ON THE ISOZYMES OF ALKALINE PHOSPHATASE OF THE SEA URCHIN (*STRONGYLOCENTROTUS PURPURATUS*)

by

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INTRODUCTION

Alkaline phosphatase is a ubiquitous enzyme found in many tissues of a wide range of organisms. It exists in multiple molecular forms or isozymes which in many cases exhibit tissue specificity. In man, for example, tissue specific isozymes of alkaline phosphatase have been reported to occur in neutrophilic leukocytes (Robinson, Pierce & Goldstein, 1965) and placentae (Robson & Harris, 1965). Best understood of the alkaline phosphatases with regard to subunit structure are those isozymes of bacterial (Schlesinger & Barrett, 1965) and human placental (Sussman, in press) origin.

Reactions of Alkaline Phosphatase

Hydrolysis

Alkaline phosphatase (orthophosphoric monester phosphohydrolase, E.C. 3.1.3.1.) is classified on the basis of its ability to catalyze the hydrolysis of organic phosphate esters. As is obvious from its common name, the velocity of the reaction is optimal at alkaline pH. The enzyme exhibits low substrate specificity as exemplified by the substrates commonly used in activity assays. Beta-glyceryl-phosphate is employed as the substrate in the Gomori method for the histochemical localization of alkaline phosphatase. A common method for quantitative assay

employs nitro-phenyl-phosphate as substrate. Naphthyl-phosphates, both simple and highly substituted, are commonly used to demonstrate the presence of alkaline phosphatase either histochemically or fluorometrically.

Phosphorylation and Transfer

Alkaline phosphatase has been reported to possess catalytic properties in addition to that of hydrolysis. In contrast to the non-specific hydrolysis reaction(s), Morton (1958) has shown that it can catalyze the phosphorylation of glucose. The phosphorylation reaction was found somewhat more specific than the hydrolysis reaction in that when glucose was phosphorylated, only glucose-6-phosphate was formed. Sussman (in press) confirmed this finding when he simultaneously assayed for the appearance of glucose-1- and glucose-6-phosphate. Morton (1958) also found that alkaline phosphatase phosphorylated other sugars, but to a lesser extent. In other words, glucose was found to be a better phosphate acceptor than, for instance, fructose. Alkaline phosphatase has been shown to phosphorylate glycerol (Sussman, in press), amino acids (Morton, 1958) and some commonly used buffers (Morton, 1958, and Dayan & Wilson, 1964). That this enzyme can catalyze phosphate transfer without the intervention of water was shown by Meyerhof and Green (1950).

Reaction Specificity

As previously noted, alkaline phosphatase will accept a wide variety of substrates. However, when glucose is supplied to the enzyme in the presence of inorganic phosphate, the formation of product is somewhat more specific than simple hydrolysis. Furthermore, Sussman (in press) has shown that, on an equimolar basis, glycerol is as good a phosphate acceptor as water. It is possible that the transfer and/or phosphorylation reaction(s) rather than hydrolysis, are the reaction(s) of biological importance. In any event, the low substrate specificity might offer a metabolic flexibility and environmental adaptability to its possessor (i.e., evolutionary advantage).

Isozymes

Numerous enzymes have been shown to be present in multiple molecular forms. This heterogeneity (microheterogeneity) has received a great deal of attention since it became known that it is a general phenomenon.

"Largely as a result of the pioneering work in the laboratories of Markert and Kaplan, techniques for analyzing the structure of isozymes have become widely disseminated." (Vesell, 1968). This analysis is no longer a mere cataloguing of the various enzymes which

exhibit molecular diversity but rather a probing by investigators of the functional significance (Fritz, 1965 and Wolin, 1964) and the underlying causes (Appella & Markert, 1961, among others) of isozymic variation.

Origin

It seems obvious that genetic duplication would confer a selective advantage on its possessor in that mutation would be less likely to inactivate, for instance, two equivalent loci as opposed to a single locus. If mutation were to result in allelic variation, a second type of selective advantage could be conferred, that of widening the adaptive range of the organism. It has been speculated that isozymes may have arisen in such a fashion (Markert, 1968 and Vessel, 1968).

Function

From a more immediate point of view, it seems probable that isozymes afford a metabolic advantage in some cases. The differences in tissue localization probably reflect different metabolic needs. The same is no doubt true for the existence of isozymes within single cells (Allen, 1961; Brody & Engel, 1964; and Henderson, 1965). If these ideas of selective advantage are not valid, it becomes difficult to envision how the occurrence of cell and tissue-specific isozymes has become such a wide spread phenomenon.

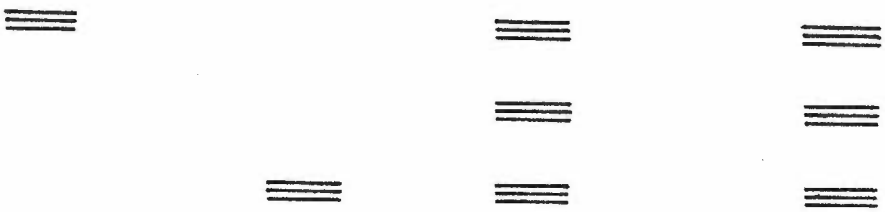
Subunit Composition of Alkaline Phosphatase

Lactic dehydrogenase was the first enzyme studied for which polypeptide subunits were shown to be responsible for the isozyme patterns observed (Markert & Møller, 1959). In this instance the five-band pattern was shown to result from the tetrameric association of two different polypeptide subunits.

It has been known since 1962, that bacterial alkaline phosphatase is a dimer of two apparently identical polypeptide subunits (Levinthal, Signer, & Fetherolf, 1962). In extracts of Escherichia coli or Serratia marcescens, three isozymes of alkaline phosphatase are observed by starch gel electrophoresis. The three 'isozymes' are known to be the product of a single non-allelic structural gene (Levinthal, et al., 1962). They have also presented evidence suggesting that the isozyme pattern is not a result of differences in peptide sequence (see also Rothman & Byrne, 1963) but may be due to alterations in either tertiary or quaternary structure. The problem of configurational change is not well understood, although Epstein and Schechter (1968) have discussed it at length.

Both E. coli and S. marcescens exhibit three isozymes of alkaline phosphatase on electrophoresis. The

isozymes of S. marcescens migrate at a considerably slower rate than those of E. coli, and are therefore readily distinguishable. If the two species are genetically crossed, three sets of three isozymes are seen on electrophoresis (Garen & Garen, 1963). Since the fastest group of isozymes has the same electrophoretic mobility as native E. coli alkaline phosphatase, and the slowest group is identical to that of S. marcescens, the intermediate group is regarded as an in vivo hybrid. The same electrophoretic pattern has been duplicated in vitro (Garen & Garen, 1963). For example:

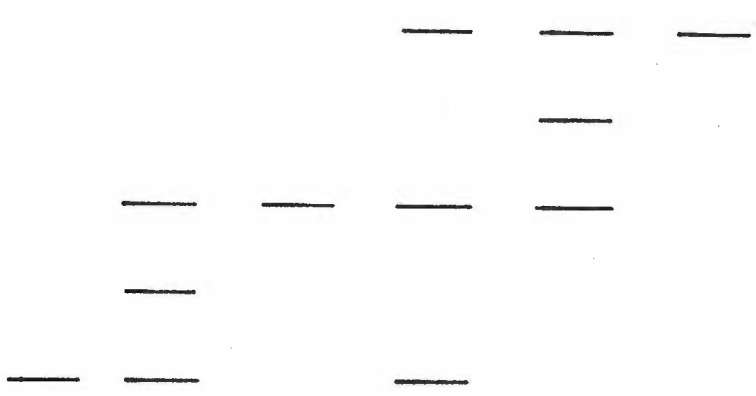


E. coli S. marcescens E. coli x S. marcescens In vitro complimentation

The in vitro complimentation technique (i.e., the ability of dissociated subunits of alkaline phosphatase, LDH, etc., to recombine with the recovery of biologic or enzymatic activity) provides an interesting framework for the

discussion of placental alkaline phosphatase isozymes.

In a study of over 1500 human placentae, Robson and Harris (1967) reported six common phenotypes with respect to isozyme patterns of alkaline phosphatase. They have shown by genetic analysis, that the six phenotypes are the product of three allelic genes. This is represented diagrammatically as follows:



SS	SI	II	SF	FI	FF
					Origin

The intermediate isozyme of the triple isozyme patterns is interpreted as "a 'hybrid' enzyme containing polypeptides characteristic of the two other components present" (Robson & Harris, 1965). In their 1967 study, several 'rare' phenotypes were reported. In each instance, the rare phenotypes exhibited triplet electrophoretic patterns and were regarded as heterozygotes for a rare allele and one of the common alleles.

Sussman (in press) has since confirmed the hypothesis that placental alkaline phosphatase is a dimer of two polypeptide subunits.

Sea Urchin Development

Considerable information dealing with the role of nucleic acids and protein synthesis in early development is available and will be considered briefly. Since it has been assumed that the increase in alkaline phosphatase activity at gastrulation is the immediate result of protein synthesis, this literature is of considerable importance.

Nucleic Acid Metabolism

A general picture of the role of DNA in early development has been elucidated by studies on the effects of enucleation, lethal hybridization, parthenogenic activation and, recently, chemical enucleation with actinomycin D. All of these experimental manipulations (i.e., partial or total enucleation) focus on the developmental role of DNA. Enucleation exerts no appreciable effect on development until gastrulation or slightly before. At the time when gastrulation would be expected a common effect of enucleation becomes apparent: gastrulation does not take place.

Traditionally this has been interpreted to indicate that the cytoplasm controls early development while the nucleus exerts its influence at the onset of tissue differentiation (i.e., at gastrulation). The apparent cytoplasmic differentiation (appearance of specific proteins) in the absence of apparent nuclear control (DNA-dependent RNA synthesis) was the basis of an academic conflict which was at its height in the early 1900's. The conflict was later resolved by the postulate of 'masked' messenger RNA later termed informosomes by Spirin (1966).

Protein Synthesis

The general pattern of protein synthesis in the sea urchin embryo corresponds to two of the morphologically observable events (i.e., fertilization and gastrulation). Two bursts of protein synthesis have been reported to occur. The first follows fertilization or 'activation' of the egg, and is not inhibited by enucleation. The second burst begins with the onset of gastrulation or slightly before and is not seen in embryos treated with actinomycin D (Gross & Cousineau, 1964).

In his review of the subject Gross (1967) states that there are two obvious ways to study the relation of protein synthesis to differentiation.

> "...by (i) following the amounts of one or more proteins, and the control of those amounts, in tissue

> cells of late embryonic stages or in differentiating cell populations of the adult organism and (ii) following the course of protein synthesis after fertilization of the egg."

He further states that the second course is advantageous only if the egg is truly at 'ground zero'* and continues to argue that it is. Moog (1965), in her review of enzymes in development, argues the opposite point of view effectively. She regards the egg as the highly differentiated end product of oogenesis.

Specific Proteins

Of the two protein species (MDH and a spindle associated protein) which have been studied in relation to protein synthesis, only one, a spindle associated protein, is the result of de novo synthesis (Stafford & Iverson, 1964). Mangan, Miki-Noumura, and Gross (1965) have shown that some newly synthesized proteins associate specifically with the microtubules of the mitotic apparatus. Further investigation would be necessary to show that the spindle associated proteins of Mangan, et al., (1965) are, in fact, a differentiated cell-product and not merely a nuclear protein found in any dividing cell.

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*I interpret his use of the term 'ground zero' to mean that he regards the egg as totally unspecified and that differentiation of the embryo is the result of immediate synthesis of cell- or tissue-specific proteins.

On the other hand, Moore and Villee (1962) have shown that malate dehydrogenase is present in the unfertilized egg of the sea urchin. By the sixteen cell stage, the macromeres retain only two of the original five isozymes, while the micromeres retain three isozymes (Moore & Villee, 1963). Subsequently there is an increase in the number of isozymes. Villee showed (1968) that neither actinomycin D nor puromycin inhibited the establishment of the appropriate 'tissue' specific patterns of malate dehydrogenase. It was found that puromycin at a concentration of 40 µg/ml depressed protein synthesis, as measured by the incorporation of C^{14}-alanine, by approximately 85% without affecting either the isozyme pattern or the specific activity of malate dehydrogenase (Villee, 1968). Actinomycin D at concentrations as high as 20 µg/ml depressed protein synthesis by approximately 10% while having no effect on the isozyme patterns and no effect on the specific activity of malate dehydrogenase (Villee, 1968). The effect of actinomycin D was not measured in terms of incorporation of labeled uridine.

Alkaline Phosphatase in Sea Urchin Development

Localization

There appears to be general agreement in the literature as to the localization of alkaline phosphatase

in the sea urchin embryo. The longstanding controversy as to whether or not alkaline phosphatase is associated with nuclei remains unresolved. The enzyme has been studied histochemically from the oocyte to late prism stages of development. Wicklund (1948) and Kruglis (1947) have both reported a distribution of activity throughout the cytoplasm of the unfertilized egg. The histochemical activity was reported to be greater in the vegetal region of the developing embryo (blastula) by Gustafson (1965).

On the other hand, some investigators have been unable to demonstrate the presence of alkaline phosphatase prior to gastrulation. It is probable that differences in the sensitivity of the histochemical techniques are responsible.

The blastula is a hollow ball of cells. At the time of gastrulation one wall of the blastula is seen to invaginate into the central cavity or blastocoele. The remnant of the blastocoele is then termed gastrocoele. The lining of the blind tube, or archenteron, thus formed is reported to be the site of the increase in alkaline phosphatase activity (Gustafson & Hasselberg, 1950, among others). Simultaneously, cells bud off of the gastrocoelic surface of the archenteron to form the primary mesenchyme cells. A somewhat lower alkaline phosphatase activity is associated with the primary mesenchyme cells than is associated with the lining of the archenteron

(Hsiao & Fujii, 1963). The primary mesenchyme cells form the skeletal elements of the pluteus (Hsiao & Fujii, 1963) and are probably the forerunners of the adult coelomocyte. By histochemical criteria, the greatest concentration of alkaline phosphatase is associated with the epithelium of the developing gut through the pluteus stage (Hsaio & Fujii, 1963).

Quantitation of Alkaline Phosphatase

There is general agreement among the various papers dealing with the quantitative appearance of alkaline phosphatase during development. Mazia, Blumenthal, and Benson (1950) reported a low level of alkaline phosphatase until just before gastrulation. From gastrulation to pluteus (ten to forty hours after fertilization respectively) they reported that alkaline phosphatase activity rose steadily to a value ten or more times that of the unfertilized egg. When the results of Pfohl (1965) are considered, the ten-fold increase reported by Mazia, et al., is probably an overestimate. Hsiao and Fujii (1963) and Pfohl (1965) report that the increase is approximately six-fold from unfertilized egg to pluteus. Pfohl found that treatment of egg homogenates with n-butanol resulted in a doubling of alkaline phosphatase activity. Mazia, et al., made no mention of using n-butanol. Therefore, if the use of n-butanol doubles the amount of alkaline

phosphatase extractable from the unfertilized egg and not later developmental stages, the ten-fold value reported by Mazia, et al., (1950) must be reduced by one-half.

