one-third.

The control blastoderms, which were incubated in media lacking exogenous substrates, reacted in either of two ways. No dye granules were present in the control blastoderms for succinate dehydrogenase and NAD and NADP diaphorase. However, the control blastoderms for the remaining dehydrogenases--lactate, alcohol, isocitrate, glutamate, glucose-6-phosphate and B-hydroxybutyrate--displayed the same patterns as the blastoderms incubated in media containing high concentrations of exogenous substrates.

### Distribution of Acid Phosphatase

Coupling of the fast red violet salt LB with the naphthol AS-BI produced very fine, red azo dye granules. Some of the acid phosphatase or azo dye diffused into the



incubating medium, for it had a faint reddish tinge at the end of the incubation period.

Dye granules were present throughout the prestreak blastoderm. Aside from the area opaca vitellina externa, which reacted slightly stronger than the rest of the blastoderm, there was no pattern of dye granule distribution: the granules were uniformly distributed. Adhering to the ventral surface of the area opaca of three blastoderms were clumps of firm, white yolk globules, which reacted with the same intensity as the area opaca vitellina externa, giving the ventral surface of the area opaca a blotched appearance.

At the definitive streak stage, Hensen's node and the primitive ridges reacted moderately, with no indication of a gradient. The primitive groove and pit, in contrast to the ridges, reacted so weakly as to be almost colorless. The rest of the area pellucida reacted more weakly than Hensen's node and the primitive ridges. At the border of the area pellucida the activity abruptly declined and then continued without further change to the area opaca vitellina externa, which appeared as a solid, red band bordering the blastoderm. The germ wall contained no dye granules except in small, randomly scattered patches, which reacted moderately.

The entire embryonic area of the head fold blastoderm, as well as that part of the extraembryonic blastoderm that had been invaded by mesoderm, reacted with remarkable uniformity. In fact, it was nearly impossible to distinguish the various structures by their histochemical reactions. The area opaca vitellina externa reacted much more strongly than the embryonic area, while the rest of the blastoderm reacted more weakly than the embryonic area. Adhering to the ventral surface of the area opaca vitellina interna of two blastoderms were clumps of firm, white yolk granules which reacted strongly, giving the region a blotched appearance. In contrast to the definitive streak blastoderm, the entire germ wall contained dye granules.

At the 7-somite stage of development, the notochord, the somites and unsegmented dorsal mesoderm, and the central nervous system, consisting of the brain, optic vesicles, spinal cord and unclosed neural folds, all reacted strongly with no indication of a gradient of activity. Slightly less reactive were the heart, omphalomesenteric veins and truncus arteriosus. The rest of the embryonic portion of the blastoderm reacted weakly. Except for the proamnion, which reacted weakly, the extraembryonic portion of the area pellucida and the area opaca vasculosa reacted moderately and had a mottled appearance. In the area opaca vitellina, the externa reacted moderately, while

the interna reacted more weakly. As in the head fold blastoderm, dye granules were present throughout the germ wall.

The histochemical reaction was negative in the control blastoderms. No dye granules appeared when the blastoderms were incubated in a medium lacking a substrate or when they were immersed for 10 minutes in distilled water at  $90^{\circ}$ C. before being incubated in a medium containing substrate.

## Distribution of Alkaline Phosphatase

The sites of alkaline phosphatase activity were marked by very fine, red granules of azo dye formed by the coupling of fast red violet salt LB with naphthol AS-MX.

Dye granules were distributed uniformly throughout the entire prestreak blastoderm making it impossible to distinguish the various regions of the blastoderm by their histochemical reactions. The primitive streak had begun to develop in one blastoderm, and it appeared as a short, broad band slightly more reactive than the rest of the blastoderm.

Hensen's node and the primitive ridges of the definitive streak blastoderm reacted moderately, while the primitive pit and primitive groove reacted very weakly. At the border of the node and ridges the activity decreased slightly before continuing without further change through

the area pellucida and area opaca vitellina interna to the area opaca vitellina externa, which appeared as a solid red band encircling the blastoderm. Strong activity also was present in the germ wall. On one blastoderm there was a row of "blisters" on the ventral surface of the area pellucida along the anterior one-third of its edge; these reacted intensely.

The head fold blastoderm displayed a surprisingly uniform pattern of dye granule distribution. The primitive groove and the neural groove reacted weakly, appearing as two almost colorless lines bisecting the blastoderm. Otherwise, the entire embryonic area, as well as that part of the blastoderm that had been invaded by the mesoderm, reacted moderately. Slightly less reactive were the proamnion and the area opaca vitellina in which the externa reacted only slightly stronger than the interna. Very coarse dye granules were present throughout the germ wall, but were most dense in its anterior one-half.

At the 7-somite stage (Figure 3) a more complex pattern of dye granule distribution appeared. All of the somites reacted strongly, in contrast to the unsegmented dorsal mesoderm, which reacted much weaker. A similar pattern was present in the notochord: that portion of the notochord lying between the two rows of somites and extending into the head reacted with the same intensity as the somites, while the base of the notochord, as well as



Hensen's node, which are flanked by the unsegmented dorsal mesoderm, reacted much more weakly. Likewise, the brain, optic vesicles, and that portion of the spinal cord lying between the two rows of somites reacted strongly; the rest of the spinal cord and the unclosed neural folds reacted much more weakly. Strong activity was also present in the heart and its associated vessels. The rest of the embryonic area, including the foregut, reacted more weakly than the unsegmented dorsal mesoderm. The most conspicuous region of the blastoderm was that in which the blood islands were developing (Figure 5). There was a gradual increase in activity from the sinus terminalis, which reacted moderately, to the more centrally located blood islands, which reacted intensely. The entire region appeared mottled. The rest of the extraembryonic area reacted uniformly but more weakly than the sinus terminalis. The area opaca vitellina externa could not be distinguished by its histochemical reaction, as it reacted with the same intensity as the rest of the interna. As in the two previous stages, the germ wall, particularly its anterior one-half, contained many coarse granules.

In both kinds of control blastoderms--those incubated in a medium lacking a substrate, and those incubated in a complete medium after being immersed for 10 minutes in distilled water at  $90^{\circ}$ C.--there were no dye granules after one hour of incubation.



## Distribution of Carboxylic Acid Esterase

The hydrolysis of 5-bromoindoxyl acetate followed by the subsequent oxidation of indoxyl produced very fine, greenish-blue granules of indigo. Because of the small size of the granules, their patterns of distribution were very sharp and precise.

In the prestreak blastoderm the area opaca vitellina externa appeared as a faint bluish band encircling the blastoderm. The rest of the blastoderm reacted uniformly but so weakly as to be almost colorless. The primitive streak had begun to develop in two blastoderms, and it appeared as a short, broad, dark blue band extending from the periphery of the area pellucida toward the center of the blastoderm.

The definitive streak blastoderm was characterized by a lack of contrast among the enzyme activities in the various structures. Hensen's node and the primitive ridges reacted uniformly with no indication of a gradient of activity. The primitive pit and primitive groove, by comparison, reacted very weakly, appearing as a light bluish line bisecting the primitive streak. From Hensen's node and the primitive ridges the activity decreased sharply and then continued, without further change, to the area opaca vitellina externa, which reacted strongly. The germ wall also reacted strongly; but, in contrast to the rest of the blastoderm, the granules here were very coarse.

At the head fold stage. Hensen's node and the primitive ridges continued to react uniformly with no indication of a gradient of activity, while the primitive pit and primitive groove reacted much more weakly. Slightly less reactive than the node and ridges were the neural folds and head fold in which the dye granules were evenly distributed. Like the primitive groove, the neural groove reacted very weakly. The activity of Hensen's node extended anteriorly into the base of the notochord and then abruptly declined, so that the rest of the notochord reacted weakly. That portion of the blastoderm which had been invaded by mesoderm reacted with the same intensity as the primitive ridges. The area opaca vitellina externa and the germ wall continued to react strongly, the dye granules being very coarse in the latter region. The rest of the blastoderm reacted weakly.

The most striking feature of the 7-somite blastoderm (Figure 4) was the abrupt change of activity in the central nervous system and the notochord at the level of the anterior intestinal portal. The brain and optic vesicles and that part of the spinal cord lying anterior to the portal reacted strongly, while the rest of the cord, as well as the unclosed neural folds, reacted much more weakly. The notochord exhibited a similar but opposite pattern; from its base to the level of the portal, it reacted moderately, but beyond the portal it reacted weakly.



