THE UNIVERSITY OF CALGARY

Isolation and characterization of a gut-specific acid phosphatase in the nematode **Caenorhabditis elegans.**

by

Christopher T. Beh

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ABSTRACT

A marker of cell differentiation in the nematode Caenorhabditis elegans was characterized. This marker, an acid phosphatase, is specifically expressed in the intestine. However, not all cells of the gut express the acid phosphatase; the six anterior-most cells of the gut are devoid of acid phosphatase activity. This pattern of acid phosphatase expression correlates with specific cellular divisions as defined by the known cell lineage.

The gut acid phosphatase is first expressed in embryogenesis when the developing gut has 16 cells. Other species of acid phosphatases are detectable in earlier developmental stages but these comprise a minute amount of the total acid phosphatase activity. During larval development, the growth rate is reflected in the level of gut acid phosphatase activity per worm.

The gut acid phosphatase enzyme was purified to apparent homogeneity and characterized. This protein is a 110 kd, homodimeric glycoprotein that, in the absence of lipid, requires detergent for stability. The purification of the acid phosphatase was complicated by the fact that this protein forms high molecular weight complexes and is easily oxidized. Acid phosphatase activity was inhibited by L-(+)-tartrate and NaF, a characteristic common to most general, high molecular weight acid phosphatases.

An attempt was made at cloning the gut acid phosphatase gene **pho-1**, via an oligonucleotide probe. The

sequence of the probe would have been based on the amino-terminal sequence of the acid phosphatase protein. However, the protein proved to be N-terminally blocked and therefore could not be sequenced.

Nonetheless, the characterization of both the expression of the gut acid phosphatase and the enzyme biochemistry will serve further genetic and molecular studies. This marker of gut differentiation is an excellent system in which to probe C. elegans development and will be useful in comparisons with other gut markers.

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CHAPTER ONE: INTRODUCTION

In the embryo, the expression of some genes is restricted to one specific tissue. In order to determine how such differential gene expression is specified, we have studied the nematode Caenorhabditis elegans, a simple and developmentally well-defined organism. In particular, I have studied the development of the gut in C. elegans. C. elegans is a very simple tissue and therefore is an excellent system in which to examine the molecular basis of development.

I. Caenorhabditis elegans

C. elegans is uniquely suited to the study of gene control during development. The developmental fate of every cell in C. elegans, from embryo to adult, has been fully documented (Sulston and Horvitz, 1977; Deppe et al., 1978; Kimble and Hirsh, 1979; Sulston et al., 1983). The development of this nematode follows a rigidly fixed program or "cell lineage". Despite the overall simplicity of C. elegans development, organogenesis is still complex because many cell lineages can give rise to a single tissue. The cell lineage that defines gut maturation, however, is particularly simple since all gut cells arise from one embryonic progenitor cell. To investigate the molecular basis of gut development, I have studied the expression and biochemistry of a gut-specific marker. The

identification of genetic elements conserved between this gut marker and other gut-expressed genes might provide an incisive approach towards understanding the spatial and temporal control of gene expression.

1. Description

Caenorhabditis elegans is a small (1 mm in length) free-living nematode that is readily amenable to genetic and molecular manipulation. Although C. elegans as many of the major tissue types of higher eukaryotes, it is composed of fewer than 1,000 somatic cells. C. elegans is translucent, so its simple cellular anatomy can be directly viewed through a light microscope. The life cycle of C. elegans lasts only 3.5 days (at 20°C); its development, from embryo through to the mature adult, can be observed in its entirety.

(a) Development

In early embryogenesis, cell divisions and cell movements proceed according to an exact schedule. At about 7 hours after fertilization, cell proliferation slows and the body elongates. About 14 hours after fertilization, the worm hatches from its egg and enters its first larval stage. After an additional three larval stages, L2, L3, and L4, lasting a total of 50 hours, the worm enters into adulthood.

(b) Genetics

Mendelian genetics has proven to be a powerful tool in the analysis of C. elegans development (Brenner, 1974). Since C. elegans is a self-fertilizing hermaphrodite (both sperm and egg are produced by a single individual), recessive mutants are easily isolated. Males can be occasionally found (0.1%) and can be used for crosses with hermaphrodites, providing genetic manipulation.