Aside from this apparent discrepancy, there is general agreement that alkaline phosphatase activity increases about six-fold from fertilization to late pluteus. The six-fold figure or estimate appears to be characteristic of the five species of sea urchins examined. In every instance the activity of alkaline phosphatase (and presumably its concentration) was described as rising gradually until gastrulation and rapidly thereafter. Pfohl (1965) observed that the rapid increase in alkaline phosphatase activity and the formation of the gut primordium corresponded to the appearance of a new isozyme of lower electrophoretic mobility.

Statement of the Problem

Alkaline phosphatase has been shown to exist in multiple molecular forms or isozymes which, in some instances, exhibit tissue specificity. Some cells and tissues, for example human neutrophilic leukocytes (Weaver, 1966) and placentae (Robson & Harris, 1965; 1967), have been shown to possess more than a single isozyme. The present study was undertaken to determine if the adult sea urchin a) exhibited isozymic variation with respect to alkaline phosphatase; and if so

b) whether or not any of these isozymes exhibited tissue specificity.

It has been demonstrated that alkaline phosphatase from E. coli and S. marcescens is a dimer of two identical polypeptide subunits (Garen & Garen, 1963). It has also been shown that human placental alkaline phosphatase is a dimer of two polypeptide subunits of identical molecular weight (Sussman, in press). In this study the isozyme patterns of sea urchin alkaline phosphatase were examined to determine if the isozyme patterns were the result of three non-allelic genes (i.e., no in vivo hybridization of polypeptide subunits), two non-allelic genes with in vivo hybridization of subunits or some combination of allelic genes.

During the course of embryonic development of the sea urchin, an exponential increase in alkaline phosphatase activity was reported to begin with the onset of gastrulation. Further, this increase was shown to coincide with the appearance of a new isozyme (Pfohl, 1965). In this study the isozyme patterns of the adult organism were examined to determine if the isozymes of the adult corresponded to those of the developing embryo, and if the appearance of the new isozyme at gastrulation could be accounted for on the basis of de novo synthesis or by a change in pre-existing enzyme or its subunits.

MATERIALS AND METHODS

Collection of Animals

Sea urchins of the species Strongylocentrotus purpuratus were collected at irregular intervals from that area of the Oregon coast between Yaquinna Head and Fogarty Creek. The time of these collections extended from the spring of 1964 to the summer of 1969.

Handling of Tissues

For the most part, emphasis was placed on sampling small gut tissue although coelomocytes, large gut, esophagus, eggs and ovaries were also examined. With the exception of the coelomocytes, the tissues were obtained by chipping away a hole in the test (shell) over a gonad and freeing the tissues from their mesenteric attachments.

The regions of gut were designated as follows: 1) esophagus--that part of the digestive tube attached to the tooth apparatus and extending for about three cm to its junction with the small gut, 2) small gut--extending to a transparent junction with the large gut, and 3) large gut--extending from this junction to the anal opening. The esophagus was always easily recognizable due to its narrow diameter and well developed muscle coat. Since the transparent junction between

small and large gut was not always obvious, an additional color criterion was employed. The epithelial surface of small gut is characteristically brown or brown-green while that of the large gut is uniformly purple.

The isolated segments of gut were divided longitudinally and the contents removed by washing in at least two changes of clean sea water. Ovarian tissue was handled in a similar fashion. All tissues except eggs were blotted on absorbant paper prior to weighing. Coelomocytes were collected by withdrawing coelomic fluid into a syringe through a 17 gauge needle. The fluid was then transferred to centrifuge tubes and the cells collected in a pellet by centrifugation at 5°C. After decantation of the fluid, the cells were weighed. Eggs were washed in filtered sea water and concentrated either by gentle centrifugation or by allowing them to settle by gravity. Since it was impractical to blot the eggs, n-butanol and buffer were added in a ratio of 1 volume of n-butanol and 3 volumes of buffer to each volume of eggs.

Extraction of Alkaline Phosphatase

Extraction of enzyme was accomplished by homogenization of material in glass flasks with a Virtis homogenizer connected to a variable voltage power supply. With the Virtis set at maximum voltage, the rheostat was set at

30 volts for 60 seconds, 20 volts for 180 seconds and 10 volts for 300 seconds in that order. Departures from this schedule are noted in appendix table I. The tissue was extracted at concentrations of 20% wet weight (weight to volume) in a mixture of ice chilled 0.05 M tris-HCl (pH 7.4 or 8.3) to which n-butanol was added to a concentration of 20% (volume to volume) with respect to the tris-HCl. Following homogenization, the resultant brei was further treated by either vacuum filtration through diatomaceus earth or by centrifugation at 37,000 x g for one hour at 5°C (Sorvall refrigerated centrifuge). The organic phase was discarded and the aqueous filtrate or supernatant which contained the alkaline phosphatase would usually retain its enzymatic activity and isozyme pattern for about two months. Initially the extracts were dialyzed to remove the dissolved n-butanol but this was found to enhance growth of microorganisms and was therefore discontinued. The extracts were always stored in the refrigerator at 15°C.

Starch Gel Electrophoresis

Buffers

Analysis of the isozyme content of samples of the extracts was performed by starch gel electrophoresis. Electrode buffer was made up in stock solution at either five or ten times the concentration used for

electrophoresis. The electrode buffer was composed of 0.03 M boric acid titrated to a pH of 8.6 with NaOH (approximately 2 gm of NaOH was required). The gel buffer was made up at the concentration used for electrophoresis. It was composed of 0.076 M tris base titrated to a pH of 8.6 with citric acid. No bacterial growth was observed in the gel buffer; however, microorganisms were observed to grow in the electrode buffer at the concentrations used during electrophoresis. It was also observed that resolution was lost if the electrode buffer was not replaced regularly. By replacing the electrode buffer at weekly intervals, both the loss of resolution and the growth of microorganisms were prevented.

Preparation of Gels

Normally, starch gels are heated by swirling the starch and buffer mixture over a Bunsen burner. In an attempt to standardize the consistency of the gels, a more uniform method of heating was devised. Thirty-two gm of hydrolyzed potato starch (Conaught) was added to 300 ml of tris-citrate buffer (pH 8.6) in a one liter side-arm flask. A pan of water was heated to boiling on a hot-plate which contained a magnetic stirring device. The filter flask containing starch, buffer, and a teflon coated magnetic stirring bar was immersed in the boiling water bath. When the temperature of the starch slurry

reached 86-87°C, the air was rapidly aspirated by vacuum and the hot gel was poured. Gels were poured into molds 12 cm by approximately 28 cm and by 1.0 cm on a 0.4 cm thick glass plate. The sides of the mold were formed with square bar stock aluminum cut to the appropriate lengths. The bar stock was affixed to the glass and to each other with Dow-Corning silicone stopcock grease. The edges which were to be in contact with the gel were also coated to prevent sticking. Gels thus formed were allowed to cool to room temperature and either covered with Saran Wrap or coated with silicone spray and stored in the refrigerator for twelve hours prior to use.

Application of Sample

The 12 by 28 cm gelatin slab was cut vertically through its 1 cm thickness at a distance 5 cm from and parallel to one end. One cm wide paper strips were cut from Whatmans No. 1 filter paper. When six extracts containing alkaline phosphatase were to be analyzed on the same gel, the 1 cm wide paper strip was cut into six segments which were 1.5 cm long. From the 1 cm wide strip of paper, seven segments 0.5 cm in length were also cut. Each of the 1 by 1.5 cm segments was dipped in enzyme extract and placed on edge between the cut surfaces of the gel slab. Between each of the paper segments containing enzyme extract was placed one of the

smaller segments which had been dipped in gel buffer. The final result of the above procedure was a single thickness of paper between the cut surfaces of the gel on which was absorbed different extracts containing alkaline phosphatase, each separated from the other by a 0.5 cm wide segment containing gel buffer. If it was advantageous to increase the amount of the extract loaded (e.g., extracts with low alkaline phosphatase content) on a particular electrophoretic run, more than a single thickness of paper strip was used. For preparative purposes, (i.e., partial purification of the enzyme) up to eight thicknesses of 1 by 12 cm paper strips were used. When the paper strip was not divided into smaller segments with spacers between, only a single extract could be separated into isozymic components.

Separation of Isozymes

The cut edges of the gel, separated by the filter paper, were reopposed as tightly as possible and the separation of isozymic components was effected by the application of 10 volts per cm potential measured within the gel by a servo power supply designed and built by Research Instruments Service. The starch gel apparatus was designed by R. B. Lyons and built by E. J. Hage. The apparatus was supplied with a baffled aluminum cooling plate through which a water and ethylene glycol mixture was circulated. The mixture was cooled to 5°C

with a constant temperature bath. Only four to five hours were required for separation of extracts into isozymic components.

Visualization of Activity

The localization of the isozymes was visualized by the simultaneous coupling reaction of beta-naphthol and Fast Blue RR. The reaction mixture was composed of 40 mg of beta-naphthyl-phosphate, 50 mg Fast Blue RR with 100 mg of $MgSO_4.7H_2O$ added as an activator in 100 ml of gel buffer (tris-citrate, pH 8.6). Incubation was allowed to occur for 2-4 hours at $15^{o}C$.

Elution of Enzyme

Enzyme referred to as partially purified was obtained by loading the gel with a single extract. The extract was applied to the gel with 6-8 thicknesses of paper strips. In this way several times the normal load of enzyme could be separated. Following electrophoresis, narrow strips were cut from the origin to the buffer line of the gel. Two edge and one central strips were stained in the reaction mixture to determine the location of the isozyme(s). The stained strips were replaced in their original position and horizontal strips of the gel were cut at the site of the migration of the alkaline phosphatase isozymes. The strips were minced in ice chilled

0.05 M tris-HCl (pH 7.4 or 8.3) and stirred in an ice bath until a homogenous slurry was formed. The enzyme containing supernatant was collected by centrifugation for 30 minutes at 12,000 x g at 5°C.

Acrylamide Gel Electrophoresis

Polyacrylamide disc electrophoresis (Davis, 1964) was occasionally employed in the study. Although it was a superior method for determination of the protein patterns, resolution of the isozymic components of alkaline phosphatase was not as good as that obtainable by starch gel techniques. Several modifications of the buffer system resulted in no improvement in resolution. Buffers tested were 1) the same as described for starch gel, 2) tris-glycine at pH 8.8-9.0, 3) tris-phosphate pH 8.8-9.0 and 4) tris-HCl. Different buffers were tested for inclusion in the polymerization mixture with a similar lack of success. Concentrations of 7% acrylamide were used throughout. The sample and spacer gels were discontinued when they were found to provide no advantage.

In both starch and acrylamide gel electrophoresis, the migration of echinochrome pigment was used to determine the buffer front during electrophoresis. In the case of the starch gels, this corresponded to the buffer line.

Filter Fluorometry

Excitation and Emission Data

Enzymatic activity was determined by the reaction of alkaline phosphatase with alpha-naphthyl-phosphate which produces the fluorescent species, alpha-naphthol. The alpha-naphthol has excitation maxima of 250 and 335 mu and an emission peak of 455 mu. The following filters were employed: a Corning 7-60 narrow pass filter for excitation (360 mu), a Wratten 2A sharp cut filter for emission (415 mu) and a 0.60 neutral density filter on the emission side of the fluorometer. With the exception of the neutral density filter obtained from Kodak, the filters were supplied with the Turner model 111 fluorometer. The temperature regulated door was used throughout the study (10°C). The output of the fluorometer was connected to a Brush recorder model Mark 10. The initial linear portion of the tracings thus obtained was used for determination of the initial velocities of the reactions.

Standards

Quinine sulfate in 0.1 N sulfuric acid was used as a primary standard since its excitation and emission spectra resemble those of alpha-naphthol. Volumetric dilutions of quinine were made up and a standard curve constructed (graphs 1 & 2). These standards were quite

Graph 1

The concentration of quinine sulfate (——) and naphthol (----) is x 10^{-2} µg/ml. The percent fluorescence in the first graph was measured with the 1X slit.

Graph 2

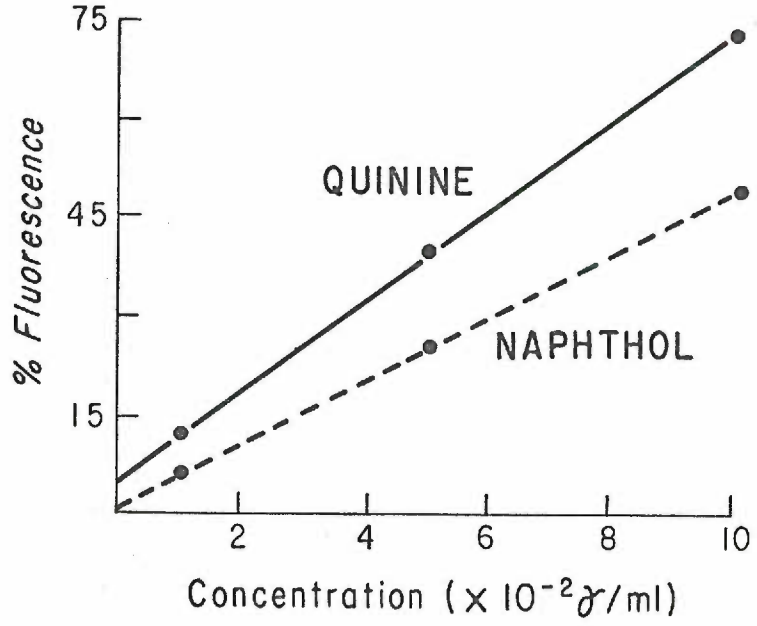
The concentration of quinine sulfate (——) and naphthol (----) is x 10^{-3} µg/ml. The percent fluorescence in the second graph was measured with the 3X slit.