The somites also displayed an interesting pattern of activity. The unsegmented dorsal mesoderm and the most recently formed pair of somites reacted so weakly as to be scarcely distinguishable from the surrounding tissue: the next pair of somites had slightly stronger activity; while the remaining five pairs of somites all reacted strongly. Moderate activity was present in the foregut and in the heart with its associated vessels. The rest of the embryonic portion of the blastoderm reacted weakly. In the extraembryonic part of the blastoderm (Figure 5). the activity in the area opaca vitellina externa was only slightly stronger than that in the interna and the proamnion, which reacted so weakly as to be almost colorless. By comparison. that part of the blastoderm in which the blood islands were developing reacted intensely and had a mottled appearance. The germ wall contained many, very coarse dve granules.

No indigo dye was present in the control blastoderms which had been immersed in distilled water at  $90^{\circ}C$ . for 10 minutes before being incubated in the histochemical medium.

#### Distribution of Phosphorylase

Deposits of glycogen appeared as very fine, dark blue granules at the sites of phosphorylase activity.

Dye granules were present throughout the prestreak blastoderm. The most intense activity was present in the area opaca vitellina externa, which appeared as a solid,

dark blue band encircling the blastoderm. From the externa to the border of the area pellucida the activity in the area opaca gradually decreased before continuing into the area pellucida, which reacted uniformly throughout.

The pattern of dye granule distribution in the definitive streak blastoderm was identical to that in the prestreak blastoderm with two exceptions. First, Hensen's node and the primitive ridges reacted more strongly and the primitive groove and primitive pit reacted more weakly than the rest of the area pellucida. And second, the germ wall reacted intensely; in fact, it was the most reactive part of the entire blastoderm.

By the time the head fold had developed, dye granules were evenly distributed throughout the entire embryonic region of the blastoderm, except in the neural and primitive grooves where they were very sparse. That part of the blastoderm which had been invaded by the lateral mesoderm reacted uniformly, but more weakly than the embryonic region. Still weaker was the activity in the proamnion. From the inner border of the area opaca vitellina to the outer edge of the blastoderm, the activity gradually increased to reach a maximum in the area opaca vitellina externa. The entire germ wall continued to react intensely.

The most notable feature of the 7-somite blastoderm was the uniform activity in the embryonic area.

Moderate activity was present throughout the central nervous system, including the optic vesicles and unclosed neural folds; the somites and unsegmented dorsal mesoderm; and the notochord. In none of these structures was there any indication of a gradient of activity. Reacting uniformly but more weakly than the above structures was the rest of the embryonic area, including the heart with its associated vessels and the foregut. That part of the blastoderm in which the blood islands were forming reacted moderately and had a mottled appearance. The activity in the area opaca vitellina interna gradually increased from its inner border, which reacted weakly (as did the proamnion), to the area opaca vitellina externa, which continued to react strongly. As in the two previous stages, the germ wall reacted intensely.

The control blastoderms, which were incubated in a medium lacking glucose-l-phosphate and then treated with Gram's iodine, contained no dark blue granules but were colored a light, diffuse orange.



# DISCUSSION

# Validity of Histochemical Tests

Before discussing the significance of the results, it would be wise to examine critically the procedures used to obtain them.

There are several obvious difficulties in interpreting the observations made in this study. The first is that the observed density of dye granules was influenced by the thickness of the tissue. For example, the absolute density of dye granules (the number of granules per unit volume of tissue) may have been the same in two different tissues; yet the thicker tissue would have appeared to have a higher density of granules than the thinner one. Whenever there was any question as to whether an enzyme pattern was due to variations in the thickness of the tissue, the blastoderms were compared with blastoderms that had been stained with carmalum. Obviously many of the variations in dye granule density can be explained in this manner. But with some tissues, the variations were so great that they cannot be attributed solely to differences in thickness; these will be discussed later.

Another difficulty is that these tests reveal the relative concentrations and not the in vivo activities of



the enzymes. Although the results of histochemical studies are customarily described in terms of "enzyme activity," this expression is misleading and should be replaced with "relative enzyme concentration." The reason for this is that each of the histochemical media was designed to ensure a zero order reaction in which the velocity of the reaction is a function only of the concentration of the enzyme. It is doubtful that similar conditions prevail in vivo.

In addition to these general difficulties, there are others that are common only to the individual histochemical tests.

# Dehydrogenases and Diaphorases

The histochemical tests for NAD and NADP linked dehydrogenases obviously failed because the control blastoderms, which had been incubated in media lacking substrates, not only reacted positively but displayed the same patterns of dye granule distribution as the experimental blastoderms. This reduction of the tetrazolium salts in the absence of exogenous substrates might be attributed to "nothing dehydrogenase" (Zimmermann and Pearse, 1959). However, the pH's of the incubating media were rigidly maintained between 6.8 and 7.0, a pH range in which "nothing dehydrogenase" activity is so slight that it is customarily ignored. Thus, it seems improbable that the strong activity in the control blastoderms was due to



"nothing dehydrogenase." Incidentally, Shaw and Koen (1965) have recently shown that "nothing dehydrogenase" activity is due, at least partially, to several isozymes of alcohol dehydrogenase. A more likely explanation is that the concentration of endogenous substrates was so high as to completely overshadow the effects of the individual exogenous substrates. Consequently, the patterns would not be those of specific enzymes but of general reducing activity under the conditions that prevailed during the histochemical tests. Whatever the original source of electrons, they apparently passed through NAD or NADP on their way to the tetrazolium salt, for when the coenzyme, as well as the substrate, was omitted from the histochemical medium (as in the control media for succinate dehydrogenase and the diaphorases), no dye granules appeared in the blastoderm.

After only a short incubation period the same patterns were observed in frozen blastoderms with Nitro-BT and MTT and in unfrozen blastoderms with MTT. Nitro-BT, however, reacted very slowly in unfrozen blastoderms, producing erratic patterns. These results can be explained by the different physical properties of the two tetrazolium salts (Pearse and Hess, 1961). Nitro-BT, a ditetrazolium salt, is a relatively large molecule and has a strong affinity for lipoprotein membranes. Thus, cell membranes and mitochondrial membranes are barriers that hinder the salt in



reaching the enzyme sites. Only when these membranes have been damaged (by freezing, for example) does the salt rapidly penetrate to the enzyme sites. MTT, a monotetrazolium salt, is a smaller molecule that has no appreciable affinity for lipoprotein membranes. Therefore, it easily penetrates both unfrozen and frozen membranes and quickly attains a high enough concentration at the enzyme sites to satisfy the kinetics of the reaction. Beck (1965) encountered this same difficulty in localizing acid phosphatase in 10-somite blastoderms. He used Barka's (1960) azo dye technique in which the blastoderms were incubated for 2 1/2 hours at 37°C. in a medium containing naphthyl phosphate as a substrate and hexazonium pararosaniline as a coupler. Following incubation, the blastoderms were washed and fixed in calcium formol. However, Beck discovered that the incubation time could be reduced to 30 minutes by either fixing or freezing and thawing the blastoderms before incubation. Apparently the substrate, the coupler, or both had difficulty in passing through undamaged membranes.

Although both tetrazolium salts were rapidly reduced by frozen blastoderms, it was necessary to incubate the blastoderms for 45 minutes in MTT in order to obtain patterns of the same intensity as those produced by Nitro-BT after only 30 minutes of incubation. This difference is probably due, in part, to the fact that Nitro-BT has a


higher redox potential (E 1/2 = -0.05 V.) and consequently is more easily reduced than MTT (E 1/2 = -0.11 V.) (Pearse, 1960).

The color of the granules formed by the reduction of MTT and the subsequent chelation of cobalt ions by the formazan was puzzling. At first the granules were violet colored, but if the blastoderms remained overnight in Ringer's solution, the granules turned blue-black. Pearse (1957) had a similar experience with this tetrazolium salt and concluded that the color of the dye granules is influenced by the substance in which the granules are located. For example, he observed that cobalt-formazan is slightly soluble in the neutral fat globules in the Purkinje cells of the rat cerebellum and imparts a lilac color to them, while in the white matter of the brain the granules are green. Such may be the case in the present study. In 1956 Fraser explanted streak forming blastoderms onto a medium containing the Nadi reagent and, upon sectioning the blastoderms, observed a blue coloring on the surface of small droplets that stained with Sudan III. More recently, Bellairs (1963) observed with the electron microscope yolk droplets in the cells of both the area pellucida and area opaca. It is quite possible that many of the cobalt-formazan granules may have dissolved in these droplets and taken on a violet color. As the chemical



composition of the droplets slowly changed overnight, the dye granules assumed their normal blue-black color.