The haploid genome of C. elegans is small (1×10^8 base pairs), only 20 times the genome size of **E. coli** (Sulston and Brenner, 1974). The small genome size and the well defined genetic map has made feasible the construction of a physical map of the C. elegans genome (Coulson et al., 1986). The physical map will ultimately consist of overlapping cosmids covering all of the six linkage groups. This facilitates the cloning of genes identifiable only by genetic phenotype; the cosmid that corresponds to a particular genetic locus can be located and tested for its ability to rescue the mutant phenotype. To date, 65% of the genome has been mapped in contiguous cosmids (A.Coulson, J.Sulston, Y.Kohara, D.Albertson, R.Fishpool*[1]).

II. Lineage specific gene expression in C. elegans

The development of the C. elegans embryo starts with a set of unequal cell divisions that gives rise to six "founder cells": the AB, C, D, E, MS and P_4 cells. In order to study the process of determination in founder

cells, several markers have been characterized. In particular, differentiation markers of the muscle lineage (comprising AB, MS, C, and D lineages), the cuticle (AB and C lineages), the germ line (the P_4 cell lineage) and the gut lineage (the E cell lineage) have been studied. These markers provide a convenient assay system in which to analyze factors required for lineage determination.

1. Muscle lineage markers

The muscle in C. elegans is derived from the daughter cells of nearly all founder-cells (see above). However, some muscle subtypes are expressed only in lineages of specific founder-cells (Sulston et al., 1983). For example, all progeny of the D cell will form body muscle. Pharyngeal muscle is only composed of cells from the AB and MS cell lineages. Thus, some muscle specific markers are also appropriate markers of lineage specific gene expression. However, if muscle specific genes are coordinately controlled, sequence comparisons have failed to identify the mode by which this is accomplished. As of yet, no conserved sequence elements between any of the muscles genes has been reported.

(a) Actin

Three of the C. elegans actin genes (**act-1**, **act-2**, **act-3**) are muscle specific and clustered on linkage group V, while the fourth gene (**act-4**) is a ubiquitous cytoplasmic actin located on the X chromosome (Files et

al., 1983; Waterston et al., 1984; Landel et al., 1984). Although all mutations in the muscle specific actin genes reduce motility and affect myofilament organization, some of the mutations effect specific muscle groups as well (Waterston et al., 1984); mutation in **act-2** results in abnormalities of pharyngeal musculature while mutation in **act-1** and **act-2** results in major dysfunction of body wall musculature (Waterston et al., 1984; Landel et al., 1984). The coding sequences of all the C. elegans actins are similar to one another; however the putative regulatory sequences upstream from these genes are dissimilar. Even **act-1** and **act-3**, which share identical coding regions, radically diverge upstream of position -28 (Files et al., 1983). The muscle specific actin genes may be regulated on an individual basis or they may be coordinately regulated through mechanisms involving the entire chromosomal domains. No common mode of regulation is apparent.

(b) Myosin

As a major component of body-wall and pharyngeal muscle, myosin is an excellent marker of muscle differentiation. There are four genes that encode myosin heavy chain protein: **unc-54** and **myo-3** encode the major and minor body-wall myosins respectively; **myo-1** and **myo-2** encode pharyngeal myosins. Sequence analysis of the 5' flanking sequences of all the members of the C. elegans myosin heavy chain gene family has identified no common

regulatory element (Dibb et al., 1989). Moreover, these genes have no TATA or other known transcriptional regulatory element. Several myosin plasmid constructs have been introduced into worms by injection and tested for the ability to complement myosin mutations (A.Fire and S.Harrison*[1]). Deletion analysis of these constructs has identified several tissue specific enhancers in **unc-54**, **myo-2** and **myo-3**. However, none of these enhancers are conserved among the various myosin genes.

(c) Paramyosin

Paramyosin is another abundant component of C. elegans muscle (reviewed by Waterston, 1988). In body-wall muscle, paramyosin is present in equimolar ratios with myosin (Waterston et al., 1974). Unlike myosin, however, paramyosin is the product of only one gene, **unc-15**. Within its coding region, **unc-15** shows extensive homology with the myosin heavy chain genes but upstream sequences share no similarity (Kagawa et al., 1989). As in the myosin genes, paramyosin has no apparent TATA or other regulatory sequence upstream of the gene start site (Kagawa et al., 1989).