Experimental Details

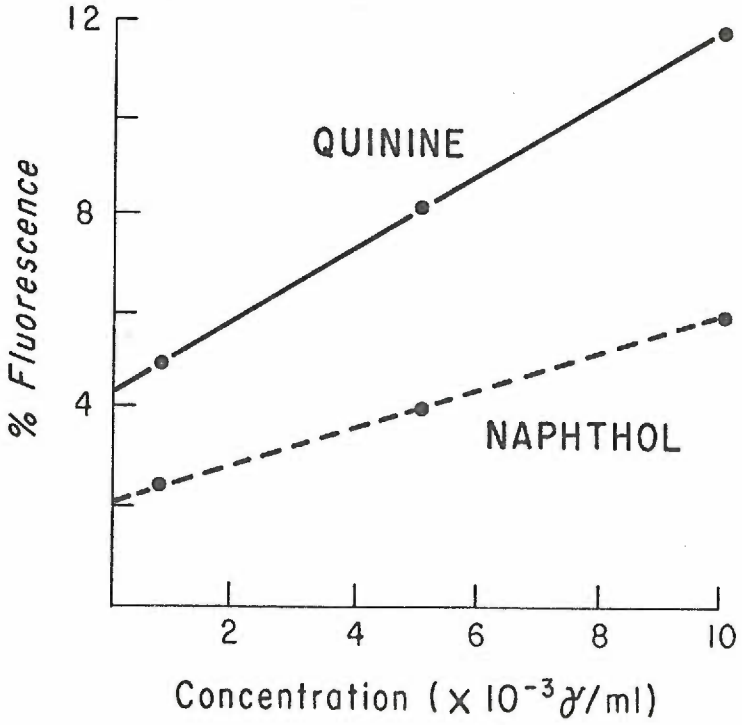
The standard curve plots the percent fluorescence against the concentration of quinine sulfate in 0.1 N H_2SO_4, alpha- and beta-naphthol. The percent fluorescence was read directly from the dial of the Turner Model 111 fluorometer. The fluorescence of the reagents used in the assays prevented the use of 10X (except in a few experiments not presented here) and 30X slit widths. The values graphed are representative of values obtained during the course of the experiments. Since most of the experiments (some of the kinetic experiments excepted) were quantitated with the 3X slit, the standard curves were prepared from data obtained with the 3X slit.

The standard curves for both alpha- and beta-naphthol were identical. This is somewhat surprising since they have dissimilar excitation and fluorescence spectra (Udenfriend, 1962). However, the excitation and fluorescence spectra of these two species overlap in the areas affected by the filters used in these experiments. In any event, quantitative dilution of alpha- and beta-naphthol exhibited no significant variation in results. The identity of these two compounds, with respect to concentration-producing fluorescence, was checked twice. From that time onward, only alpha-naphthol standards were prepared.

Graph 1



Graph 2



stable when stored in the dark and they were used prior and subsequent to each run. No appreciable variation was noticed during the usual 6-8 hour's time that the fluorometer was in use. Alpha-naphthol standards are not stable enough to be used as a primary standard. The accompanying standard curve for reaction product was constructed and correlated to the quinine curve so that the reaction could be quantitated. New naphthol and quinine curves were constructed whenever a departure from linearity (in the quinine standards) became apparent. The quinine standards gave uniform results for periods over six months. Usually a single tube would be in question, but since each curve was composed of three points, new standards were prepared whenever this occurred.

Assay Conditions

Assays were always carried out in triplicate. The reaction was run in 5 ml pyrex test tubes and contained from 0.1 to 0.3 ml of enzyme solution (depending on activity), 0.1 ml of 4.5×10^{-3} M sodium alpha-naphthyl-phosphate (Sigma) and 3.0 ml of 0.05 M tris at pH 10.1. The final concentration of substrate was 10^{-4} M. In the kinetic assays, the substrate concentration was varied below inhibiting concentrations. The reactions were observed to be linear during the time of measurement (up to 10 minutes in some cases).

Kinetic Analysis of Isozymes

Kinetic analysis was carried out on samples of partially purified enzyme obtained by elution of the isozymes from starch gels. Protein concentration was estimated spectrophotometrically. Magnesium was added to the assay buffer such that the final magnesium concentration was 10^{-3} M. In all cases the concentration range of the substrate (alpha- or beta-naphthyl-phosphate) was tested prior to analysis to be certain that it was below inhibiting concentrations.

The tracings from the recorder were measured in percent per minute, converted to moles per minute by reference to graphs 1 or 2, and the specific activity of the samples calculated from the A_{280} measured on an aliquot of partially purified enzyme. The specific activity at the corresponding substrate concentration was punched on data cards and the kinetic parameters calculated on an I.B.M. 1130 computer. The program was written by Dr. V. Weimar to whom grateful thanks are extended for its use. Statistical analysis was a part of the program (appendix table II).

Total Protein Estimations

The protein concentrations of the partially purified and crude extracts were estimated by measurement of

absorbance at 280 mu (Beckman DU spectrophotometer). The concentration of protein was estimated assuming that a value of 1.0 equaled 1 mg/ml (1.0 x O.D. 280 mu = mg protein/ml). The O.D. 280 mu method was employed for a variety of reasons. It is a simple technique requiring few manipulations and for this reason several samples may be estimated in a short time. Also, the absolute protein concentrations were not important in this study but rather the relative concentration of alkaline phosphatase. Apparently very little of the material absorbing at 280 mu could be dialized out, since the change in volume resulting from dialysis was proportional to the change in absorbance whenever checked. In any event, the samples measured were diluted such that the actual O.D. value measured was between 0.02 and 1.0.

Subunit Complimentation

Dissociation of Subunits

Dithiothreitol concentrations from 10^{-1} to 10^{-4} M were diluted from 1 M stock solution (made up fresh for each experiment) such that 1 volume of dithiothreitol was added to 9 volumes of enzyme extract or starch gel eluent. Normally, 0.1 ml of the reagent at the appropriate dilution was added to 0.9 ml of enzyme. To this mixture, 0.2 ml of 5 M NaCl was added so that the final concentration of NaCl was 1 M.

Reassociation of Subunits

Since inactivation was essentially instantaneous, the above described mixture of enzyme extract, dithiothreitol and NaCl was transferred to washed dialysis sacs. The sacs were dialyzed against three changes of 0.05 M tris-HCl (pH 8.3) in the refrigerator (15°). The three changes of buffer were made within a 24-hour period. Dialysis sacs containing different concentrations of dithiothreitol were kept separate during dialysis.

Histochemistry

Samples of tissue for histochemical localization of alkaline phosphatase were removed from the animal, washed twice in cold filtered sea water, and fixed for 30 minutes in cold 10% v/v formalin-sea water. Although other techniques were employed, (e.g., frozen sections and paraffin sections) the best results were obtained with nitrocellulose embedded tissue. The only departure from the standard technique for nitrocellulose embedding was that infiltration and embedding were carried out at room temperature. Sectioning was done on a sliding microtome and the sections were mounted on albuminized slides and stored in 70% ethanol. Alkaline phosphatase activity was detected by the calcium-cobalt method of Gomori as described by Pearse (1960). The sites of enzymatic activity became apparent after 1-2 hours incubation. Control sections

were made simultaneously in incubation medium lacking substrate. Following reaction, the sections were counterstained with nuclear fast red.

RESULTS

Isozymes of Alkaline Phosphatase

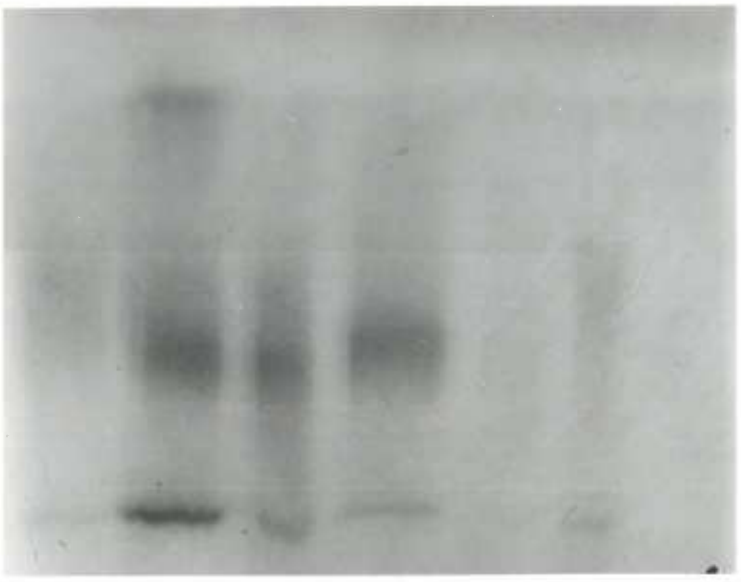
Observation of sea urchin tissue extracts for the presence of alkaline phosphatase activity over a period of months resulted in the identification of three isozymes with consistently distinct electrophoretic mobilities (plate 1). For the sake of convenience these isozymes are designated as slow, intermediate and fast in relation to anodal mobility. Plate 1 shows that the intermediate isozyme is located slightly closer to the slow isozyme than it is to the fast isozyme.

Isozymes of Small Gut

Graph 3 shows that small gut extracts were always observed to contain at least two forms of the enzyme. During the period from February through June, all three isozymes were present in extracts of small gut. The intermediate isozyme disappeared from gut extracts in June. From that time until September only the fast and slow isozymes were observed in the small gut extracts. In October the intermediate isozyme reappeared and the fast isozyme apparently disappeared. Conflicting data were obtained with regard to the fast isozyme at this time. No animals were extracted during late October through December. In

Plate 1

Zymogram of starch gel reacted to show the isozymes of alkaline phosphatase. The second sample (from left) shows all three isozymes of small gut. This extract was prepared 2-19-69 from an urchin collected on 2-17-69 at Boiler Bay, Oregon. The other samples were prepared on 2-14-69 from urchins which had been maintained in the laboratory for 2-3 weeks.



through December. In January, the intermediate isozyme had reappeared while the fast isozyme was absent. (Again, there is conflicting data about the fast isozyme.)

Isozymes of Large Gut and Coelomocytes

Concurrent observation of large gut and coelomocyte extracts revealed identical patterns (graph 4). In addition, the pattern was identical to that seen in the small gut for the fast and intermediate isozymes (compare graphs 3 & 4). However, the slow isozyme was never observed in any extracts other than small gut.

Histochemical Localization of the Isozymes

The absence of slow isozyme in large gut and coelomocyte extracts prompted histochemical examination of large gut and its comparison with small gut to determine if slow isozyme exhibited a localization different from the fast isozyme. This examination was made during July and August when only fast and slow isozymes were present in small gut and only the fast isozyme was present in large gut. The photomicrograph of small gut tissue (plate 2) shows two discrete localizations of alkaline phosphatase. The epithelial border shows an intense reaction. A second localization is confined to the deep portion of the lamina propria within the coelomocytes. In sections of large gut tissue (plate 3) reacted for alkaline phosphatase, only this second localization

Variations in Isozyme Patterns

Graph 3

This graph shows a compilation of the data recorded in appendix table 1. No animals were examined for isozyme content in November, December or January. The lines representing the various isozymes during those months were connected with the lines of the preceding and following months. Extracts of 58 animals are represented.

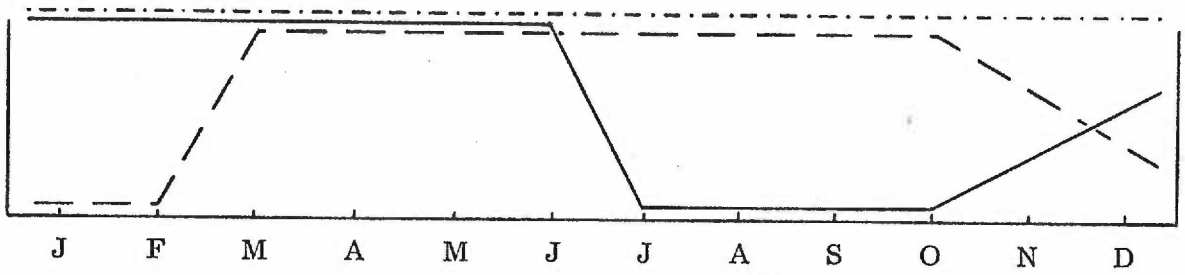
Graph 4

This graph shows a compilation of the data recorded in appendix table 1. This graph of large gut and coelomocyte isozymes has the added limitation that it is derived from only 32 different animals.

Variation in Isozyme Patterns

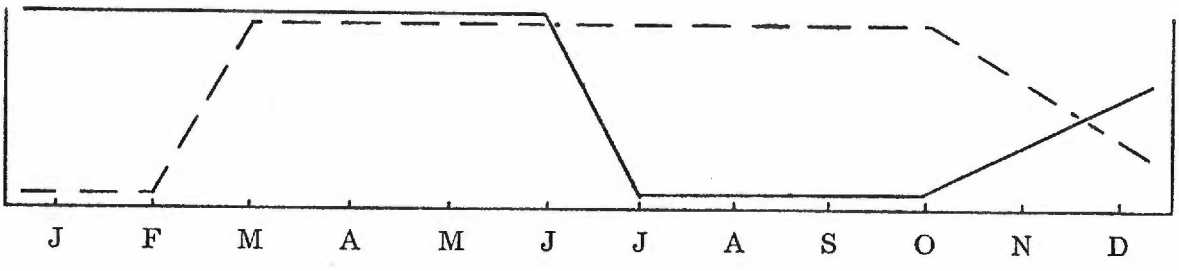
Tissue Specificity

3. Small Gut Isozymes



Fast — — — —
Intermediate ———
Slow ·—·—·—·—·—·

4. Large Gut and Coelomocyte Isozymes



Fast — — — —
Intermediate ———

was observed. Both small and large gut have coelomcytes present which contain enzyme and both contain fast isozyme. The epithelial localization of alkaline phosphatase in small gut corresponds to the presence of slow isozyme in extracts of this tissue.

Isozymes of Eggs and Ovaries

In extracts of egg with electrophoretically detectable alkaline phosphatase, only the intermediate isozyme was apparent. Extracts made from ovaries contained fast and a slight amount of intermediate isozymes. However, most of the enzymatic activity of the gels was due to the fast isozyme. The ovarian extracts were prepared during the late summer months when the gonads appeared ripe. At this time the coelomocytes contained only the fast isozyme.

Michaelis-Menten Constants of the Isozymes

Partially purified isozymes (eluted from starch gels after electrophoresis) were subjected to kinetic analysis. It is seen from table 1, that the variation in Vmax is so great as to render the value meaningless. However the Km values exhibit very little variability. In no instance is there an overlap in Km values among the isozymes with either substrate investigated.

The purpose of these experiments is to provide additional evidence that the isozymes are different, and not preparation artifacts.

Plate 2

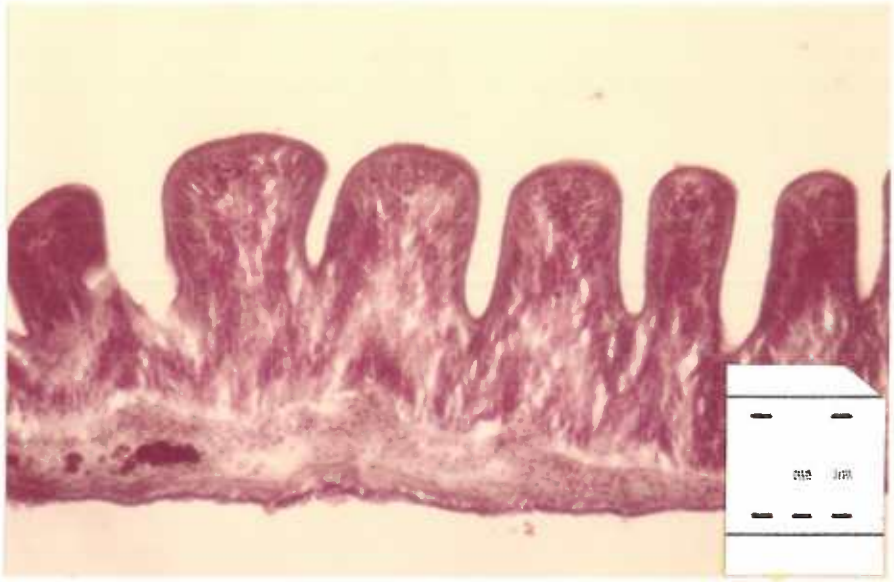
The photomicrograph shows a section of small gut reacted for the localization of alkaline phosphatase. The tissue was washed twice in cold sea water and fixed in 10% formalin-sea water. Following dehydration, the tissue was embedded in celloidin and sectioned on a sliding microtome. Beta-glyceryl-phosphate was used as substrate and $MgSO_4$ was added as an activator.