## Acid and Alkaline Phosphatase and Carboxylic Acid Esterase

The weakest step in the procedures for localizing acid and alkaline phosphatase and carboxylic acid esterase was fixing the enzymes. These enzymes, particularly acid phosphatase, are water soluble and easily diffuse out of the blastoderm into aqueous solutions. To prevent this, acid phosphatase was fixed with acetone, and alkaline phosphatase and esterase with 10% formalin. (How these fixatives were selected is explained in Appendix B.) Nevertheless, some enzyme probably was lost by diffusion into the fixing solution and some through inactivation by the fixative. This loss was minimized in the following ways. (a) The enzymes were fixed just long enough to prevent their diffusion into the incubating medium. (b) Sucrose and calcium chloride were added to the fixatives for alkaline phosphatase and esterase, respectively. These substances, for unknown reasons, help protect the enzymes from inactivation by the fixative (Hannibal and Nachlas, 1959). (c) To further reduce inactivation of the enzymes, all three were fixed at a low temperature  $(4^{\circ}C.)$ .

Acid and alkaline phosphatase were localized with a simultaneous coupling azo dye method using substituted naphthol esters (Burstone, 1961). The substrates, naphthol



AS-BI phosphate and naphthol AS-MX phosphate, are rapidly hydrolyzed by these enzymes. The naphthols released by the hydrolysis are highly stable compounds that are very insoluble in the incubating medium and have a high affinity for protein. Therefore, they do not readily diffuse away from the enzyme sites. In contrast to the unsubstituted naphthols, which couple rapidly with diazonium salts only at alkaline pH's, the substituted naphthols couple rapidly at acid pH's as well (Burstone, 1958a). The diazonium salt, fast red violet LB, which was used as a coupler, is a very stable compound that couples rapidly at both acid and alkaline pH's to form intensely colored azo dye particles (Burstone, 1958b). No other method for localizing acid and alkaline phosphatase combines so many advantages.

Nevertheless, this method has two drawbacks (Pearse, 1960). The more serious is that naphthol AS-BI phosphate and naphthol AS-MX phosphate are less soluble in aqueous media than unsubstituted naphthol esters, making it difficult, if not impossible, to achieve an optimum concentration of substrate. Until the kinetics of the reaction have been studied, one cannot be certain that the concentration of substrate recommended by Burstone (1961) and used in this study is sufficient to ensure a zero order reaction. The other drawback is that substituted naphthols have a lower coupling rate and, therefore, a greater



tendency to diffuse away from the enzyme sites than unsubstituted naphthols. This defect, however, is partly offset by the low solubility and high substantivity of the substituted naphthols.

Interpreting the results of the phosphatase tests is difficult because the exact identities of the <u>in vivo</u> substrates are unknown. Although these enzymes catalyze the hydrolysis of monophosphoric acid esters, the alcoholic portions of the esters have not been identified, and it is this portion that is responsible for the specificity of the substrate.

In 1954 Zacks localized carboxylic acid esterase in unincubated to 8-somite blastoderms with a simultaneous coupling azo dye method. The blastoderms were incubated in a medium containing 6-bromo-2-naphthyl acetate as a substrate and diazo blue B as a coupler and were then fixed in 10% neutral formalin. With this method, Zacks observed almost no activity in the area opaca, and in the older blastoderms most of the dye granules were located on the surfaces of the various structures rather than within them. From these observations Zacks conceded that at least one component of the incubating medium apparently failed to penetrate, or penetrated very slowly, to the enzyme sites. To check the validity of his results, carboxylic acid esterase was localized in this study with Holt and Withers' (1952) indoxyl acetate method. Although

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a popular and seemingly reliable method, it has been criticized on two counts. First, trypsin, chymotrypsin and possibly other proteolytic enzymes are known to catalyze the hydrolysis of the substrate, 5-bromoindoxyl acetate. However, the amount of dye formed by the action of these enzymes is probably so small that it can be neglected, for Holt (1959) has shown that whole rat liver catalyzes the hydrolysis of 5-bromoindoxyl acetate about 100 times faster than does the same amount of crystalline chymotrypsin. The second, more serious and more controversial criticism of this technique is that potassium ferricyanide and potassium ferrocyanide inhibit at least some of the esterases. In a comprehensive study of the esterases of the rat kidney and liver, Shnitka and Seligman (1961) discovered that esterases respond in two different ways to the potassium ferri-ferrocyanide. Some are inhibited by the redox system, and therefore cannot be demonstrated with 6-bromoindoxyl acetate; the others are resistant to the redox system and can be demonstrated with this method. Furthermore, the latter were localized exclusively in perinuclear droplets of the kidney cells and in peribiliary droplets of the liver cells, an observation from which it is reasonable to conclude that the "droplets" were really lysosomes (DeDuve, 1959). If this study is reliable, then the indoxyl acetate method may demonstrate only esterases that are confined to lysosomes.



Tsou and Su (1963) have since shown that potassium ferricyanide reduces the rate at which 5-bromoindoxyl is oxidized to 5,5' dibromoindigo. But whether the redox system also inhibits the enzymes is still unknown.

Despite these criticisms, Holt (1963) has steadfastly maintained that the potassium ferri-ferrocyanide redox system does not significantly inhibit the esterases. According to his studies, not more than 2% of these enzymes in rat liver are inhibited by the system. Also, he has been unable to obtain the same results as Shnitka and Seligman (1961) upon repeating some of their studies.

Another difficulty of this method is that the substrate, 6-bromoindoxyl acetate, is hydrolyzed not by a single specific enzyme but by several enzymes, which include non-specific esterase, lipase, acetylcholinesterase and non-specific cholinesterase. These same enzymes also catalyze the hydrolysis of 6-bromo-2-naphthyl acetate, the substrate used by Zacks (1954) (Holt, 1958).

The validity of the histochemical tests for acid and alkaline phosphatase and the esterases was upheld by the failure of dye granules to appear in the control blastoderms. The tests for acid and alkaline phosphatase were controlled by incubating fixed blastoderms in histochemical media lacking substrates. No dye granules were seen in these blastoderms, thus eliminating the possibility that the diazonium salt coupled with a naphthol other than



that produced by the hydrolysis of the naphthol AS phosphate. In addition to these controls, the tests for all three enzymes were controlled by immersing fixed blastoderms in distilled water at 90°C. for 10 minutes in order to denature the enzymes (Burstone, 1958a and b; Holt, 1958), and then incubating them in complete histochemical media. These controls also were negative, supporting the belief that the production of dye in the experimental blastoderms was indeed catalyzed by these enzymes. However, such controls do not exclude the possibility that other hydrolases, which also are inactivated by this treatment, are involved.

## Phosphorylase

Eranko and Palkama's (1961) improved method for localizing phosphorylase was modified in three ways. First, the blastoderms were quickly frozen and thawed before being incubated in the histochemical medium. If this was not done, the histochemical reaction was so slow that only a few randomly scattered dye granules were faintly visible in blastoderms that had been incubated 90 minutes. Apparently one or more components of the incubating medium was unable to penetrate quickly to the enzyme sites in unfrozen blastoderms. Second, mercuric chloride was substituted for absolute ethyl alcohol in the incubating medium, because the former is a stronger



inhibitor of the branching enzyme than the latter (Takeuchi, 1958). The third modification was to transfer the blastoderms, after the glycogen granules had been stained with Gram's iodine-sucrose, into a dilute Gram's iodine-sucrose solution. If the blastoderms were instead transferred into a solution that did not contain a small amount of iodine, the staining faded within minutes.

There are several reasons for believing that the dye granules do indeed represent sites of phosphorylase activity. The control blastoderms, which were incubated in media lacking glucose-l-phosphate, turned a diffuse orange but contained no discrete, dark blue granules. This means that the granules seen in the experimental blastoderms could not have been present prior to the histochemical test, but were synthesized from the exogenous glucose-l-phosphate. (The glycogen that is in the blastoderms at the beginning of the histochemical tests diffuses out while the blastoderms are incubating in the histochemical solution.) The deep blue color of the granules indicated that each was an aggregate of unbranched amylose chains whose synthesis was catalyzed by phosphorylase. Had any branching enzyme escaped inhibition by the mercuric chloride, some branched amylopectin chains would have been formed and would have stained reddish brown (Takeuchi, 1958). This histochemical test may also be controlled by immersing fixed blastoderms in a solution



of amylase before staining them with Gram's iodine. Alpha amylase catalyzes the hydrolysis of amylose and amylopectin to a mixture of unbranched and branched oligosaccharides, which are not stained by Gram's iodine. Beta amylase catalyzes the hydrolysis of amylose to maltose units, which are not stained by Gram's iodine, and amylopectin to dextrins, which stain reddish brown, indicating sites of the branching enzyme (Takeuchi, 1958). Preliminary tests revealed that the blastoderms were so fragile they could not withstand enzymatic digestion in addition to freezing and thawing and a 40 minute incubation in the histochemical medium. For this reason this type of control was not used.