2. Hypodermal lineage markers

The hypodermis is comprised of cells exclusively of the AB and C cell lineages (Sulston et al., 1983). The hypodermis secretes a cuticle that covers the body and forms pharynx and rectum lining (White, 1988). Prior to

each postembryonic molt, new cuticle is synthesized and old cuticle is shed. The expression of cuticle proteins, such as collagen, reflects the developmental regulation of cuticle synthesis during each molt (Kramer et al., 1985). Therefore, collagen is an excellent marker of hypodermal differentiation.

C. elegans collagens are encoded by a family of 40 to 150 related genes (Cox et al., 1984). These genes fall into three groups, each having distinct developmental patterns of expression (Cox and Hirsh, 1985). Sequence comparisons between the collagen genes have identified specific 5'-flanking sequences that may control their developmental expression (Cox et al., 1989). Two upstream pyrimidine-rich sequences are conserved between **col-2** and **col-6.** Both these genes are expressed only in worms molting into dauer stage larvae. Another upstream region is conserved between **col-7** and **col-19.** Both of these genes are expressed in worms molting into adults. The fact that certain sequences are coincident with distinct developmental stages suggests that these regions may regulate the temporal expression of these genes (Cox et al., 1989). Most of the collagen genes yet analyzed also share at least one of two 5' homologous flanking sequences (Cox et al., 1989). These sequences may designate tissue or lineage specificity.

3. Germ cell lineage markers

The P_4 cell gives rise to the germ cell line

exclusively (Sulston et al., 1983). Unlike the other tissues yet discussed, the germ line is homogeneous in its lineage ancestry. Markers of germ line differentiation are markers only of the P_4 cell lineage. By fluorescent antibody staining, cytoplasmic granules unique to germ line cells have been identified (Strome and Wood, 1982). These elements, P granules, have been exploited as convenient markers of the P cell lineage.

P granules are observed in all germ line cells except mature sperm (Strome and Wood, 1982). During embryogenesis, these elements are actively segregated to the germ line precursors (Strome and Wood, 1983). P granules appear not to be produced by the cells of the P_4 lineage, but rather appear to be maternally derived and transported to the cells of the P lineage. P granules may be excellent markers of factor segregation into the P lineage but perhaps not appropriate markers of zygotic gene activation within that lineage.

P granules are similar to other elements called nuage or germ plasm found in other animals (Strome and Wood, 1982). In Drosophila, these elements appear to be responsible for germ line cell determination (Illmensee and Mahowald, 1974). The true function of the P granules, however, remains unknown. Monoclonal antibodies directed against a 40 kd protein have been produced that also recognize P granules (Strome and Wood, 1983), but neither the protein nor its gene have been isolated.

4. The intestinal cell lineage

All cells of the gut arise from one founder cell, the E cell; the E cell generates no other tissue (Sulston et al., 1983). The E cell lineage is unusually simple; five, nearly synchronous, rounds of cell division generate the roughly 20 cells (not rigidly fixed) observed in the embryo (Sulston et al., 1983). Fifteen minutes after L1 lethargus, all but the six anterior-most nuclei divide producing the 30-34 gut nuclei normally in the adult (Sulston and Horvitz, 1977). Thus, there are only five rounds of cell division (six nuclear divisions) during which gut specific markers can be expressed. For these reasons, the gut provides the best tissue system in C. elegans for examining lineage specific gene expression.

(a) Vitellogenins

Vitellogenins, or yolk proteins, are synthesized exclusively by all the cells of the hermaphrodite gut (Wood et al., 1985). These proteins are then secreted and taken up by the gonad to be incorporated into oocytes (Kimble and Sharrock, 1983). Vitellogenin mRNA is highly enriched in intestine but is not detectable in RNA isolated from gonads (Blumenthal et al., 1984). The synthesis of yolk protein is not dependent on the gonad since hermaphrodites whose gonads have been ablated still produce vitellogenins (Kimble and Sharrock, 1983). However, yolk protein synthesis may still be positionally influenced rather than independently controlled by the

cells of the E lineage (Wood et al., 1985).