The diagram of a composite starch gel shows (left) the isozyme pattern of small gut at the time of the histochemical examination, (middle) the isozyme pattern observed in February, and (right) the isozyme pattern from late February through June.

Plate 3

This photomicrograph shows a section of large gut tissue reacted for the localization of alkaline phosphatase. The experimental details are the same as those described for the previous plate.

As above, the diagram of a composite starch gel shows (left) the isozyme pattern of large gut at the time of the histochemical examination, (middle) the isozyme pattern observed in February, and (right) the pattern observed from late February through June.



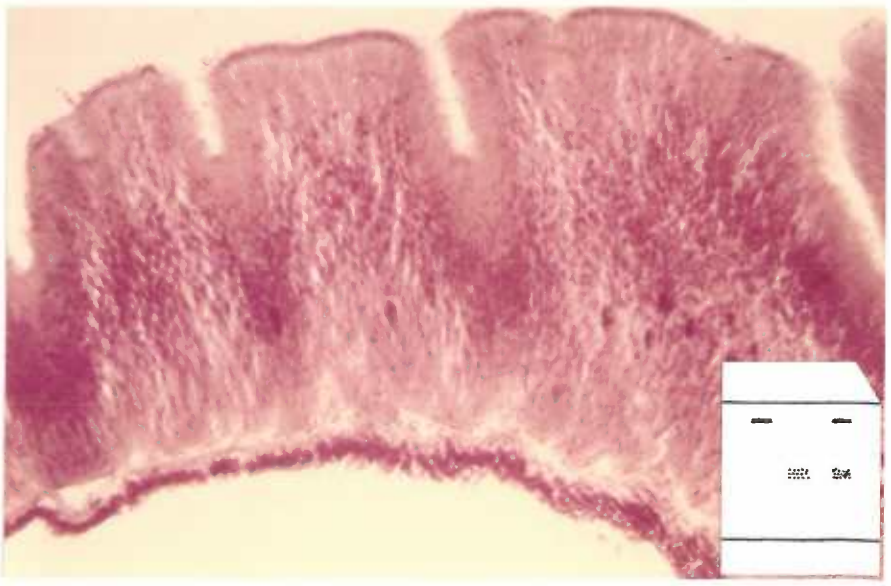


Plate 4

The photomicrograph shows a control section of small gut. The experimental details are the same as those presented on the previous page, except that the substrate, beta-glyceryl-phosphate, was omitted from the reaction medium.

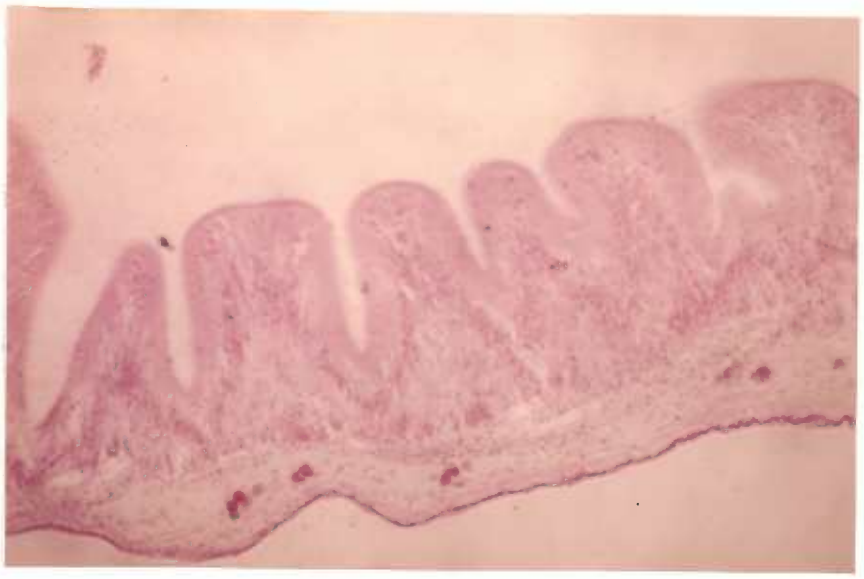


Table 1

The Michaelis-Menten (Km) and maximum velocity values are x 10^{-5} moles and x 10^{-8} moles min^{-1}/mg respectively. The df and F refer to the regression analysis which was performed on the data to arrive at the line of best fit. Reference to an F table shows that for significance at the 1% level with 10 degrees of freedom, a value of 10.04 must be exceeded. The value of F calculated in each case exceeds 10.04.

Representative Lineweaver-Burke plots are shown in the appendix. The six graphs show each isozyme with either alpha- or beta-naphthyl-phosphate.

Table 1

Michaelis-Menten Constants of Isozymes

alpha-naphthyl-phosphate Lineweaver-Burke				beta-naphthyl-phosphate Lineweaver-Burke			
Km	Vmax	df	F	Km	Vmax	df	F
	Slow isozyme				Slow isozyme		
8.7	10.5	14	1991	5.9	175.6	12	50
8.8	10.1	14	2045	6.0	8.8	12	49
8.8	13.6	14	2104	6.0	8.8	12	50
8.8	273.3	14	2101				
	Intermediate isozyme				Intermediate isozyme		
8.0	12.6	14	2922	4.5	129.7	14	2265
7.9	1.0	14	2963	4.5	11.4	14	2255
7.9	185.9	14	2772	4.6	8.5	14	1701
7.9	16.3	14	2592				
	Fast isozyme				Fast isozyme		
6.4	7.4	11	468	7.5	22.5	10	270
6.2	25.3	13	257	8.1	10.4	14	465
6.5	123.1	11	505	7.8	372.9	14	455
6.4	9.7	11	489	8.2	27.8	14	450
6.4	7.4	11	486				

Subunit Composition of Alkaline Phosphatase

Irreversible Inactivation

As was previously noted, techniques for the reversible dissociation of bacterial alkaline phosphatase have been reported (Schlesinger, et al., 1963). When treated with disulfide reducing agents, guanidine HCl, and acid pH, the bacterial isozymes of alkaline phosphatase dissociated into polypeptide subunits. If the disulfide reducing agent or guanidine HCl were removed by dialysis or the pH adjusted to pH 8.0, the enzymatic activity and the characteristic electrophoretic mobility were recovered. However, Sussman (in press) was unable to recover enzymatic activity of the placental enzyme with these methods. He did show that mercaptoethanol treatment caused dissociation of placental alkaline phosphatase in polypeptide subunits.

Similarly, treatment with mercaptoethanol, guanidine HCl, and acidification to pH 4.0 resulted in the irreversible loss of alkaline phosphatase activity from extracts of sea urchin tissue. After treatment with these reagents, no alkaline phosphatase activity was detectible by electrophoresis.

Reversible Inactivation

As an hypothesis, it was assumed that the sea urchin alkaline phosphatase was a dimer of polypeptide subunits.

In the simplest situation, the three-isozyme pattern could result from the possible associations of two different polypeptide subunits.

Dithiothreitol was known to reduce disulfide linkages to sulfhydril groups and protect these groups against further reduction by forming an oxidized ring compound (Cleland, 1963). Treatment with 10^{-2} M DTT was found to denature the sea urchin enzyme reversibly. However, treatment of crude extracts which contained fast and slow isozymes did not produce the expected pattern of three isozymes.

If there were two different polypeptide subunits dissociated by DTT, one would expect all three isozymes to appear following dissociation and reaggregation of the two homozygous isozymes. Only the two original isozymes were recovered. It was reasoned that it could be possible to reduce the disulfide linkages without disrupting the quaternary structure of the enzymes, (i.e., disruption of the tertiary structure without dissociation of the subunits). For this reason, 1 M NaCl was included to minimize hydrogen bonding, etc. Whether or not the reasoning was correct, with the inclusion of 1 M NaCl, the expected intermediate isozyme was found on electrophoresis (plate 5 & 6).

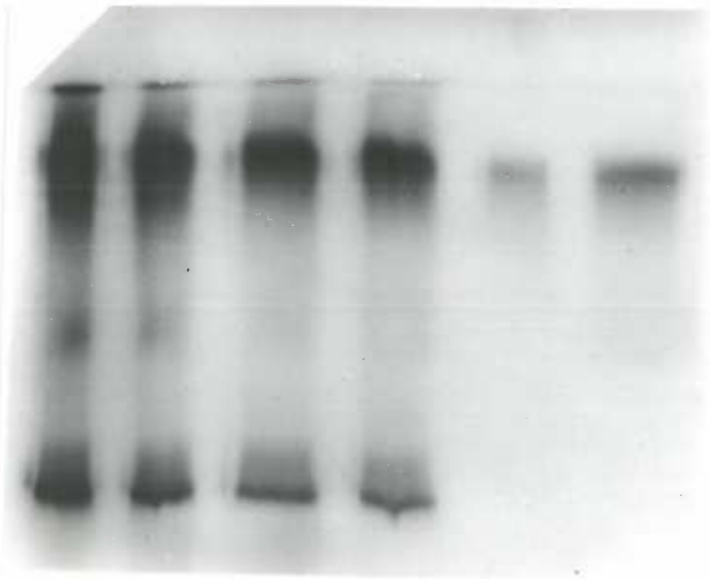
Treatment of either fast or slow isozymes separately

Plate 5

Zymogram of starch gel to show localization of alkaline phosphatase isozymes. The four samples on the left show the effect of increasing concentrations of NaCl while the concentration of dithiothreitol is held constant. All except the sample at the extreme right were treated with 10^{-2} M DTT prior to dialysis. The four samples on the left were aliquots of a small gut extract, known from prior electrophoresis to contain fast and slow isozymes. Similarly, the two samples on the right were aliquots of a partially purified (eluted from preparative gel) large gut extract and contained only fast isozyme.

Plate 6

Drawing of gel pattern obtained by dithiothreitol treatment. Three different samples are represented. All were treated with 1 M NaCl. The two samples on the left represent an extract of small gut which contained only fast and slow isozymes. The sample at the extreme left was treated with 10^{-2} M DTT while the second represents the control (lacking DTT). The remaining four samples were purified by elution from a previous starch gel. The left sample of each pair represents treatment with 10^{-2} M DTT and the right, control (lacking DTT).



with DTT and NaCl produced no change in electrophoretic mobility. Unfortunately, no intermediate isozyme was available at the time and the reciprocal experiment was not performed. According to the hypothesis of two different polypeptide subunits producing three isozymes, the intermediate (hybrid) isozyme, if tested, should have produced all three isozymes following dissociation and reaggregation.

Quantitative Effect of DTT on Small Gut Extracts

The effect of increasing the DTT concentration on crude extracts (broken lines of graph 5 through 11) is quite apparent. All show a precipitous decrease in specific activity following the addition of DTT. By DTT concentration of 10^{-2} M, well over 99% of the enzymatic activity is lost. By comparison of graphs 5 and 6 with graphs 7 and 8, it is seen that dilution of the extracts followed by dialysis results in a loss of specific activity. It is noted in the legend of these graphs (5-8) that the specific activity of both 1:10 and 1:100 dilutions of the crude extracts was identical prior to dialysis.

All four of the graphs (5-8) showing the effects of DTT treatment have common features. Of primary interest is that DTT treatment followed by dialysis

Graphs 5 & 6

The graphs on the opposing page represent two experiments carried out on different extracts of small gut tissue. Extract I was prepared from small gut tissue of five sea urchins. Small gut tissue was pooled from four sea urchins in Extract II. The specific activity of the undialyzed extracts was 11 x 10^{-3}m moles min^{-1}/mg and 12 x 10^{-3}m moles min^{-1}/mg for Extract I and II respectively.

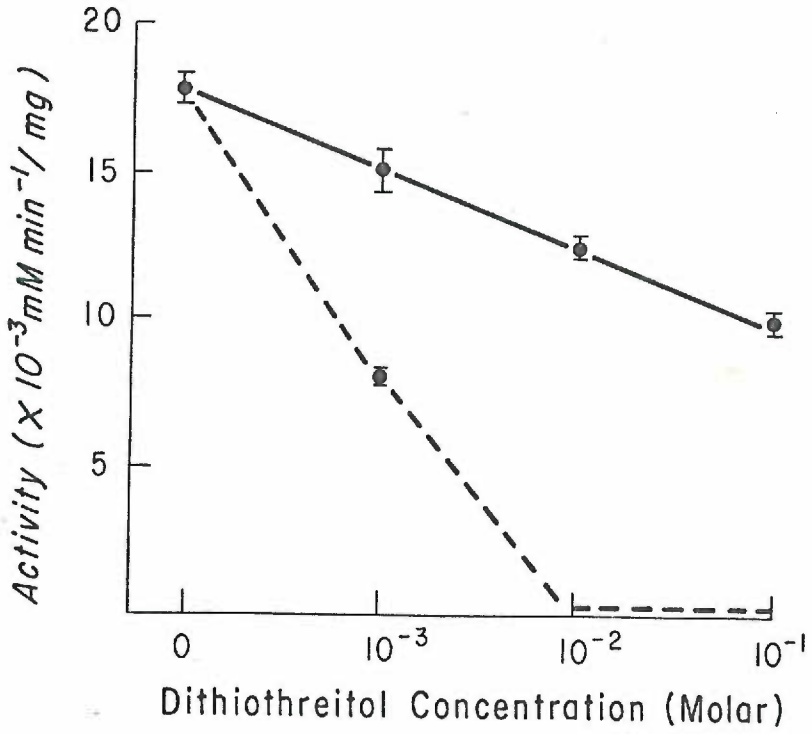
The broken line (----) shows the effect of increasing DTT concentrations. The solid line (——) shows the recovery of alkaline phosphatase activity following the removal of DTT by dialysis against three changes of buffer. The brackets show the range of variation of the experimental measurements.

The activity is x 10^{-3}m moles of alpha-naphthyl-phosphate hydrolyzed min^{-1}/mg of protein.