The results of the phosphorylase test, nevertheless, must be interpreted cautiously, for this enzyme may exist in two forms: an active form known as phosphorylase a, and an inactive form, phosphorylase b. If the enzyme is similar to that in vertebrate muscle cells, phosphorylase b would have been activated by the high concentration of adenosine monophosphate in the incubating medium, and the synthesis of glycogen would have been catalyzed by the combined action of phosphorylases a and b. But if the enzyme is similar to that in liver cells, phosphorylase b would have been unaffected by adenosine monophosphate, and the synthesis of glycogen would have been catalyzed only



by phosphorylase a (White, <u>et al.</u>, 1964). Of course, it is possible that during these early stages of development, the enzyme exists only in an active form. Unfortunately, nothing is known about the nature of this enzyme in the early chick blastoderm.

Despite the difficulties discussed above, the results of these tests are not without value. They provide considerable insight into the chemistry of development, and they raise some interesting questions, which could serve nicely as bases for further investigations.

## Significance of Enzyme Patterns

Unquestionably the most interesting patterns were those observed in the axial structures of the 7-somite blastoderms. The patterns of the NAD and NADP linked dehydrogenases are meaningless, since the histochemical tests could not be controlled. The pattern of succinate dehydrogenase, however, is not only reliable but informative. This enzyme is firmly bound to mitochondria (Green, 1959), and, providing the mitochondria are protected by incubating the blastoderms in a slightly hypertonic medium containing magnesium ions, each dye granule can be assumed to represent a single mitochondrion (Pearse, 1960). Thus, the distribution of dye granules reflects the distribution of mitochondria, assuming, of course, that each mitochondrion contains enough active succinate dehydrogenase to

produce a visible dye granule. Accordingly, there appear to be three mitochondrial gradients in the axial structures of the 7-somite blastoderm. The mitochondria are highly concentrated in the unclosed neural folds, in Hensen's node and that portion of the notochord that lies directly under the sinus rhomboidalis, and in the unsegmented dorsal mesoderm and the most recently formed pair of somites. Proceeding anteriorly, their concentration in the spinal cord, the notochord and the somites gradually decreases; proceeding posteriorly through the primitive streak, their concentration also decreases. The third gradient is in the brain where the concentration of mitochondria increases from the posterior border of the brain to the anterior neuropore.

These gradients have been reported by other investigators, one of the earliest being Hyman (1927). She recorded the progressive death of cells in 5- to 8-somite blastoderms that had been treated with potassium cyanide, ammonium hydroxide or sodium hydroxide. The first cells to die and disintegrate were those of Hensen's node and those of the neural folds where they had fused first. From Hensen's node, disintegration spread posteriorly through the primitive streak and anteriorly through the central nervous system and the somites. From the point of closure of the neural folds, disintegration spread anteriorly to the neuropore and posteriorly through the

central nervous system. Hyman does not describe the disintegration of the notochord in 5- to 8-somite blastoderms; but in head fold blastoderms, disintegration began at the anterior tip and proceeded posteriorly. From these observations Hyman concluded that Hensen's node and the point of closure of the neural folds are centers of high metabolism.

A similar pattern was observed by Rulon (1935), who recorded the anaerobic reduction of Janus green to diethylsafranin. This stain, according to Lazarow and Cooperstein (1953), is reduced by FADH<sub>2</sub> and, therefore, is an indicator of the reduced form of this prosthetic group and its apoprotein. In the axial structures of blastoderms of 5 to 6 somites, red coloring first appeared in Hensen's node from which it spread anteriorly through the central nervous system and somites and posteriorly through the primitive streak. Rulon (1935) does not describe reduction in the notochord. When the coloring had reached half way between Hensen's node and the hindbrain, the edges of the anterior neuropore turned red, and this coloring began spreading posteriorly through the central nervous system.

Moog (1943a) observed two of these gradients in studying the distribution of cytochrome oxidase in the chick blastoderm. After treating blastoderms of 5 to 10 somites with the Nadi reagent, she observed indophenol

blue in the edges of the anterior neuropore and in Hensen's node. From these two places, the coloring gradually spread posteriorly through the axial structures. When the blastoderms were treated with sodium azide, which inhibits cytochrome oxidase, the anterior gradient was affected only slightly, while the posterior gradient was appreciably repressed. Why she failed to observe the other gradient, which extends anteriorly from Hensen's node, is puzzling.

The most extensive study of the gradients is that of Spratt (1958). He immersed blastoderms of 15 to 20 somites in Ringer's solution, buffered at pH 7.8 to 8.0 and containing one of the following indicators: potassium tellurite, neotetrazolium chloride, triphenyl tetrazolium chloride, methylene blue or Janus green. The methylene blue and Janus green were reduced under anaerobic conditions, while the other indicators were reduced aerobically. All five were reduced along the three gradients just described; also, they were rapidly reduced in and around the posterior end of the primitive streak. Spratt also explanted blastoderms of 3 to 12 somites onto Ringer-agar media containing neotetrazolium chloride and specific substrates. When 0.02 to 0.2 M sodium cyanide was included in the media, he discovered that the reducing activity in Hensen's node and in the forebrain were affected differently according to the substrate used. For example, when

glucose or mannose was the substrate, reducing activity of the brain but not Hensen's node was stimulated; with fructose or maltose as the substrate, reducing activity of both the brain and Hensen's node was inhibited. These results, along with those of Moog (1943a), strongly suggest that the origins of the gradients may not be identical.

During at least the first week of incubation, glucose is the principle source of energy to the blastoderm (Spratt, 1949). Thus, it is not surprising that phosphorylase is present in a relatively high concentration in the axial structures. Its uniform distribution, however, is surprising, in view of the mitochondrial gradients just described. Of course, it is possible, as already mentioned, that some of the phosphorylase may be inactive and that the active form may be distributed in a pattern entirely different from that observed. Another reason why it is difficult to interpret this pattern is that there is no accurate and detailed description of the distribution of its substrate, glycogen, in the axial structures of blastoderms of this age. Finally, it is not known how much of the glucose-l-phosphate, released by the hydrolysis of glycogen, eventually undergoes glycolysis and how much enters the pentose shunt. More might be known about this if the tests for lactate dehydrogenase and glucose-6-phosphate dehydrogenase had succeeded, for



the presences of these enzymes are, respectively, reliable indicators of parts of these two pathways (Pearse, 1960).

Alkaline phosphatase activity abruptly changed at the level between the unsegmented dorsal mesoderm and the most recently formed pair of somites: anterior to this level the central nervous system, the somites, and the notochord reacted strongly and uniformly, while posteriorly much weaker activity was present in the base of the spinal cord and the unclosed neural folds, the unsegmented dorsal mesoderm, and the base of the notochord and Hensen's node. An identical pattern was observed in the central nervous system by Moog (1943b), who localized this enzyme with Gomori's (1941a) method. In a quantitative study of adenosine triphosphatase (ATPase) activity in the dorsal mesoderm of 13-somite blastoderms, Deuchar (1960a) discovered that the somites have a higher calcium activated ATPase activity than the unsegmented mesoderm. Magnesium-activated ATPase activity, however, was the same throughout the dorsal mesoderm. Additional studies (Deuchar, 1960b) supported her conclusion that the increased ATPase activity in the somites represents the synthesis of myosin or a precursor of myosin as the somites are molded from the dorsal mesoderm.

Acid phosphatase reacted strongly and uniformly in the notochord, the somites and unsegmented dorsal

mesoderm, and the central nervous system, including the unclosed neural folds. It is surprising that the distribution of this enzyme, with its low optimum pH, should completely overlap that of alkaline phosphatase, which has a much higher optimum pH. This paradox can be explained by assuming that only one of the two enzymes is active at any given moment, depending upon the prevailing pH. A more likely explanation, however, is that acid phosphatase is confined to lysosomes within which the pH may be acid (De Duve, 1959), while alkaline phosphatase is located outside the lysosomes where the pH may be much higher. Under these conditions both enzymes could be active simultaneously.

Especially intriguing is the abrupt change in the activity of the carboxylic acid esterases in the central nervous system and notochord at the level of the anterior intestinal portal. The central nervous system reacted strongly anterior to the portal but weakly posterior to the portal. The notochord, on the other hand, reacted weakly anterior to the portal but strongly posterior to the portal. These results suggest that there may be some kind of interaction between the central nervous system, the notochord and the developing foregut at this stage of development.