The vitellogenins are encoded by a family of six genes, **vit-1** through to **vit-6** respectively (Blumenthal et al., 1984; Spieth and Blumenthal, 1985). In order to identify putative regulatory elements, the 5′ flanking regions of five of the six **vit** genes have been sequenced (Spieth et al., 1985). Small repeats of the sequence CTGATAA were found upstream of all members of the vitellogenin gene family. It has been proposed that these sequences are responsible for the activation of **vit** genes in the gut (Spieth et al., 1985). Worms transformed with a truncated **vit-2** plasmid construct, containing only 247 bp of 5′ flanking DNA, show proper developmental expression (Spieth et al., 1988). This conserved sequence is contained within the 247 bp of flanking sequence.

In addition to the stage and tissue specificity of yolk protein synthesis, yolk proteins are also synthesized in a sex specific manner. Vitellogenins are only produced in the intestines of hermaphrodites, not in males (Kimble and Sharrock, 1983). The **vit** genes may be poor markers of early gut differentiation since their regulation is contingent upon temporal and spatial factors, as well as sex determination factors.

(b) Gut granules

When exposed to ultraviolet light, tryptophan catabolites, collected in the C. elegans intestine, exhibit a blue autofluorescence. These "gut granules" are

the product of at least four genes, **flu-1** to **flu-4** (reviewed by Siddiqui and von Ehrenstein, 1980). **flu-1** and **flu-2** encode kynurenine hydroxylase and kynureninase, respectively. Both are enzymes involved in the catabolic pathway of tryptophan. During C. elegans embryogenesis, the onset of gut granule expression coincides with the first detectable synthesis of nuclear poly(A) RNA (Edgar and McGhee, 1988). Gut granules (expressed at the 2 E cell stage) appear to be among the earliest markers expressed in C. elegans development (Edgar and McGhee, 1988).

In genetic mosaics induced by X-irradiation, **flu-3** alleles segregate in accord with the known E cell lineage (Siddiqui and Babu, 1980). This suggests gut granule expression is E cell autonomous. The developmental regulation of gut granule expression may be quite simple. Since gut granules are expressed early in development, the potential number of cell divisions during which gut transcription factors can act on the **flu** genes is limited. As of yet, however, the **flu** genes have not been cloned nor have their enzyme products been isolated.

(c) The gut esterase

Digestive hydrolases have proven to be excellent markers of the gut cell lineage. A gut esterase has been described that is completely localized to both the embryonic and adult intestine (Edgar and McGhee, 1986). E-lineage cells express the gut esterase even in the

absence of the other embryonic cells. Even in embryos where cytokinesis has been arrested by cytochalasin D, only gut lineage cells express esterase activity (Edgar and McGhee, 1986). Clearly, esterase expression is E-lineage specific.

The gut esterase is first detectable by histochemical staining at the 4 E cell stage (Edgar and McGhee, 1986). If embryos are incubated with the RNA polymerase II inhibitor ∝-amanitin, prior to the 4 E cell stage, esterase activity is abolished. Transcriptional activation of the esterase gene appears to be nearly concomitant with the first detection of esterase activity staining during the 4 E cell stage.

The DNA polymerase inhibitor aphidicolin has been used to investigate the dependence of esterase expression on DNA synthesis (Edgar and McGhee, 1988). Inhibition of the round of DNA synthesis immediately preceding esterase transcription does not prevent marker expression. However, if DNA synthesis is inhibited just prior to the appearance of the E cell, esterase expression is abolished. The clonal establishment of the E cell may signify a round of DNA replication in which gut lineage specific genes are given "permission" for later expression (Edgar and McGhee, 1988).