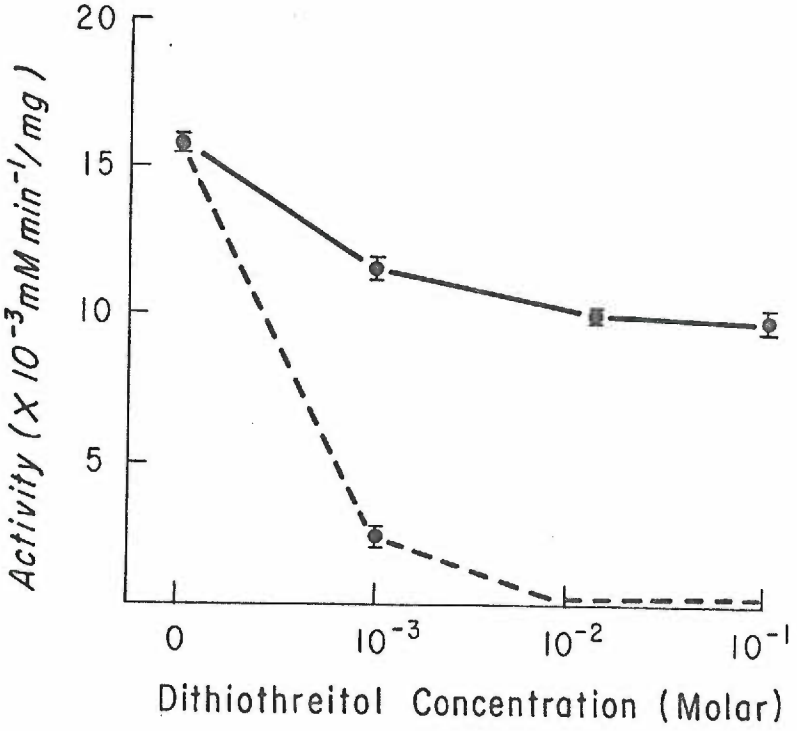
Experimental Details

In each of the experiments which describe the quantitative effect of DTT on crude extracts of sea urchin alkaline phosphatase, the experimental procedure was the same. The crude extracts were dialyzed against three changes of 0.05 M tris-HCl (pH 8.3) in 24 hours to remove the n-butanol remaining from the extraction procedure. To 0.9 ml of extract was added 0.1 ml of either distilled water or DTT at the appropriate dilution. Two samples were prepared at each DTT concentration. After the addition of 0.2 ml of 5 M NaCl, one sample at each DTT concentration was dialyzed against three changes of 0.05 M tris-HCl (pH 8.3) in 24 hours to remove the DTT. The activity of the samples containing DTT and the samples from which the DTT had been removed by dialysis was measured fluorometrically. This activity in %/min was converted to m moles of naphthol/min by reference to the standard curve (graphs 1 & 2). An aliquot of each sample was reserved for A_{280} measurement. From the A_{280}, the specific activity was calculated.

Graph 5



Graph 6



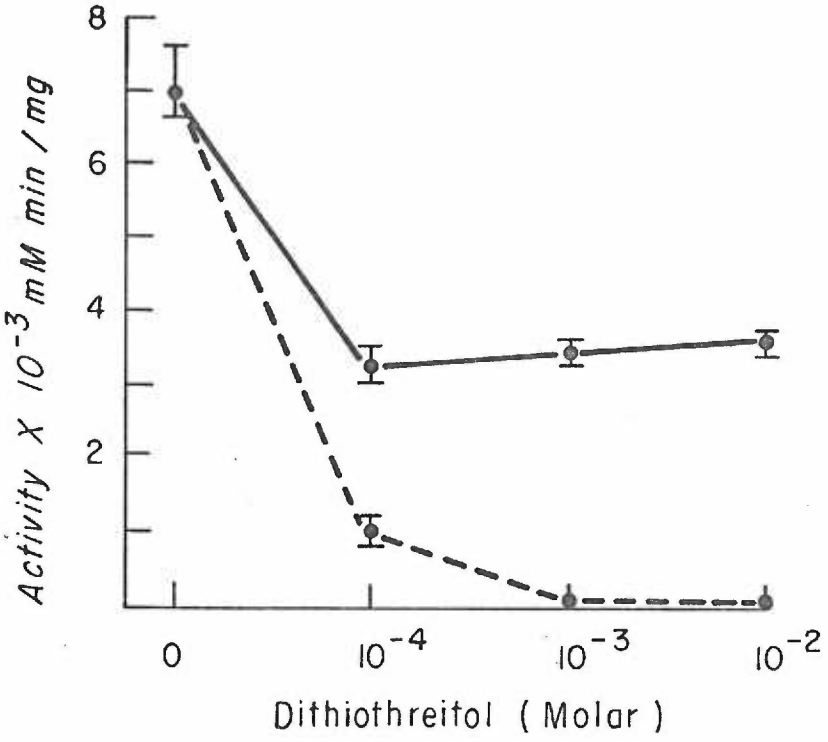
Graphs 7 & 8

These graphs represent the results of two experiments where the same extracts represented in graphs 5 and 6 were diluted 1:100 prior to dialysis. The specific activity of the extracts did not change when a sample was diluted 1:100 prior to dialysis (i.e., 11 and 12 x 10^{-3}m moles min^{-1}/mg of protein).

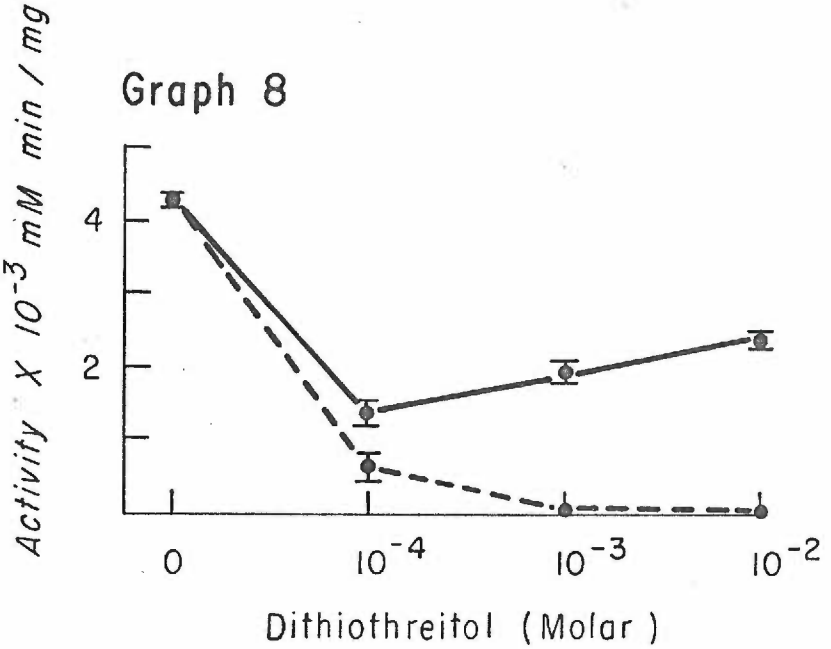
The broken line (----) shows the effect of adding DTT to the diluted extracts. The solid line (——) shows the recovery of alkaline phosphatase activity when the DTT is removed by dialysis. The brackets show the range of variation of the experimental measurements.

For experimental details, see legend of graphs 5 and 6.

Graph 7



Graph 8



results in a loss of specific activity. Although activity is recoverable, it never reaches the original value of the extract.

Quantitative Effect of DTT on Egg Extracts

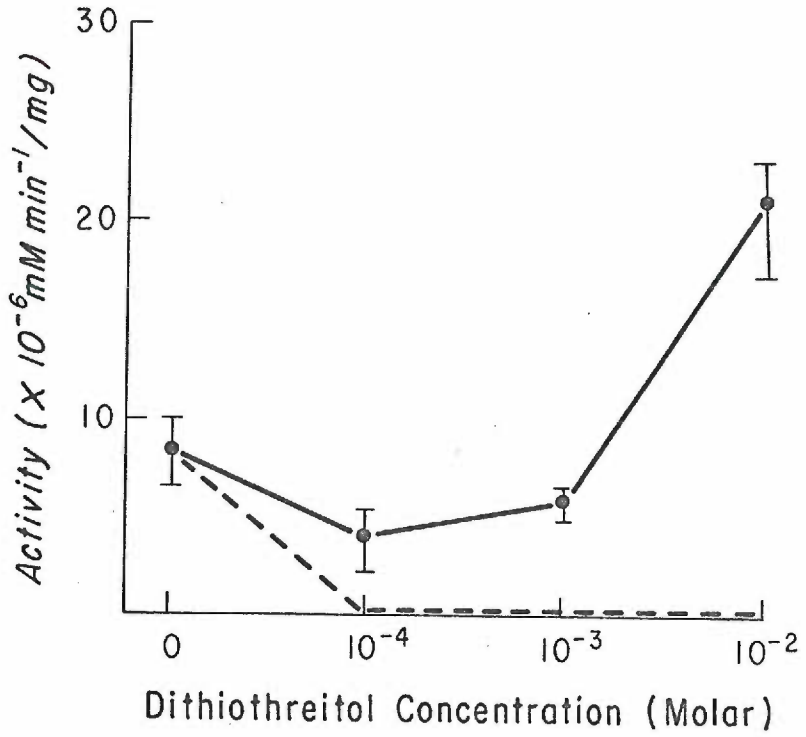
When DTT is added to unfertilized egg extracts, the loss of fluorometrically measured activity is more rapid than in small gut extracts (i.e., compare graphs 5 through 8 with graphs 9 through 11). The more rapid loss of activity is not surprising since the concentration of alkaline phosphatase in the egg extracts is much lower. However, when the DTT is removed by dialysis, instead of the expected partial recovery in specific activity, an increase over that present in the original extract is apparent (graphs 9-11). The first portion of the graphs is similar to those of small gut extracts in that following dialysis not all of the activity is recovered. When DTT concentrations of 10^{-2} M are reached, the activity recovered exceeds that of the original extract prior to DTT treatment. The average increase in alkaline phosphatase activity is slightly over two and one-half fold.

Graphs 9 & 10

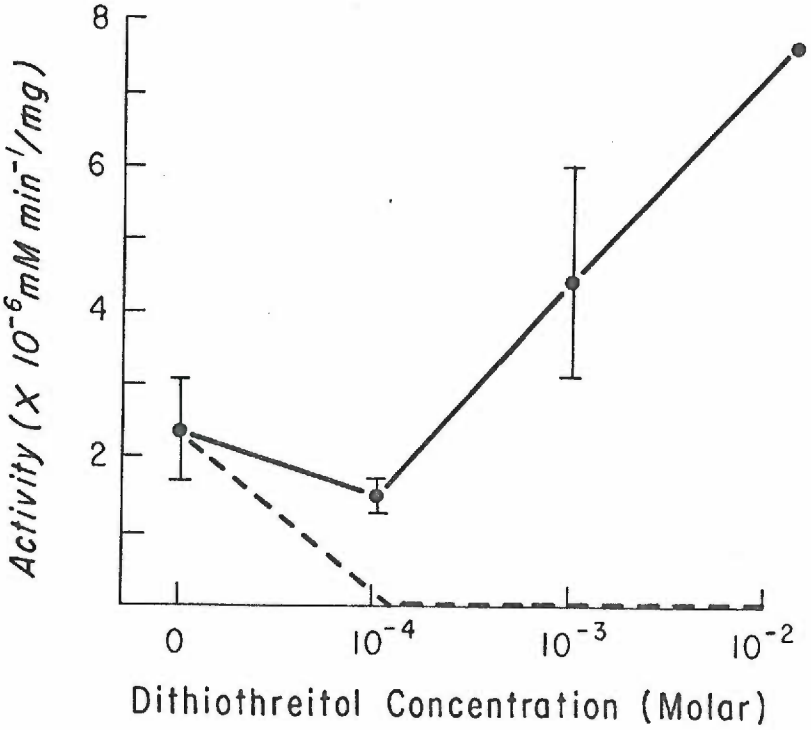
These graphs represent the results of two experiments with unfertilized egg extracts. Eggs for the first experiment were obtained from a single female. In the second experiment it was necessary to pool the eggs of two females to obtain sufficient material for extraction.

The broken line (----) shows the effect of increasing DTT concentrations on the specific activity of the egg extracts. The solid line (——) represents the recovery of alkaline phosphatase activity when the DTT is removed by dialysis. The brackets show the range of variation of the experimental measurements.

Graph 9



Graph 10

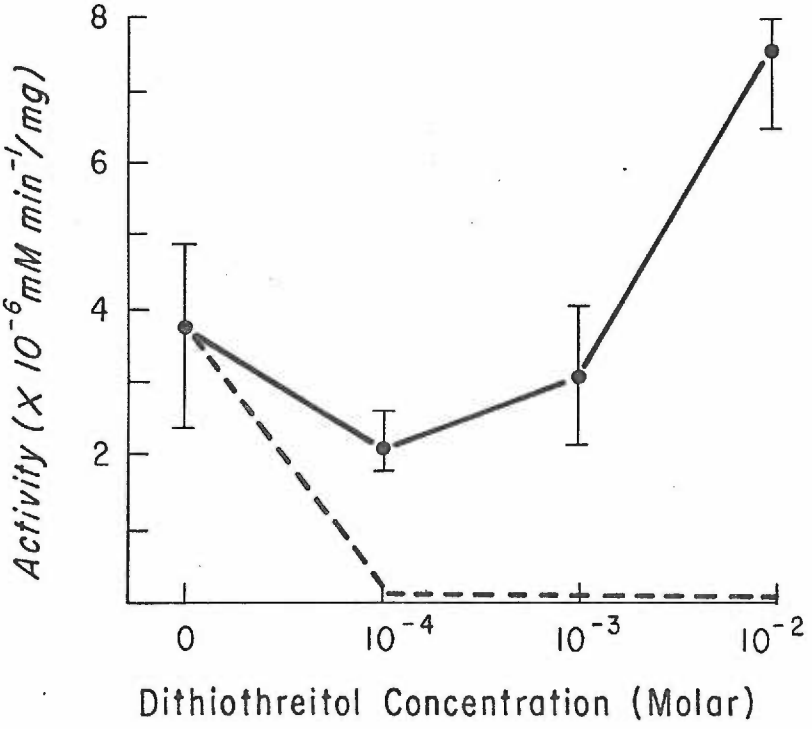


Graph 11

This graph represents the result of an experiment with unfertilized egg extract. Eggs were pooled from two females to obtain sufficient material for extraction.

The broken line (----) shows the effect of increasing DTT concentrations. The solid line (——) represents the recovery of alkaline phosphatase activity when the DTT is removed by dialysis. The brackets show the range of variation of the experimental measurements.