The importance of the overlying tissues to the development of the foregut is illustrated by two of



Waddington's (1932) experiments. In one experiment, he explanted the endoderm of 16- to 24-hour blastoderms onto a clot; 2 days later there was no indication of a foregut. In the other experiment, he rotated  $90^{\circ}$  the epiblast of short, medium and long primitive streak blastoderms; when the foregut subsequently developed, it too had been rotated 90°, lying in its normal relationship to the other axial structures. Which of the axial structures is responsible for inducing the development of the foregut is still a controversial question. However, Fraser (1954) observed in the 30-hour (6-somite) blastoderm that the notochord is attached to the endoderm directly above the anterior intestinal portal, but anterior and posterior to this point of attachment it is separated from the endoderm by mesenchyme. This observation, along with the pattern of esterase distribution in the notochord, strongly suggests that the notochord may be intimately involved in the molding of the foregut.

Although scarcely anything is known about the relationship between the notochord and the central nervous system in the 7-somite blastoderm, this has been carefully studied in 21-somite blastoderms exhibiting duplicitas anterior (Watterson, et al., 1955). Such blastoderms clearly show that the notochord causes the floor of the neural tube to become thinner and that the larger the notochord, the thinner the wall becomes. But in order



for this to occur, the two structures must be in contact, for if they are separated by just a few strands of mesenchyme, the notochord has no effect upon the neural tube. The somites have an opposite effect upon the neural tube, causing its wall to thicken and the cells to polarize so they are perpendicular to the somites. Also, the larger the somites, the greater their effect upon the neural tube. In another series of experiments, Watterson and his colleagues (1955) extirpated sections of neural tube and notochord from blastoderms of 12 to 28 somites. In several blastoderms they accidentally removed some notochord from the rest of the blastoderm; yet the floor of the neural tube subsequently became thinner even though the notochord was missing. Thus, the notochord apparently exerts its effect upon the neural tube before the 12-somite stage. It is tempting to speculate that whatever this influence is, it is manifested by the changes in esterase activity in the spinal cord and notochord of the 7-somite blastoderm.

The patterns of enzyme activity in the area opaca at all four stages also are enlightening. One of the most interesting, yet unexplainable, observations was the marked increase of enzyme activity in the area opaca vitellina externa between the prestreak and the definitive streak stages. In the prestreak blastoderm, the activities of succinate dehydrogenase, NAD diaphorase, NADP diaphorase and alkaline phosphatase were uniform throughout the entire

area opaca; the activities of acid phosphatase and carboxylic acid esterase were slightly stronger in the vitellina externa than in the vitellina interna. But in the defiitive streak, head fold, and 7-somite blastoderms, the activities of all of these enzymes were so strong in the externa that it appeared as a solid, colored band encircling the blastoderm, in contrast to the interna, which reacted with nearly the same intensity as it did in the prestreak blastoderm. The only enzyme not to exhibit these patterns was phosphorylase, which at all four stages was distributed along a radial gradient, its activity gradually increasing from the inner edge of the interna to the outer edge of the blastoderm.

The area opaca vitellina externa is the principle site of growth of the area opaca, for Feulgen staining has revealed a preponderance of mitotic figures just inside its outer edge (New, 1959). Although their exact location has not been described, they probably are located in the margin of overgrowth along its border with the zone of junction. As the margin of overgrowth expands, the cells forming its outer edge move outward upon the inner surface of the vitelline membrane to which they adhere, while the cells along its inner edge differentiate into ectoderm and endoderm cells of the zone of junction.

Bellairs and New (1962) have shown that the cells of the margin of overgrowth phagocytize yolk as they move

outward upon its surface. They covered explanted blastoderms (early streak to 2-somites) with colloidal suspensions of gold and carmine, and, several hours later, discovered gold and carmine particles inside yolk droplets in the cells of only the margin of overgrowth. They also discovered that phagocytosis stops if the movement of the cells is stopped. Beck (1965) obtained similar results upon bathing blastoderms in ovo with Ringer's solution containing trypan blue; 12 hours later, at the 10-somite stage, he observed the dye inside the cells of the margin of overgrowth. With the electron microscope, Bellairs (1963) observed processes, devoid of endoplasmic reticulum and other cell organelles, extending from the cells of the margin of overgrowth into the yolk. These cells, but not their processes, contain a high concentration of acid phosphatase (Beck, 1965).

The zone of junction apparently does not phagocytize yolk, but instead exchanges materials with the yolk by means of active transport. Bellairs (1963) has observed microvilli on the ventral surfaces of the endoderm cells of the zone of junction. She also has observed long processes extending from the yolk cells up between the cells of the zone of junction to the vitelline membrane, as if to facilitate exchange between the yolk and the ectoderm cells and possibly between the yolk and the

albumin. The high concentration of alkaline phosphatase in the vitellina externa, as revealed by the present study, also suggests that active transport is occurring at a high rate in the zone of junction. This enzyme almost invariably is present in cell membranes through which active transport is taking place (Moog, 1959), particularly membranes covering microvilli (Clark, 1961).

The endoderm cells of the zone of junction contain large yolk droplets (Bellairs, 1963) that most likely were phagocytized when the cells were part of the margin of overgrowth. Much of the acid phosphatase activity of the vitellina externa is probably confined to these droplets, as well as those in the margin of overgrowth, where it is catalyzing the breakdown of yolk, for Barka (1962) has shown that this enzyme invariably is present in phagocytotic vacuoles of rat tissues. Some of the esterases also may be associated with the phagocytotic vacuoles (Holt, 1963).

Among the substances being secreted into the yolk by the zone of junction is acid phosphatase. This enzyme was localized in clumps of white yolk adhering to the area opaca of prestreak blastoderms and in yolk globules adhering to the zone of junction of head fold blastoderm. Its presence in the yolk underlying the vitellina externa has been detected also by Beck (1963) in 10-somite blastoderms. The enzyme probably is synthesized by the endoderm


cells of the zone of junction, stored in the Golgi complexes, and eventually secreted into the yolk where it helps catalyze the breakdown of yolk into particles that can be taken into the cells by methods other than phagocytosis. According to Needham (1950), the pH of the yolk lies between 4.5 and 6.0, a range that coincides exactly with the optimum pH range of acid phosphatase.

Much of the energy for these various activities probably is supplied by aerobic respiration, as indicated by the high concentration of succinate dehydrogenase granules, and therefore mitochondria, in the vitellina externa. According to Bellairs (1963), the mitochondria in the zone of junction are more concentrated in the ectoderm cells than in the endoderm cells. The present evidence points to glycogen as a major source of this energy: (a) Bellairs (1963) has observed clusters of granules resembling glycogen granules in the ectoderm cells of the zone of junction; (b) as the present study has shown, there is a high concentration of phosphorylase in the vitellina externa; and (c) at this particular stage of development, glucose is essential for the continued growth and development of the blastoderm (Spratt, 1949).

The much weaker enzymatic activity of the area opaca vitellina interna, as well as its morphology, suggests



that this region is relatively quiescent. Although the endoderm cells contain yolk droplets (Bellairs, 1963), there is no evidence that they are engaged in phagocytosis (Bellairs & New, 1962) -- the droplets probably were phagocytized when the cells were part of the margin of overgrowth. The absence of microvilli on the ventral surfaces of the endoderm cells and the absence of yolk processes extending through the blastoderm to the vitelline membrane (Bellairs, 1963) indicate a reduced rate of active transport. The interna grows little, if at all, by the addition of new cells, for there are few mitotic figures in the region (New, 1959). Finally, there is no evidence of any significant differentiation taking place here. Aside from the absence of villi on the endoderm cells, the presence of a basement membrane separating the endoderm and ectoderm, and the fact that the endoderm cells are closer together (Bellairs, 1963), the interna appears to be morphologically identical to the zone of junction.

The enzyme patterns of the area opaca vasculosa were different in the head fold and 7-somite blastoderms. In the head fold blastoderm, the activities of succinate dehydrogenase, NAD diaphorase, NADP diaphorase and alkaline phosphatase were uniform throughout the area opaca vasculosa and the vitellina interna, making it impossible to discern the boundary between the two areas by their



histochemical reactions. The activities of acid phosphatase, carboxylic acid esterase and phosphorylase, on the other hand, were appreciably stronger in the area opaca vasculosa than in the vitellina interna. In the 7-somite blastoderm, however, the activities of all seven enzymes were stronger in the area opaca vasculosa than in the vitellina interna. Furthermore, the dye granules were distributed so as to give the vasculosa a mottled appearance.