The esterase is the product of a single gene, **ges-1**, mapped to the left end of chromosome V (McGhee and Cottrell, 1986). Based on the N-terminal amino acid sequence of purified gut esterase (McGhee, 1987), an

oligonucleotide probe was constructed and used to clone the esterase gene (Kennedy and McGhee, manuscript in preparation). Sequence analysis of gut esterase 5′ flanking regions in C. elegans and in C. briggsae (a related, sister species), has identified a conserved 17 bp sequence. The functional significance of this sequence has been analyzed by transient transformation of **ges-1** null mutants with esterase plasmid constructs (Aamodt, Chung and McGhee, manuscript in preparation). The exogenous esterase is expressed in the gut and some expression in seen in the pharynx (unlike the endogenous esterase, which is only gut localized). Deletions extending into the conserved 17mer abolish all esterase expression in the gut but strong expression in the pharynx remains. When most of the 5′ flanking region is removed, pharyngeal expression fades and random expression of esterase in all tissues is observed (Aamodt, Chung and McGhee, op. cit.). As shown by band-shift assay (see Fried and Crothers, 1981; Garner and Revzin, 1981), the conserved 17mer is also bound by a (presumably regulatory) protein (Kennedy and McGhee, manuscript in preparation).

I have partially characterized a second gut localized hydrolase, an acid phosphatase. My objective is to compare the molecular and developmental biology of two genes expressed in the same cell lineage. As a beginning step towards this goal, I describe the developmental expression of this acid phosphatase and its biochemistry.

III. Acid phosphatases

1. General properties

Acid phosphatases (ACPase; EC 3.1.3.2) are a large group of enzymes that share the property of hydrolyzing phosphomonoesters under acidic conditions (Hollander, 1971). ACPases share a surprising lack of homology. However, ACPases can be grouped according to molecular weight, tissue and subcellular distribution, as well as substrate and inhibitor specificities.

Several C. elegans ACPase activities have been previously characterized (Bolanowski et al., 1983). Unfortunately, these activities were not studied in great depth. Some kinetic parameters of these ACPases were investigated (K_ms for the substrate 4-methylumbelliferyl phosphate, some inhibitors) but a ACPase pH activity curve demonstrating the acid optimum of the enzymes was not performed. It still remains to be shown whether the enzymatic activities Bolanowski et al. (1983) have described are indeed ACPase activities.

2. Range of substrates, inhibitors

One way to categorize ACPases is by their sensitivity to the inhibitors tartaric acid and fluoride (Hollander, 1971). Low molecular weight ($\leq$25 kd) ACPases are not inhibited either by tartrate or fluoride, which are potent inhibitors of the high molecular weight ($\geq$100 kd) form (Taga and van Etten, 1982). Another common characteristic of ACPases is their low substrate specificity (Hollander,

1971). Although low molecular weight ACPases only hydrolyze p-nitrophenyl phosphate and riboflavin phosphate efficiently, high molecular weight ACPase hydrolyze a large number of acyl, aryl, and alkyl phosphates as well as some phosphoproteins (Hollander, 1971; Taga and van Etten, 1982; Camici et al., 1989; Pavlovic et al., 1985).

3. Function/Structure

Physical characteristics of the ACPases vary widely. However, many species of ACPase are glycosylated (eg. Feigen et al., 1980; Waheed et al., 1988) and most high molecular weight forms are homodimeric (eg. Feigen et al., 1980; Barbaric et al., 1984; Gieselmann et al., 1984). Glycosylation is consistent with the subcellular localization of many ACPases. In vertebrates, glycosylated soluble and membrane-bound forms of ACPase are found in lysosomes (eg. Waheed et al., 1988; Gieselmann et al., 1984; Lemansky et al., 1985). Like other hydrolases in lysosomes, presumably these ACPases participate in general catabolism. Prostatic ACPase is synthesized under androgen control and secreted into seminal fluid. Despite its abundance in seminal fluid (1 mg per ml), the physiological importance of this ACPase is unknown (reviewed by Hollander, 1971). Invertebrate ACPases have diverse functions. Acid phosphatase-1, the major phosphomonoesterase in Drosophila melanogaster, seems to function in yolk digestion and cell movement during early embryogenesis (Sawicki and MacIntyre, 1978).

ACPases in prokaryotes and unicellular eukaryotes are not required during balanced growth but during periods of inorganic phosphate starvation, enzyme synthesis is stimulated (Dassa et al., 1982; Bostian et al., 1980). These ACPases are exocellular or periplasmic proteins that scavenge for phosphate in response to the cellular needs.

4. Regulatory circuits

The regulation of ACPases in response to developmental or nutritional cues has made these enzymes model systems for the analysis of gene expression. ACPases in two invertebrate systems, Drosophila melanogaster and Saccharomyces cerevisiae, have been notably well studied.