Graph II


8
6
4
2
Activity (X 10⁻⁶mM min⁻¹/mg)
0
10⁻⁴
10⁻³
10⁻²
Dithiothreitol Concentration (Molar)

DISCUSSION

Variation in Isozyme Patterns

The data represented by graphs 3 and 4 (derived from appendix table I) show that the isozyme patterns of small gut, large gut and coelomocytes, exhibit fluctuations when examined by starch gel electrophoresis. Lyons, et al., (1968), Weaver (1966) and others, have shown that the alkaline phosphatase isozyme patterns of human leukocytes vary with respect to time, physiologic state, etc. Considering these reports it is not surprising that the isozyme patterns of specific tissues of the sea urchin exhibit fluctuations. However, too many variables were introduced by the collecting procedure to allow for interpretation of these data (e.g., site of collection, availability of food, condition and sex of animal, etc.). It would be of considerable interest to follow this phenomenon with attention to control and measurement of these kinds of variables.

Tissue Localization of Isozymes

The localization of the isozymes within the tissues studied is demonstrated by comparison of the tissue isozyme patterns with the histochemical localization of alkaline phosphatase. Throughout the study, the gel patterns of large gut and coelomocyte extracts were

identical. Histochemical localization of alkaline phosphatase showed that only the coelomocytes possessed detectable enzyme. This is in agreement with the report of Lyons and Weaver (1962). Their data, as well as the data presented here, show that the large gut has no intrinsic alkaline phosphatase and that the alkaline phosphatase activity of large gut homogenates is due to the presence of the ubiquitous coelomocyte.

Small gut, on the other hand, exhibits a consistantly different isozyme pattern (graph 3). The intermediate and fast isozymes are seen in small gut extracts only when these isozymes are present in large gut and coelomocyte extracts. However, an additional isozyme, the slow isozyme, is found only in small gut extracts. Plate 4 shows the histochemical localization of alkaline phosphatase in small gut. It is quite obvious by comparison of plate 4, showing small gut localization, with plate 5, showing large gut alkaline phosphatase localization, that the epithelial alkaline phosphatase represents enzyme intrinsic to small gut. Since it is logical to assume that the coelomocytes possess the same isozymes whether observed in large gut, small gut or free in the coelomic fluid, it is suggested that the epithelial localization is due to the presence of the slow migrating isozyme. Furthermore, since it appears only in small gut epithelium,

it is regarded as a tissue-specific isozyme. This distinction may not appear important at this point, but its relevance will become clear when the development of the sea urchin larvae is considered.

In unfertilized eggs, only intermediate isozyme has been seen by starch gel electrophoresis (appendix table I). During the late summer months (i.e., July and August, graph 3 & 4), coelomocytes do not contain detectable amounts of intermediate isozyme. Although at some times of the year the coelomocytes contain both fast and intermediate isozymes, extracts of ovary were prepared when the coelomocytes contained only fast isozyme. When subjected to electrophoretic analysis, ovarian extracts exhibited both fast and a slight amount of intermediate isozymes. Since coelomocytes, at the time, contained only fast isozyme and the unfertilized eggs contained only the intermediate isozyme, it is suggested that ovarian tissue, like large gut, contains no intrinsic enzyme. No attempt was made to histochemically localize the alkaline phosphatase of the ovary and, therefore, this conclusion must be regarded as tentative. Numerous other cells are present in the ovary besides eggs, oocytes, and coelomocytes.

Michaelis-Menten Constants of the Isozymes (Table 1)

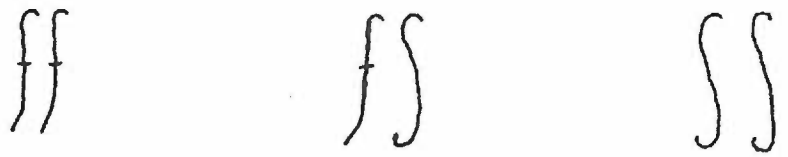
In spite of the variability of Vmax, the Km values computed were quite uniform. The most probable explanations for the variability of Vmax are that either non-competitive inhibitors or variable quantities of contaminating proteins were present in the samples analyzed. More importantly, the uniformity of the Km values support the argument that there are, in fact, three different isozymes present. The differences in substrate affinity of the three isozymes are more pronounced when beta-naphthyl-phosphate is the substrate.

Subunit Complimentation

A basic assumption which underlies the use of the technique of complimentation is that the 'blueprints' for the assemblage and folding of complex proteins are contained within the primary structure or amino acid sequence of the constituent polypeptides. This assumption is no doubt true for most if not all proteins. It has been documented for alkaline phosphatase, at least at the bacterial level of complexity. A considerable difference in molecular weight is known to exist between bacterial (c. 86,000, Schlesinger, 1965) and human placental (c. 120,000, Sussman, in press) alkaline phosphatase. In both instances, disulfide reduction

results in the dissociation of the enzyme into two polypeptide subunits of equal molecular weight. With bacterial alkaline phosphatase, strong reducing agents were required (i.e., thioglycolic acid). Human placental alkaline phosphatase did not require such a strong reducing agent (mercaptoethanol, Sussman, in press) for this dissociation. However, Sussman was unable to reactivate the dissociated enzyme when using mercaptoethanol.

In the study of sea urchin alkaline phosphatase dithiothreitol was employed. As an hypothesis it was assumed that sea urchin alkaline phosphatase is a dimer of polypeptide subunits. With this assumption it is possible to explain the fast, intermediate and slow isozymes as the in vivo association product of two non-allelic genes. This may be diagramed as follows:



Fast Intermediate Slow

If the hypothesis is correct, one would expect that if fast or slow isozymes were dissociated separately into polypeptide subunits and that these subunits were allowed to reassociate at random, only fast or slow isozyme would be produced. This is shown in plate 3. With the same

hypothesis, if fast and slow isozymes were dissociated together and allowed to reassociate in a random fashion, one would expect the appearance of an intermediate, hybrid-type, isozyme. The experimental results (plate 3) show this also.

Efficiency of Dissociation and Reassociation

Small Gut versus Egg Extracts

Graphs 5-8 show that the recovery of active enzyme from DTT-treated small gut extracts is considerably less than 100%. However, when unfertilized egg extracts are treated in a similar fashion, instead of the expected loss, a substantial increase in activity is observed.

It will be recalled that previous investigators (e.g., Pfohl, 1965, among others) measured a six-fold increase in alkaline phosphatase activity during development from the unfertilized egg to the pluteus stage. Further, since a new isozyme of different electrophoretic mobility appeared to coincide with the beginning of gastrulation, Pfohl (1965) interpreted his data to indicate alkaline phosphatase synthesis began at gastrulation.

It appears to be a general practice of investigators in this area of research to normalize the data. Due to this practice, actual values cannot be compared. However,

Flickinger (1958) presented his results in specific activity. Although his assays required incubation for two hours to develop sufficient product for measurement, the specific activity he reported can be extrapolated to 10^{-6}m moles min^{-1}/mg of protein for unfertilized egg extracts. This is the same range of specific activity as measured in graphs 9-11.

There is general agreement in the literature (Pfohl, 1965; Hsaio & Fujii, 1963; and Mazia et al., 1950) that alkaline phosphatase activity increases six-fold from the unfertilized egg to the pluteus stage. Gastrulation occurs about 10 hours after fertilization and has been suggested to be the beginning of de novo synthesis of alkaline phosphatase (Pfohl, 1965). The data presented in graphs 9-11 suggest that there may be sufficient latent alkaline phosphatase present in the unfertilized egg to carry it nearly to the pluteus stage without the necessity of synthesis.

In the three experiments described by graphs 9-11 a loss of about 50% of the original activity was expected to result from DTT treatment. However, an increase in activity, which averages 2.5 times the untreated value was observed. These data suggest that there is sufficient alkaline phosphatase activity in the egg to account for most, if not all, of the increase in alkaline

phosphatase activity reported in the literature.

There are several hypotheses which could explain the observed increase in alkaline phosphatase activity following DTT treatment of unfertilized egg extracts. The most attractive interpretation would appear to be that there are inactive or masked polypeptide subunits or enzyme present in the egg prior to fertilization. This would explain the results of DTT treatment on unfertilized egg extracts taking into consideration what is known of the effects of disulfide reduction on alkaline phosphatase.

A second possibility is the selective activation of the intermediate isozyme. A selective effect on the intermediate isozyme (e.g., allosteric change) would require a dramatic change in the catalytic properties of the intermediate isozyme. Aside from the data in table 1, graphs 5-8 suggest that no such change occurs. Intermediate isozyme is produced by DTT treatment of small gut extracts containing fast and slow isozymes (plates 2 & 3). Quantitative measurement of the effect of DTT on these extracts show a substantial loss of activity resulting from this treatment.

Another alternative might be release from specific inhibition. One specific inhibitor of alkaline phosphatase (i.e., phosphate) is known to be concentrated against a gradient in the unfertilized egg (Chambers

& Whitely, 1968). Evidence has been discussed suggesting that there are inhibitors of alkaline phosphatase present in crude extracts of small gut. However, it seems unlikely that DTT treatment would provide specific release from inhibition only in the egg extracts.

Malate Dehydrogenase versus Alkaline Phosphatase

Studies by other investigators on protein synthesis in early development were described in the introduction. Most of these studies dealt with patterns of proteins rather than specifically identified proteins. With regard to malate dehydrogenase, Villee (1968) was unable to prevent the appearance of MDH isozymes or to alter their normal isozyme patterns with puromycin, an inhibitor of protein synthesis, or with actinomycin D. He did not offer an explanation of this phenomenon. If protein synthesis was stopped by puromycin without altering either the appearance of MDH or its specific isozyme patterns, it must be concluded that the appearance of MDH and its specific isozyme patterns during early development are not dependent on de novo synthesis. One interpretation of these data is that either the intact enzyme or its subunits were present in an inactive or masked form. This is the same interpretation as was offered for the increase in alkaline phosphatase activity resulting from the manipulation of egg extracts.

Differential Segregation of Proteins

The problem of segregation of proteins during early development was investigated by Villee (1968). Again, the specific protein followed was malate dehydrogenase. He found that by the 16-cell stage, the blastomeres had a differing isozyme content. By the 16-cell stage, unequal partitioning of the cytoplasm has occurred. In the instance of alkaline phosphatase, Gustafson (1965) noted that the vegital region of the embryo had a higher activity. This is the region from which the micromeres form. The micromeres are the cells which at gastrulation form the primary mesenchyme. Following gastrulation these cells behave like coelomocytes (e.g., pigment formation, skeletal formation, mobility, possession of alkaline phosphatase, etc.). In the adult sea urchin, the intermediate isozyme is contained within the coelomocytes. Since this isozyme is found in the unfertilized egg and is partitioned unequally during cleavage (Gustafson, 1965) into cells destined to become coelomocytes, it is suggested that at gastrulation the intermediate isozyme is confined to those cells of the primary mesenchyme.

This line of reasoning is further supported by the appearance of the slow isozyme (small epithelial form of the adult which coincides with the appearance

of the gut primordium. It will be recalled that the archenteron is the site of the increase in alkaline phosphatase activity.

These preliminary results suggest that alkaline phosphatase may indeed provide an illustrative model for the study of early echinoderm development. The enzyme appears to be quite similar in physical properties to its placental relative which Sussman, Small and Cotlove (1968) purified. To prove conclusively the exact nature of this developmental phenomenon, it will be necessary to purify echinoderm alkaline phosphatase. Once this purification of the enzyme and its constituent subunits is accomplished, the molecular mechanism of its activation (and probably its synthesis also) is open to investigation.

A central question which cannot be answered at this time is the relative contribution to early development of synthesis or activation. A review of the literature would seem to suggest that the balance of popular opinion favors the synthetic view. In the final analysis this view is necessarily correct, however, the specific examples cited (i.e., malate dehydrogenase and spindle associated proteins), the results of this thesis research, and the classical literature suggest that the immediate mechanism may be activation.

SUMMARY AND CONCLUSIONS

In the adult purple urchin there are three isozymes of alkaline phosphatase. These isozyme patterns were shown to exhibit fluctuations during the course of the study. The factors involved in these fluctuations were not evaluated.

Several tissues were examined by starch gel electrophoresis for the presence of alkaline phosphatase isozymes. Coelomocytes were shown to contain fast and/or intermediate isozymes. Large gut was shown histochemically to contain no intrinsic alkaline phosphatase. It was shown to contain coelomocytes with their isozymes. Small gut was examined both histochemically and electrophoretically and shown to contain coelomocytes with their isozymes plus an additional epithelial localization. This epithelial localization was shown to correspond with the slow migrating isozyme. The slow isozyme was interpreted to be a tissue-specific form of alkaline phosphatase.

Unfertilized eggs were shown to contain the intermediate isozyme. Ovaries were shown to contain both fast and intermediate isozymes at a time when coelomocytes contained only fast isozyme. It is suggested that the fast isozyme of the ovary is due to the presence of the coelomocytes and the intermediate isozyme is due to the eggs. No histochemical analysis was attempted of the

ovary. It is suggested that the ovary, like large gut, contains no intrinsic alkaline phosphatase.

Kinetic analysis of the three isozymes show that each isozyme has a Km value which is different from the other isozymes. Two different substrates were tested, alpha- and beta-naphthyl-phosphate. The Km values of the three isozymes were more readily distinguishable when beta-naphthyl-phosphate was used as the substrate.

Data drawn from over 100 different sea urchins (appendix table I) show that the three isozyme pattern is consistent with the interpretation of two non-allelic genes. A complimentation test using DTT was described, the results of which suggest that the fast isozyme is composed of two identical fast-type subunits, the slow isozyme is composed of two slow-type subunits, and the intermediate isozyme is a hybrid-dimer of one fast- and one slow-type subunits. This test comprises necessary but not sufficient evidence for conclusion. However, evidence from the literature supports and strongly suggests that this is the correct conclusion.

When adult tissue extracts were subjected to disulfide reduction with subsequent reoxidation, between 30 and 50% of the alkaline phosphatase activity was lost. Identical treatment of unfertilized egg extracts resulted in an increase in activity, which averaged 2.5 times the original activity. The increase following treatment was

taken to indicate that the unfertilized egg contained sufficient latent enzyme to account for nearly all of the activity which arises by the pluteus stage. Three different kinds of mechanism by which this increase could occur were considered.

In conclusion, the data referred to in the literature and the data presented in this work are in close agreement. (The same cannot be said of the interpretation placed on it.) The few specific proteins studied during early development of the sea urchin agree closely with the classical interpretation. The unequal partitioning of materials described by the classical embryologists at the light microscope level extends to the molecular level with the unequal segregation of isozymes, even in 'regulative' eggs.

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List of the selected extracts upon

which conclusions in this paper are based.