The relatively strong activities of acid phosphatase, carboxylic acid esterase and phosphorylase in the head fold blastoderm suggest that these enzymes are involved in the formation of the exocoelom. At the head fold stage, the exocoelom is just beginning to develop (Sabin, 1920). It consists of vesicles between the upper two cell layers of the mesoderm; in the medial part of the vasculosa, the vesicles are small and separated, whereas in the distal part of the vasculosa, they are larger and have begun to coalesce to form a plexus.

All seven enzymes seem to be involved in the development of the blood vessels and blood cells of the yolk sac as evidenced by their relatively strong activities and mottled patterns in the 7-somite blastoderm. Development of the blood vessels and blood cells does not begin until about the 2-somite stage (Sabin, 1920). At this



time isolated clumps of cells in the lower lavers of the mesoderm differentiate into angioblasts. These cells have a highly granular and strongly basophilic cytoplasm. As the clumps of angioblasts enlarge and become more numerous, they coalesce to form a plexus. The angioblasts that lie on the surfaces of the plexus form an endothelium, while some of the deeper angioblasts liquify to form plasma. The remaining angioblasts, which adhere in clumps (blood islands) to the endothelium, ultimately give rise to primitive erythrocytes. Some investigators believe that some of the angioblasts of the blood islands differentiate into stem cells from which leucocytes, as well as erythrocytes, may develop. However, no cells in blastoderms of the second day of incubation have been identified as ancestors of the leucocytes. In the 7-somite blastoderm, the area pellucida and area opaca vasculosa each can be divided into two concentric zones. In the area pellucida, the medial zone, which borders the embryonic area, contains undifferentiated mesoderm, whereas the distal zone contains isolated clumps of angioblasts. In the area opaca vasculosa, the medial zone contains a solid plexus of angioblasts, in contrast to the distal zone, which contains a plexus of endothelial tubes filled with plasma and clumps of primitive erythroblasts.

The intense enzymatic activity in the area opaca vasculosa of the 7-somite blastoderms correlates well



with the morphology of this region as revealed with the electron microscope (Bellairs, 1963). The ectoderm of the vasculosa is several cells thick and is underlain by mesoderm cells in which angioblasts are differentiating. Although glycogen granules and ribosomes are present throughout the ectoderm, they are concentrated in the deeper cells and in the mesodermal cells. The concentration of ribosomes, along with the fact that collagenlike fibrils appear in the interstitial spaces around the cells just after circulation begins, indicates that these cells are engaged in a relatively high rate of protein synthesis. Because of its identical distribution, glycogen may also be involved in this process. Its principle function probably is to supply glucose-6phosphate, of which some may feed into the pentose shunt to provide pentoses for RNA synthesis, and some may enter the glycolytic pathway and subsequently the Krebs cycle to provide energy. This could easily account for the high concentration of phosphorylase and succinate dehydrogenase (mitochondria) in the vasculosa. The endoderm of the vasculosa in some respects resembles intestinal epithelium. It consists of a single layer of columnar cells resting upon a dorsal basement membrane; the cells are closely packed and seem to adhere to one another by means of desmosomes. The free surfaces of the cells are deeply indented, a specialization which suggests that the

cells are engaged in active transport, in which case some of the alkaline phosphatase is probably localized in the membranes of their free surfaces (Moog, 1959). Also, the cells contain yolk droplets within which are tiny vacuoles. This is the first morphological indication that the droplets may be undergoing catabolism. Beck (1965) has shown that most of the acid phosphatase in the vasculosa is present in the endoderm, where it, along with some of the esterases, probably is confined to the yolk droplets.

The germ wall consists of a ring of cells, exclusive of the ectoderm, which forms the inner edge of the area opaca. Its depth gradates from at least six cell diameters posteriorly to one or two cell diameters anteriorly. The cells are spherical and heavily laden with large yolk droplets (Spratt and Haas, 1965). As in the area opaca vitellina externa, there was an appreciable increase in enzyme activity in the germ wall between the prestreak and definitive streak stages. In the prestreak blastoderms the germ wall reacted with the same intensity as the adjacent opaca and pellucida. But by the definitive streak stage, all of the enzymes, except acid phosphatase, reacted strongly along the entire germ wall; acid phosphatase activity was confined to small, randomly scattered patches of the wall, and not until the head fold stage was the enzyme present along the entire wall.

Between the prestreak and definitive streak stages the area pellucida changes from an oval- to a pear-shaped region and its circumference increases considerably. If the germ wall is a circular blastema which contributes cells to the extraembryonic endoderm and mesoderm, as Spratt and Haas (1965) maintain, then it should become very active during this phase of development. This may account for its increase in enzyme activity.

Several enzymes displayed gradients of activity in the germ wall of the definitive streak, head fold and 7-somite blastoderms. Alkaline phosphatase was more active in the anterior one-half of the germ wall than in the posterior one-half; succinate dehydrogenase and the two diaphorases, on the other hand, were more active in the posterior one-third than in the anterior two-thirds.

Blastoderms stained with carmalum displayed gradients in the germ wall identical to those of succinate dehydrogenase and the diaphorases, indicating that the latter are not true enzyme gradients but instead reflect the depth of the germ wall. It is surprising, therefore, that the remaining enzymes also did not display this gradient. Although these enzymes probably are present throughout the germ wall, they may be most active in only the lowest one or two cell layers, which would account for their uniform distribution.



Finally, in the three latter stages of development, the azo dye granules produced by the test for alkaline phosphatase and the indigo dye granules produced by the test for carboxylic acid esterase were much coarser in the germ wall than elsewhere in the blastoderm. In contrast, the tests for the dehydrogenases and diaphorases did not produce dye granules in the germ wall; instead the wall was colored a diffuse violet (MTT formazan) or blue-black (Nitro-ET diformazan). These variations in the sizes of the dye granules underscore the difference between the cells of the germ wall and the adjacent cells of the area opaca.

One last observation deserves mention. In the definitive streak blastoderm, succinate dehydrogenase, NAD diaphorase and NADP diaphorase were uniformly distributed in Hensen's node and along the primitive ridges. But with the appearance of the notochord, there was a marked increase in the concentration of dye granules in the node and in the anterior ends of the ridges. This increase in reducing activity had been observed previously by Spratt (1958) in blastoderms that had been explanted at early ages onto albumin-agar containing 0.1% neotetrazolium chloride. Spratt also observed a simultaneous increase in reducing activity in and around the posterior end of the streak, which probably is related to the regression of the streak. The increase in



succinate dehydrogenase activity in Hensen's node and in the anterior ends of the ridges almost certainly reflects an increase in the concentration of mitochondria in these tissues, for this enzyme seems to be associated exclusively with these organelles (Green, 1959). Assuming that the primitive streak is not a blastopore but rather a blastema (Spratt and Haas, 1965) and that the notochord develops from cells of Hensen's node (Spratt, 1955), such an increase in the mitochondrial population is reasonable. The principle source of energy for the cells of the node appears to be glycogen, for this compound is highly concentrated in all cells of the node except those forming the lowest layer (endoderm) where it is scarce (McCallion and Wong, 1956).



## SUMMARY

I. The objectives of this study were to localize 13 enzymes in whole chick blastoderms at four stages of development: prestreak (6 hours), definitive streak (18 hours), head fold (24 hours), and 7-somite (33 hours). The enzymes were the following dehydrogenases: lactate, alcohol, isocitrate, succinate, glutamate, glucose-6-phosphate, and B-hydroxybutyrate; NAD diaphorase and NADP diaphorase; acid and alkaline phosphatase; carboxylic acid esterase; and phosphorylase.

II. The dehydrogenases and diaphorases were localized with the method of Thomas and Pearse (1961); both Nitro-BT and MTT were used as electron acceptors. Acid and alkaline phosphatase were localized with the method of Burstone (1961); naphthol AS-BI and naphthol AS-MX phosphoric acids, respectively, were used as substrates and fast red violet salt LB as a coupler. Carboxylic acid esterase was localized with the method of Holt and Withers (1952), using 6-bromo-indoxyl acetate as a substrate. And phosphorylase was localized with the method of Eranko and Palkama (1961).



III. The tests for the NAD- and NADP-linked dehydrogenases--lactate, alcohol, isocitrate, glutamate, glucose-6-phosphate and B-hydroxybutyrate--could not be controlled, so the patterns of these enzymes are invalid.

IV. The remaining seven enzymes--succinate dehydrogenase, NAD diaphorase, NADP diaphorase, acid and alkaline phosphatase, carboxylic acid esterase and phosphorylase-were satisfactorily localized. Their patterns are summarized below.