Appendix Table I

Date	Tissue	Band Patterns	Remarks
3-2-65	small gut	F+S	Smear often appeared between F and S bands.
3-17-65	large gut	F only	0.005 M tris--20% wet wt/vol--n-butanol,
4-7-65	large gut	F only	20% vol/vol.
4-7-64	coelomocytes	F only	No rheostat used with Virtis--set at 30 for 3 minutes--extract
4-7-65	small gut	F + S	collected by filtration through diatomaceous
4-11-65	small gut	F + S	earth.
4-11-65	small gut	F + S	
4-12-65	small gut	F + S	
4-12-65	small gut	F only	
4-12-65	small gut	F + S	
4-12-65	large gut	F only	
5-13-65	small gut	F + S	
5-13-65	large gut	F only	
6-23-66	small gut	F + S	Buffer and n-butanol concentrations as above.
6-24-66	small gut	F + S	Virtis settings changed to 30 for 60
6-30-66	large gut	F only	seconds followed by 0 for 3 minutes.
6-30-66	coelomocytes	F only	
7-1-66	small gut	F + S	
7-1-66	large gut	F only	
7-1-66	coelomocytes	F only	
7-26-66	small gut	F + S	
7-26-66	large gut	F only	
7-26-66	coelomocytes	F only	

Date	Tissue	Band Patterns	Remarks
5-2-68	pooled gut	F, some S	Extraction procedure identical to above.
5-2-68	pooled gut	F, some S	
6-18-68	pooled gut	F + S	Some of pooled gut extracts exhibited smearing between S and F bands.
6-18-68	pooled gut	F, some S	
7-1-68	pooled gut	F, some S	
7-1-68	pooled gut	F, some S	
7-1-68	pooled gut	F + S	
7-22-68	small gut	F + S	
9-3-68	small gut	F + S	
9-3-68	small gut	F + S	
9-30-68	small gut	F + S	Tris replaced with filtered sea water in homogenation mixture. Resulted in lowering of activity.
9-30-68	esophagus	F only	
9-30-68	large gut	F only	
9-30-68	large gut	F only	
9-30-68	coelomocytes	F only	
10-15-68	small gut	F + S	
10-15-68	small gut	F + S	
10-15-68	small gut	F + S	
10-15-68	small gut	F + S	
10-18-68	small gut	F + S (I?)	
10-18-68	large gut	F + (I?)	
10-18-68	coelomocytes	F + I	
1-23-69	small gut	S + I	Extraction procedure identical to above, i.e., 1966.
1-23-69	small gut	S + I	
1-23-69	small gut	S + I	
1-23-69	small gut	S + I	

Date	Tissue	Band Patterns	Remarks
1-23-69	coelomocytes	I only	
1-23-69	coelomocytes	I only	
1-23-69	large gut	I only	
1-23-69	large gut	I only	
1-26-69	pooled gut	I, some S	
1-26-69	pooled gut	I, some S	
1-26-69	pooled gut	I + S	
1-26-69	pooled gut	I, some S	
1-26-69	eggs	I only	eggs were unfertilized
1-26-69	eggs	I only	
1-26-69	eggs	I only	
1-26-69	eggs	I only	
1-28-69	small gut	I + S	
1-28-69	large gut	I only	
1-28-69	large gut	I only	
1-28-69	coelomocytes	I only	
1-28-69	eggs	I only	
1-28-69	eggs	I only	
1-28-69	eggs	I only	
1-28-69	eggs	I only	
2-14-69	small gut	I + S	Virtis set at maximum and connected through rheostat. Rheostat settings varied systematically from 20V for 60 seconds through 100V for 3 minutes--optimal setting found to be 30V for 60 seconds, 20V for 180 seconds and 10V for 300 seconds.
2-14-69	small gut	I + S	
2-14-69	coelomocytes	I	
2-14-69	small gut	I + S	
2-14-69	small gut	I + S	

Date	Tissue	Band Patterns	Remarks
2-14-69	small gut	I + S	Between 2-14-69 and 2-18-69, 70 extracts of tissues from individual urchins were prepared. Those with sufficient activity (most) showed either I or I + S band activity.
2-14-69	eggs	I only	
2-14-69	small gut	I + S(F?)	
2-14-69	small gut	I + S	
2-19-69	small gut	S + I + F	Fresh urchins were collected 2-17-69 at Boiler Bay. Animals extracted 2-14-69 had been in the laboratory for 2-3 weeks.
2-19-69	small gut	S + I + F	
2-19-69	small gut	S + I + F	
2-19-69	small gut	S + I + F	
2-19-69	small gut	S + I + F	
2-19-69	small gut	S + I + F	
2-19-69	small gut	S + I + F	
2-26-69	large gut	I + F	
2-26-69	eggs	I only	
4-7-69	small gut	S + I + F	
4-7-69	large gut	I + F	
4-8-69	large gut	I only	
4-8-69	large gut	I + F	
4-8-69	small gut	S + I + F	
4-8-69	eggs	I only	
5-6-69	small gut	S + I + F	
5-6-69	small gut	S + I + F	
5-6-69	small gut	S + I + F	
5-6-69	large gut	I + F	

Date	Tissue	Band Patterns	Remarks
6-12-69	small gut	S + I + F	
6-12-69	ovaries	I + F	
6-12-69	ovaries	I + F	
6-12-69	eggs	I only	
7-1-69	eggs	I only	
7-1-69	eggs	I only	
7-1-69	eggs	I only	
7-1-69	small gut	F + S	
7-1-69	large gut	F only	
7-1-69	large gut	F only	
7-1-69	coelomocytes	F only	

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PAGE   1

// JOB 740

LOG DRIVE   CART SPEC   CART AVAIL  PHY DRIVE
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// FOR
*NAME RJLWH
*LIST ALL
*ONE WORD INTEGERS
*IOCS(1132 PRINTER,CARD)
*EXTENDED PRECISION
      INTEGER T
      REAL MSR, MSDR
      DIMENSION X(200), Y(200), CF(200), ITITL(78)
    1 FORMAT (I3)
    2 FORMAT (2E10.2)
    9 FORMAT (5H     )
   17 FORMAT (F2.1)
   18 FORMAT (I4)
   32 FORMAT (10F7.0)
   71 FORMAT (' 1/VMAX =', E20.7)
   72 FORMAT (' VMAX =', E20.7)
   73 FORMAT (' KM =', E20.7)
   74 FORMAT (' KM/VMAX =', E20.7)
   75 FORMAT (' -1/KM =', E20.7)
   81 FORMAT (' -KM =', E20.7)
   85 FORMAT (' VMAX/KM =', E20.7)
   86 FORMAT (' COMPUTED -1/KM =', E20.7)
   87 FORMAT (' COMPUTED -KM =', E20.7)
   88 FORMAT (' COMPUTED VMAX/KM =', E20.7)
  203 FORMAT (39A2)
  204 FORMAT (1H1, 39A2/)
  207 FORMAT (1H1,10X, '1/S', 20X, '1/V'////)
  208 FORMAT (2E20.7)
  209 FORMAT (1H1,10X, 'S', 20X, 'S/V'////)
  211 FORMAT (1H1, 10X, 'V/S', 20X, 'V'////)
  213 FORMAT (10X,'S', 20X, 'V'//)
  214 FORMAT (////, 10X, 'LINEWEAVER-BURKE PLOT'////)
  215 FORMAT (////, 10X, 'WOOLF PLOT'////)
  216 FORMAT (////, 10X, 'HOFSTEE PLOT'////)
  217 FORMAT (5X, 39A2//)
      READ (2,17) R
   60 READ (2,18) T
      READ (2,203) (ITITL(I),I=1,39)
      WRITE (3,204) (ITITL(I),I=1,39)
      WRITE (3,9)
      IF (T - 9999) 50, 40, 50
   50 READ (2,1) N
      WRITE (3,213)
      READ (2,2) (X(I), Y(I), I = 1,N)
      READ (2,32) (CF(I), I = 1,N)
      WRITE (3,208) (X(I), Y(I), I = 1,N)
      DO 193 I = 1,N
      Y(I) = Y(I) - CF(I)
  193 CONTINUE
      Z = 1./R
      DO 10 I = 1,N
      Y(I) = Z/Y(I)
```

```
   10 CONTINUE
      WRITE (3,207)
      DO 163 I = 1,N
      X(I) = 1./X(I)
      WRITE (3,206) X(I), Y(I)
  163 CONTINUE
      WRITE (3,214)
      WRITE (3,217) (ITITL(I), I = 1,39)
      CALL RLJ (N, X, Y, MSR, MSDR, AYX, BYX)
      VMAX = 1./AYX
      AKM = BYX*VMAX
      XINT = -1./AKM
      WRITE (3,71) AYX
      WRITE (3,72) VMAX
      WRITE (3,73) AKM
      WRITE (3,74) BYX
      WRITE (3,75) XINT
      XKINT = -AYX/BYX
      WRITE (3,86) XKINT
      WRITE (3,209)
      DO 70 K = 1,N
      X(K) = 1./X(K)
      Y(K) = Y(K)*X(K)
      WRITE (3,206) X(K), Y(K)
   70 CONTINUE
      WRITE (3,215)
      WRITE (3,217) (ITITL(I), I = 1,39)
      CALL RLJ (N, X, Y, MSR, MSDR, AYX, BYX)
      VMAX = 1./BYX
      AKM = AYX*VMAX
      XINT = -AKM
      WRITE (3,71) BYX
      WRITE (3,74) AYX
      WRITE (3,72) VMAX
      WRITE (3,73) AKM
      WRITE (3,81) XINT
      XKINT = -AYX/BYX
      WRITE (3,87) XKINT
      WRITE (3,211)
      DO 126 I = 1,N
      Y(I) = 1./ (Y(I)*(1./X(I)))
      X(I) = Y(I)*(1./X(I))
      WRITE (3,208) X(I), Y(I)
  126 CONTINUE
      WRITE (3,216)
      WRITE (3,217) (ITITL(I), I = 1,39)
      CALL RLJ (N, X, Y, MSR, MSDR, AYX, BYX)
      AKM = -BYX
      XINT = AYX/AKM
      WRITE (3,72) AYX
      WRITE (3,73) AKM
      WRITE (3,81) BYX
      WRITE (3,85) XINT
      XKINT = -AYX/BYX
      WRITE (3,88) XKINT
      GO TO 60
   40 CALL EXIT
      END
VARIABLE ALLOCATIONS
     XIR )=0255-0000     YIR )=04AD-0258    CFIR )=0705-0480   MSRIR )=0708       MSDRIR )=0708          RIR )=070E
```

```
PAGE    3

     Z(R )=0711          AYX(R )=0714          BYX(R )=0717          VMAX(R )=071A          AKM(R )=071D          XINT(R )=0720
 XKINT(R )=0723       ITITL(I )=0773-0726        T(I )=0774            I(I )=0775            N(I )=0776            K(I )=0777

STATEMENT ALLOCATIONS
 1     =0792  2     =0784  9     =0787  17    =078C  18    =078E  32    =0790  71    =0793  72    =0798  73    =07A2  74    =07A8
 75    =07B0  81    =07B7  85    =07BD  86    =07C5  87    =07D1  88    =07DC  203   =07E9  204   =07EC  207   =07F2  208   =0801
 209   =0804  211   =0812  213   =0820  214   =0829  215   =083F  216   =084F  217   =0860  60    =0882  50    =08BE  193   =0910
 10    =093B  163   =0961  70    =09F1  126   =0A85  40    =0AE4

FEATURES SUPPORTED
 ONE WORD INTEGERS
 EXTENDED PRECISION
 IOCS

CALLED SUBPROGRAMS
 RLJ     ESUBX   EMPY    EMPYX   EDIV    EDIVX   ELD     ELDX    ESTO    ESTOX   EDVR    CARDZ   PRNTZ   SHED    SHRT
 SCOMP   SFIO    SIOFX   SIOIX   SIOF    SIOI    SUBSC   SNR

REAL CONSTANTS
   .100000000E 01=077A

INTEGER CONSTANTS
     2=077D       1=077E      39=077F       3=0780    9999=0781

CORE REQUIREMENTS FOR RJLWH
 COMMON       0  VARIABLES   1914  PROGRAM    876

 END OF COMPILATION

// DUP

*STORE      WS  UA  RJLWH
CART ID 0740   DB ADDR  1BD9   DB CNT   003E

// XEQ RJLWH
```

Graph 12

Graph 12 is a representative Lineweaver-Burke plot of the slow isozyme with alpha-naphthyl-phosphate as substrate. 1/v is x 10^{7} moles min^{-1}/mg protein and 1/[S] is x 10^{4} M. The points represent experimental measurements.

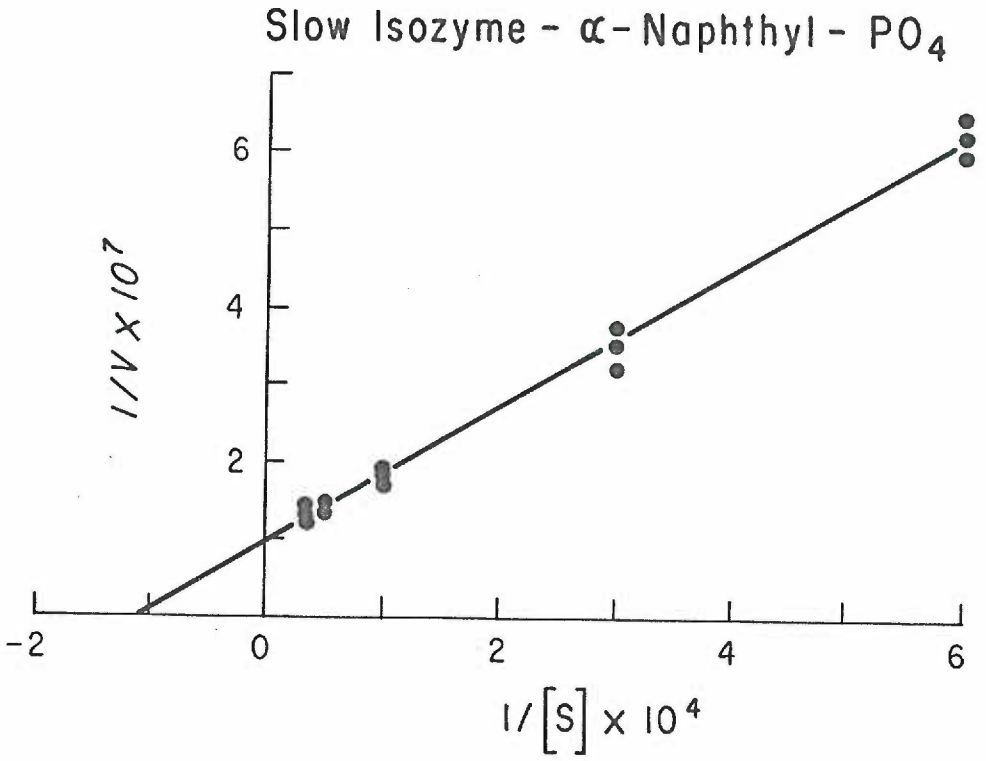
Graph 13

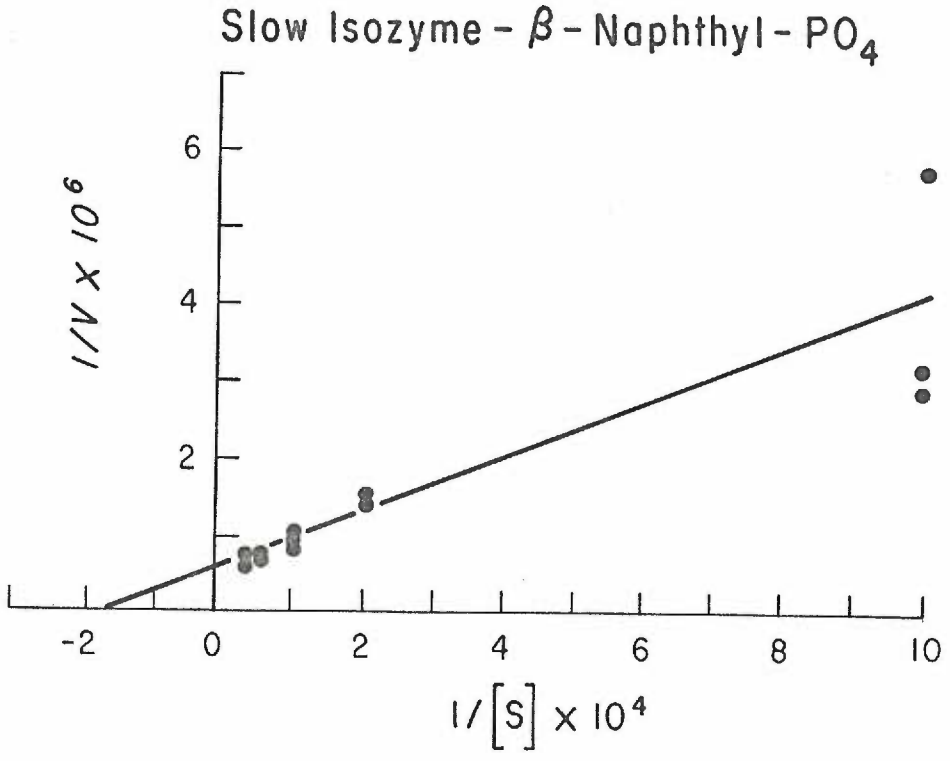
Graph 13 is a representative Lineweaver-Burke plot of the slow isozyme with beta-naphthyl-phosphate as substrate. 1/v is x 10^{6} moles min^{-1}/mg of protein and 1/[S] is x 10^{4} M. The points represent experimental measurements.

Experimental Details

Graphs 12-17 are representative of the data from which table 1 was constructed. The isozymes were separated and partially purified by elution from starch gels after electrophoresis as described in the methods section. The reaction mixture had a final volume of 4.5 ml and contained alpha- or beta-naphthyl-phosphate (diluted from 4.5 x 10^{-3} M stock solution), 0.1 to 0.3 ml of partially purified enzyme (depending on the activity of the eluent) and 0.05 M tris-HCl buffer (pH 10.1). Both the enzyme and the substrate solutions were prepared in the same buffer. In each case, the tris buffer contained 2 x 10^{-3} M $MgSO_4$.

The reaction velocity was measured fluorometrically, and the moles/min of substrate hydrolyzed was calculated by reference to the standard curve (graphs 1 & 2). Although the calculation of specific activity is unnecessary for Km determination, the concentration of protein in the partially purified enzyme eluent was estimated by its absorbance at 280 mμ.


Slow Isozyme - α - Naphthyl - PO4
1/V X 10^7
6
4
2
-2
0
2
4
6
1/[S] X 10^4

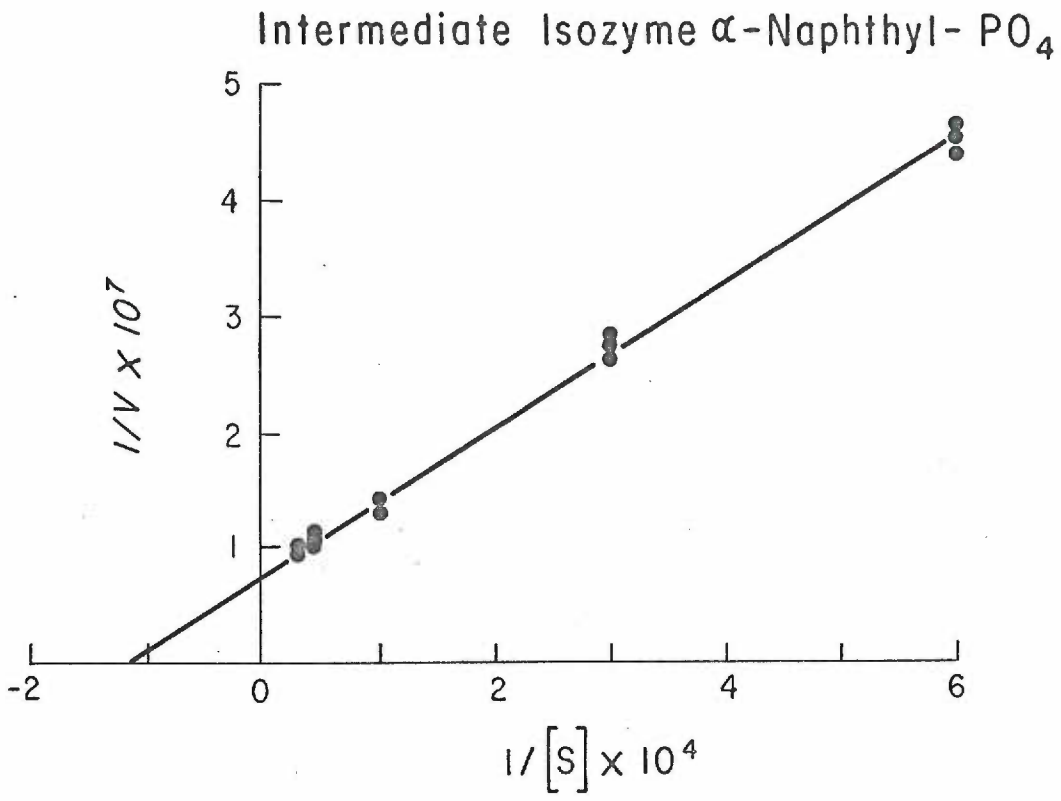

Slow Isozyme - β - Naphthyl - PO4
1/V X 10^6
6
4
2
-2
0
2
4
6
8
10
1/[S] X 10^4

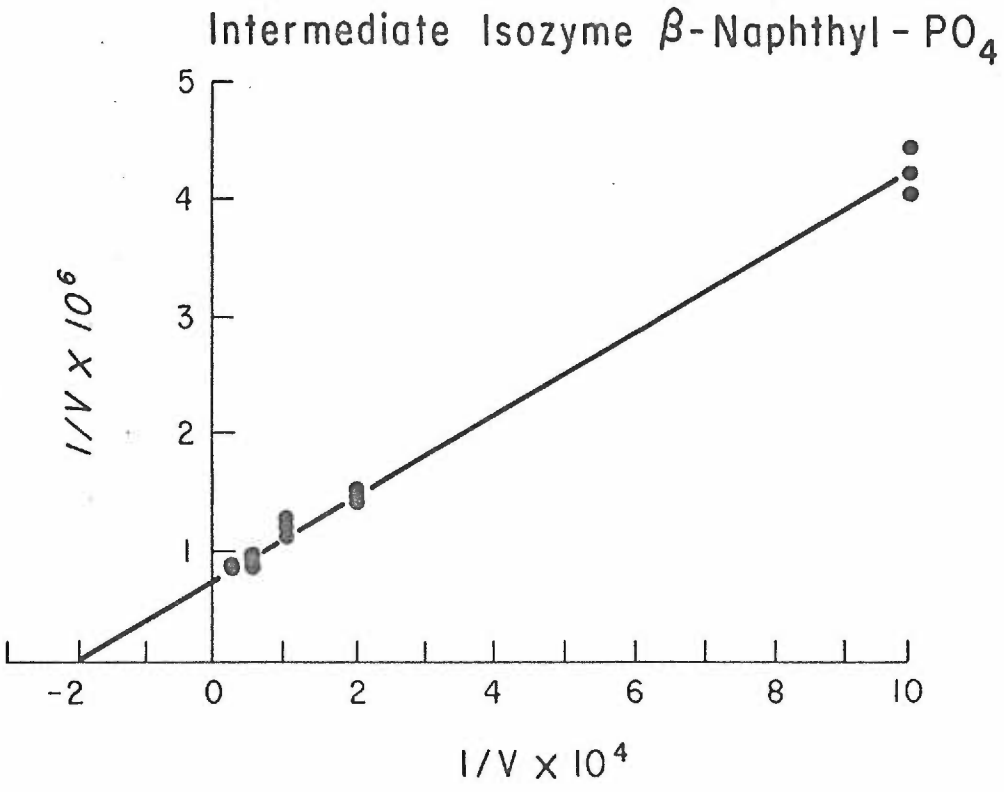
Graph 14

Graph 14 is a Lineweaver-Burke plot of the intermediate isozyme with alpha-naphthyl-phosphate as substrate. 1/v is x 10^7 moles min^{-1}/mg of protein and 1/[S] is x 10^4 M. The points represent experimental measurements.

Graph 15

Graph 15 is a Lineweaver-Burke plot of the intermediate isozyme with beta-naphthyl-phosphate as substrate. 1/v is x 10^6 moles min^{-1}/mg of protein and 1/[S] is x 10^4 M. The points represent experimental measurements.


Intermediate Isozyme α-Naphthyl- PO4
1/V X 10^7
5
4
3
2
1
-2
0
2
4
6
1/[S] X 10^4

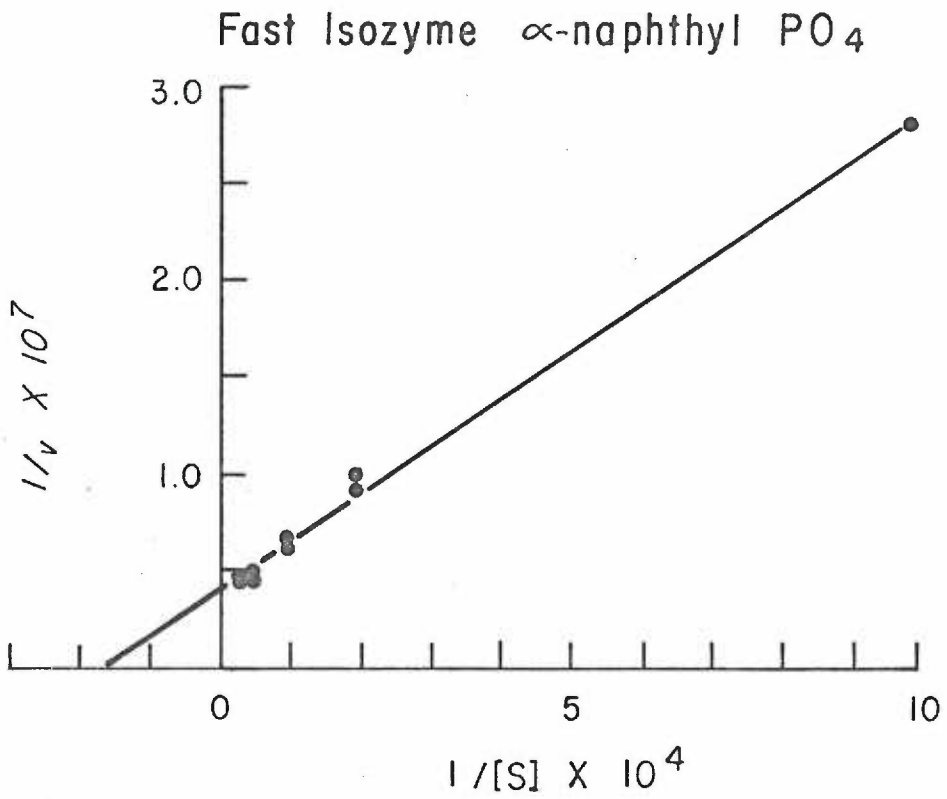

Intermediate Isozyme β-Naphthyl - PO4
1/V X 10^6
5
4
3
2
1
-2
0
2
4
6
8
10
1/V X 10^4

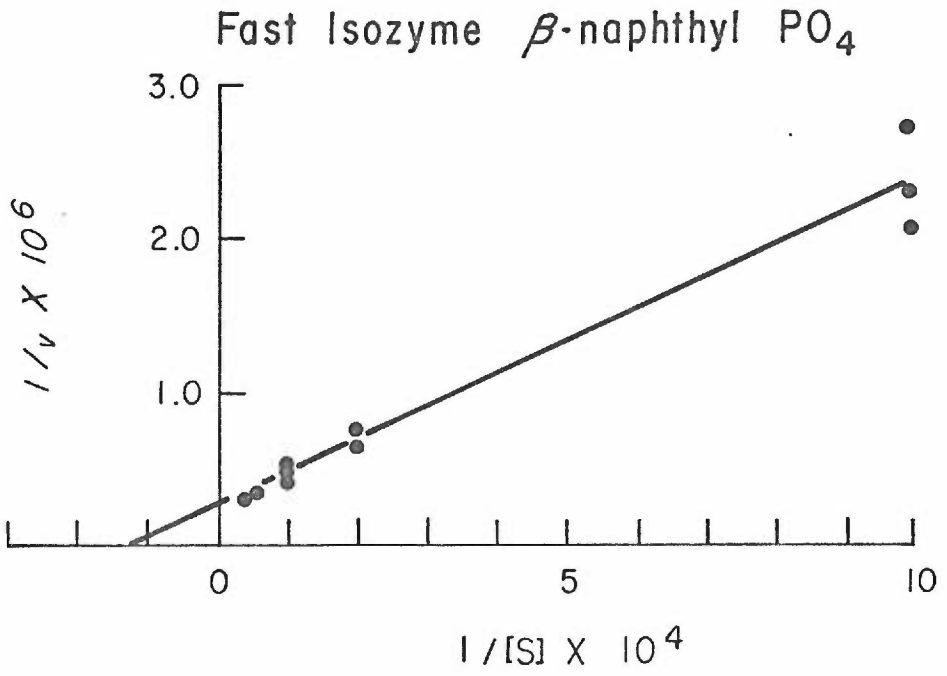
Graph 16

Graph 16 is a Lineweaver-Burke plot of the fast isozyme with alpha-naphthyl-phosphate as substrate. 1/v is x 10^7 moles min^{-1}/mg of protein and 1/[S] is x 10^4 M. The points represent experimental measurements.

Graph 17

Graph 17 is a Lineweaver-Burke plot of the fast isozyme with beta-naphthyl-phosphate as substrate. 1/v is x 10^6 moles min^{-1}/mg of protein and 1/[S] is x 10^4 M. The points represent experimental measurements.


Fast Isozyme ∝-naphthyl PO4
3.0
2.0
1.0
1/v X 10^7
0
5
10
1/[S] X 10^4


Fast Isozyme β-naphthyl PO4
3.0
2.0
1.0
1/v X 10^6
0
5
10
1/[S] X 10^4