V. The activities of acid phosphatase and carboxylic acid esterase were uniform throughout the entire prestreak blastoderm except in the area opaca vitellina externa where they were slightly stronger. Phosphorylase activity was uniform in the area pellucida but increased from medial to distal in the area opaca vitellina interna, becoming intense in the area opaca vitellina externa. The activities of the remaining enzymes were uniform throughout the entire blastoderm.

VI. In the primitive streak blastoderm, the enzyme activities were slightly stronger in Hensen's node and the primitive ridges and much weaker in the primitive pit and groove than in the rest of the area pellucida. In the area opaca, phosphorylase activity increased from medial to distal in the vitellina interna, becoming intense in the vitellina externa. For the rest of the



enzymes, the interna reacted uniformly with much stronger activity in the externa. Intense activity was present throughout the germ wall, except in the case of acid phosphatase, which was present only in randomly scattered patches. The activities of succinate dehydrogenase and the two diaphorases were stronger in the posterior onethird than in the anterior two-thirds of the germ wall. The formazan dye granules (succinate dehydrogenase and the two diaphorases) were much finer and the indigo dye granules (carboxylic acid esterase) were much coarser in the germ wall than elsewhere in the blastoderm.

VII. Some blastoderms in which the dehydrogenases and diaphorases were localized had begun to develop notochords. In these blastoderms, the activities of succinate dehydrogenase and the two diaphorases were much stronger in Hensen's node and the anterior ends of the primitive ridges than in the remainder of the ridges.

VIII. In the head fold blastoderm, the enzyme activity in the embryonic area was generally uniform. The principle exceptions were the neural and primitive grooves in which the activity was much weaker; the notochord in which the activities of succinate dehydrogenase, the two diaphorases, and carboxylic acid esterase were much stronger in the base than in the rest of the structure; and the primitive streak, in which the activities of



succinate dehydrogenase and the two diaphorases decreased from anterior to posterior. The rest of the area pellucida reacted more weakly than the embryonic area. In the area opaca, the activities of succinate dehydrogenase and the two diaphorases were uniform throughout the vasculosa and vitellina interna, whereas the activities of acid and alkaline phosphatase, carboxylic acid esterase, and phosphorylase were stronger in the vasculosa than in the vitellina interna. Phosphorylase activity in the interna increased from medial to distal. The activities of all enzymes were intense in the vitellina externa. The entire germ wall reacted strongly, with the activities of succinate dehydrogenase and the two diaphorases being strongest in the posterior one-third and the activity of alkaline phosphatase being strongest in the anterior one-half. The formazan dye granules (succinate dehydrogenase and the two diaphorases) were much finer and the azo dye granules (alkaline phosphatase) and indigo dye granules (carboxylic acid esterase) were much coarser in the germ wall than elsewhere in the blastoderm.

IX. At the 7-somite stage, most of the enzymes had characteristic patterns of distribution in the axial structures of the embryo. Succinate dehydrogenase and the two diaphorases were distributed along three gradients. Their activities decreased from anterior to posterior in



the primitive ridges; from posterior to anterior in Hensen's node and the notochord, the unsegmented dorsal mesoderm and the somites, and the unclosed neural folds and the spinal cord; and from anterior to posterior in the brain. Alkaline phosphatase activity increased sharply at the level between the unsegmented dorsal mesoderm and the most recently formed pair of somites. Posterior to this level relatively weak activity was present in the spinal cord and unclosed neural folds, Hensen's node and the base of the notochord, and the unsegmented dorsal mesoderm; anterior to this level much stronger activity was present in the central nervous system, the notochord, and the somites. The activity of carboxylic acid esterase changed abruptly at the level of the anterior intestinal portal: the activity in the central nervous system was much stronger anterior to the portal than posterior to it, whereas the activity in the notochord was much stronger posterior to the portal than anterior to it. The unsegmented dorsal mesoderm and the two most recently formed pairs of somites reacted much more weakly than the older, five pairs of somites. The patterns of acid phosphatase and phosphorylase were uniform in the axial structures with no indications of any gradients. In the area opaca, the activities of all the enzymes were stronger in the vasculosa than in the



vitellina interna. Furthermore, the dye granules in the vasculosa were distributed so as to give the region a mottled appearance. The patterns in the vitellina and germ wall were identical to those of the head fold blastoderm.

X. The validity of the histochemical tests and the significance of the enzyme patterns are discussed.



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Figure 1.--A dorsal view of the primitive streak just after the notochord has begun to develop, showing the distributions of succinate dehydrogenase, NAD diaphorase and NADP diaphorase (x 50).



a 7-somite blastoderm, showing the distributions of succinate dehydrogenase, NAD diaphorase and NADP dia-phorase. Only the base of the notochord can be seen; the dark line of granules which bisects the central nervous system marks the line of closure of the neural tube. The heart was deliberately omitted from the figure (x 40).

Figure 2.-- A dorsal view of the axial structures of



Figure 3.--A dorsal view of the axial structures of a 7-somite blastoderm, showing the distribution of alkaline phosphatase. Only the base of the notochord can be seen; the heart was deliberately omitted from the figure. (x 40).

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Figure 4.--A dorsal view of the axial structures of a 7-somite blastoderm, showing the distribution of carboxylic acid esterase. Only the base of the noto-chord can be seen; the heart was deliberately omitted from the figure (x 40).



Figure 5.--A ventral view of a posterior segment of the area opaca of a 7-somite blastoderm, showing the distributions of alkaline phosphatase and carboxylic acid esterase (x 30). A--germ wall; B--area opaca vasculosa; C--area opaca vitellina interna; D--area opaca vitellina externa.





Nitro-BT		MTT	
Unfrozen	Frozen	Unfrozen	Frozen
7(8)	6(5)	5(6)	5(5)
8(6)	7(7)	7(5)	5(6)
10(9)	7(5)	8(5)	6(6)
11(6)	8(6)	8(7)	7(5)
	Nitro- Unfrozen 7(8) 8(6) 10(9) 11(6)	Nitro-BT        Unfrozen      Frozen        7(8)      6(5)        8(6)      7(7)        10(9)      7(5)        11(6)      8(6)	Nitro-BT      MTT        Unfrozen      Frozen      Unfrozen        7(8)      6(5)      5(6)        8(6)      7(7)      7(5)        10(9)      7(5)      8(5)        11(6)      8(6)      8(7)

TABLE 1.--The numbers of unfrozen and frozen blastoderms in which lactate dehydrogenase was localized with Nitro-BT and MTT. The numbers of control blastoderms are enclosed in parentheses.

TABLE 2.--The numbers of unfrozen and frozen blastoderms in which alcohol dehydrogenase was localized with Nitro-BT and MTT. The numbers of control blastoderms are enclosed in parentheses.

	Nitro	-BT	MTT	
Stage	Unfrozen	Frozen	Unfrozen	Frozen
Prestreak	7(6)	7(5)	6(6)	5(6)
Definitive streak	19(14)	16(12)	14(15)	12(11)
Head fold	26(15)	18(11)	21(23)	15(15)
7-somite	20(14)	14(13)	16(16)	10(8)



	Nitro-BT		MTT	
Stage	Unfrozen	Frozen	Unfrozen	Frozen
Prestreak	9(7)	8(6)	9(11)	6(5)
Definitive streak	17(18)	13(9)	14(12)	9(8)
Head fold	18(15)	13(13)	10(11)	7(6)
7-somite	15(12)	7(6)	13(10)	8(8)

TABLE 3.--The numbers of unfrozen and frozen blastoderms in which isocitrate dehydrogenase was localized with Nitro-BT and MTT. The numbers of control blastoderms are enclosed in parentheses.

TABLE 4.--The numbers of unfrozen and frozen blastoderms in which succinate dehydrogenase was localized with Nitro-BT and MTT. The numbers of control blastoderms are enclosed in parentheses.

	Nitro-	-BT	MTT	
Stage	Unfrozen	Frozen	Unfrozen	Frozen
Prestreak	12(14)	9(9)	7(6)	6(6)
Definitive streak	23(30)	25(20)	14(16)	13(11)
Head fold	20(12)	19(12)	15(15)	8(10)
7-somite	17(12)	13(8)	10(9)	7(8)



	Nitro-	-BT	MTT	
Stage	Unfrozen	Frozen	Unfrozen	Frozen
Prestreak	7(5)	8(5)	6(6)	6(7)
Definitive streak	10(11)	11(8)	13(12)	9(10)
Head Fold	11(9)	12(9)	10(11)	7(6)
7-somite	12(11)	12(9)	9(7)	11(8)

TABLE 5.--The numbers of unfrozen and frozen blastoderms in which glutamate dehydrogenase was localized with Nitro-BT and MTT. The numbers of control blastoderms are enclosed in parentheses.

TABLE 6.--The numbers of unfrozen and frozen blastoderms in which glucose-6-phosphate dehydrogenase was localized with Nitro-BT and MTT. The numbers of control blastoderms are enclosed in parentheses.

	Nitro	-BT	MTT	
Stage	Unfrozen	Frozen	Unfrozen	Frozen
Prestreak	8(9)	7(5)	9(6)	6(7)
Definitive streak	12(10)	10(10)	15(11)	9(7)
Head fold	10(12)	8(6)	9(9)	6(7)
7-somite	14(13)	12(9)	13(15)	9(11)

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	Nitro-BT		МТТ	
Stage	Unfrozen	Frozen	Unfrozen	Frozen
Prestreak	8(9)	6(6)	7(8)	6(5)
Definitive streak	14(11)	12(11)	12(13)	10(7)
He <b>a</b> d fold	12(8)	7(8)	11(14)	9(6)
7-somite	12(7)	13(10)	16(12)	8(9)

TABLE 7.--The numbers of unfrozen and frozen blastoderms in which B-hydroxybutyrate dehydrogenase was localized with Nitro-BT and MTT. The numbers of control blastoderms are enclosed in parentheses.

TABLE 8.--The numbers of unfrozen and frozen blastoderms in which NAD diaphorase was localized with Nitro-BT and MTT. The numbers of control blastoderms are enclosed in parentheses.

<b>C</b> t	Nitro-BT		MTT	
Stage	Unfrozen	Frozen	Unfrozen	Frozen
Prestreak	9(8)	7(8)	10(11)	8(6)
Definitive streak	22(15)	18(16)	20(21)	14(11)
Head fold	21(20)	17(19)	15(20)	12(9)
7-somite	14(11)	12(12)	13(9)	10(11)



	Nitro-BT		МТТ	
Stage	Unfrozen	Frozen	Unfrozen	Frozen
Prestreak	6(6)	7(5)	6(6)	6(5)
Definitive streak	13(10)	12(9)	9(10)	7(8)
Head fold	17(15)	14(10)	19(18)	13(11)
7-somite	19(9)	8(10)	11(7)	9(6)

TABLE 9.--The numbers of unfrozen and frozen blastoderms in which NADP diaphorase was localized with Nitro-BT and MTT. The numbers of control blastoderms are enclosed in parentheses.

TABLE 10.--The numbers of blastoderms in which acid and alkaline phosphatase, carboxylic acid esterase, and phosphorylase were localized. The numbers of control blastoderms are enclosed in parentheses: those that are starred (\*) were heated, the others were incubated in media lacking substrates.

Enzyme	Prestreak	Definitive Streak	Head Fold	7-Somite
Acid phosphatase	12(7-5*)	16(8-6*)	19(9-8*)	15(8-7*)
Alk. phosphatase	10(6-7*)	12(5-8*)	19(8-7*)	24(9-6*)
Esterase	9(5-5*)	14(9-8*)	15(6-7*)	29(12-9*)
Phosphorylase	11(10)	20(18)	32(23)	30(24)



Enzyme	Fixative	Optimum Period
Acid phosphatase	Acetone	45 min.
	10% Formalin	60 min.
Alkaline phosphatase	Acetone	60 min.
	10% Formalin	30 min.
Esterase	Acetone	45 min.
	10% Formalin	30 min.
Acid phosphatase Alkaline phosphatase Esterase	Acetone 10% Formalin Acetone 10% Formalin Acetone 10% Formalin	<pre>45 min. 60 min. 60 min. 30 min. 45 min. 30 min.</pre>

TABLE 11.--The optimum fixation period for acid and alkaline phosphatase and carboxylic acid esterase.

APPENDIX A



Histochemical Incubating Media

Dehydrogenases and Diaphorases (Slightly modified from Thomas and Pearse, 1961)

Standard solution:

Substrate 0.1 ml NAD or NADP, 0.1 M 0.1 ml Sodium azide or sodium cyanide, 0.1 M 0.1 ml Magnesium chloride, 0.05 M 0.1 ml Tris buffer, 0.2 M, pH 7.0 Nitro-BT or MTT, 1 mg/ml 0.25 ml 0.25 ml 1.0 ml Distilled water to make Polyvinylpyrrolidone 75 mg

Substrates:

1.0 M sodium DL lactate a. 1.0 M ethanol b. 1.0 M DL isocitric acid с. d. 0.6 M sodium succinate e. 1.0 M sodium L glutamate 1.0 M sodium DL B-hydroxybutyric acid f. 1.0 M disodium glucopyranose-6-phosphate g. 0.1 M NADH, h. 0.1 M NADPH, 1.

Notes:

- a. NAD was used as a coenzyme with substrates a,
  b, c, e, and f; NADP was used with substrate g;
  and no coenzyme was used with substrates d, h,
  and i.
- b. Sodium azide was used as an inhibitor with MTT and sodium cyanide with Nitro-BT.
- c. In localizing the diaphorases with MTT, the pH of the Tris buffer was increased to 8.0.
- d. In addition to the substances listed above, 0.05 ml of 0.5 M cobaltous chloride was included in the media containing MTT.



Acid Phosphatase (Burstone, 1961)		
Naphthol AS-BI phosphate N, N-dimethylformamide	10 0.25	mg ml
Dissolve and add:		
Acetate buffer, 0.2 M, pH 5.2 Distilled water Manganese chloride, 10%	25 25 2	ml ml drops
Mix and add:		
Fast red violet salt LB	30	mg
Shake and filter		
Alkaline Phosphatase (Burstone, 1961)		
Naphthol AS-MX phosphate N, N-dimethylformamide	10 0.25	mg ml
Dissolve and add:		
Tris buffer, 0.2 M, pH 8.3 Distilled water Manganese chloride, 10%	25 25 2	ml ml drops
Mix and add:		
Fast red violet salt LB	30	mg
Shake and filter		
Carboxylic Acid Esterase (Holt and Withers	<b>,</b> 1952	2)
5-bromoindoxyl acetate Ethanol	1.3 0.1	mg ml
Dissolve and add the following mixture	::	
Tris buffer, 0.1 M, pH 6.12 Potassium ferricyanide, 0.05 M Potassium ferrocyanide, 0.05 M Calcium chloride, 0.1 M Sodium chloride, 0.1 M	2.0 0.1 0.1 1.0 6.7	ml ml ml ml

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Phosphorylase (Slightly modified from Eranko and Palkama, 1961).		
Glucose-l-phosphate, dipotassium salt Adenosine-5-phosphate Glycogen Insulin, 40 i.u./ml Mercuric chloride, 10 <sup>-4</sup> Acetate buffer, 0.1 M, pH 5.9	50 5 1 1 5	mg mg drop ml ml


## APPENDIX B



## The Optimum Fixation Periods for Acid and Alkaline Phosphatase and Carboxylic Acid Esterase

The fixatives for acid and alkaline phosphatase and carboxylic acid esterase were selected as follows. Each of these enzymes was localized in head fold blastoderms that had been fixed for 10, 15, 20, 30, 45, 60, and 90 minutes in acetone at  $4^{\circ}$ C. If the fixation period was too short, some of the enzyme diffused into the incubating medium, and the medium turned greenish-blue (esterase) or red (phosphatase). The shortest fixation period that prevented diffusion was taken as the optimum fixation period. These tests were then repeated using 10% formalin as the fixative. The results are shown in Table 11.

Next, each enzyme was localized in two groups of head fold blastoderms. Each group had been fixed for an optimum period at 4°C., one in acetone and the other in 10% formalin. The intensities of the histochemical reactions in the two groups were compared, and the fixative responsible for the more intense reaction was chosen as the better fixative. Thus, acetone was selected as the fixative for acid phosphatase, and 10% formalin for alkaline phosphatase and esterase.

The intensity of the histochemical reaction for alkaline phosphatase was increased by adding sucrose to the formalin fixative (0.88 M sucrose in 10% formalin).

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Likewise, the addition of calcium chloride to the formalin fixative (1% calcium chloride in 10% formalin) increased the intensity of the reaction for carboxylic acid esterase (Hannibal and Nachlas, 1959). The activity of acid phosphatase was appreciably increased by shortening the fixation period from 45 minutes to 30 minutes. With the shorter period, however, there was diffusion of the enzyme into the incubating medium, but it was very slight and the patterns of dye granule distribution were readily reproducible.





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