(a) Drosophila melanogaster acid phosphatase

The acid phosphatase-1 gene (ACPH-1) in Drosophila is an excellent system for understanding gene regulation in eukaryotes. The ACPH-1 gene is under hormonal control from ecdysone and juvenile hormone in the larval salivary gland and the ovary respectively (Postlethwait and Gray, 1976). In addition, ACPH-1 is genetically well characterized (MacIntyre, 1966; Bell and MacIntyre, 1973). The ACPH-1 gene product has been purified and characterized (Feigen et al., 1980). The developmental expression of the enzyme has also been well defined (Sawicki and MacIntyre, 1978). The enzyme is expressed both maternally and zygotically (Yasbin et al., 1978).

The enzyme exists in several isozymic forms at fertilization; all but one disappears during embryogenesis. In normal embryos, ACPH-1 enzyme activity is concentrated in the yolk, tracheal invaginations, and in cells bordering the lumen of the gut (Sawicki and MacIntyre, 1978). Since yolk proteins have a high phosphate content, they may be the natural substrate for the maternal ACPH-1 enzyme activity (Sawicki and MacIntyre, 1978).

(b) Saccharomyces cerevisiae constitutive and repressible acid phosphatases

The expression of acid phosphatases in S. cerevisiae is regulated by a complex dispersed gene control system (reviewed by Oshima, 1982; Toh-e et al., 1981). Inorganic phosphate, acting through a number of regulatory genes, controls transcription of the "phosphate-repressible" ACPase **PHO5** (Bostian et al., 1980). **PHO5** is only expressed when cells are phosphate starved. Although it is tightly linked to **PHO5**, the "constitutive" ACPase **PHO3**, is synthesized regardless of the phosphate demands of the cell (Andersen et al., 1983). At least five other genes are necessary for **PHO5** transcription: **PHO2**, **PHO4**, **PHO80**, **PHO81**, and **PHO85** (see Oshima, 1982).

The overall regulatory network controlling **PHO5** expression is similar to other metabolic regulatory circuits such as the galactose system (Oshima, 1982). This similarity may reflect a higher order of control by

which many metabolic genes can be coordinately regulated. **PHO2** has been shown to mediate basal level control of amino acid and purine biosynthesis in addition to controlling basal levels of **PHO5** expression (Arndt et al., 1987). The **PHO2** gene product binds to both the **HIS4** and **PHO5** gene promoters. Perhaps in higher eukaryotes, coordinate control of gene expression in specific tissues may represent an evolutionary expansion of the global system controlling the metabolic genes in yeast.

CHAPTER TWO: MATERIALS AND METHODS

I. Materials

Aldrich Chemical Company, Inc., Milwaukee, Wisconsin, U.S.A.

3-phenoxy-1,2-propanediol, tetramethylammonium chloride **(TMAC)**, ortho-vanadate

Amicon Division, W.R. Grace and Co., Danvers, MA., U.S.A.

centricon 30 microconcentrator, centriprep 30 concentrator

Bethesda Research Laboratories, Inc., Gaithersburg, MD., U.S.A.

ammonium sulfate (enzyme grade)

Bio-Rad Laboratories, Richmond, CA., U.S.A.

acrylamide, ammonium persulfate **(APS)**, Bio-Gel HTP (DNA grade) **(HAP)**, bromophenol blue, Coomassie brilliant blue R-250, dithiothreitol **(DTT)**, glycine, β-mercaptoethanol **(β-ME)**, N,N'-methylene-bis-acrylamide **(bis)**, molecular weight standards (SDS-PAGE high), riboflavin, sodium dodecyl sulfate **(SDS)**, N,N,N',N'-tetramethylethylenediamine **(TEMED)**

Boehringer Mannheim Biochemicals, Indianapolis, Indiana, U.S.A.

4',6-diamidino-2-phenylindol-dihydrochloride **(DAPI)**, tris(hydroxymethyl)-aminomethane crystallized **(Tris)**,

Calbiochem Corporation, La Jolla, CA., U.S.A.

(bis-(2-hydroxyethyl)-imino)-tris(hydroxymethyl)-methane **(bis-Tris)**, Genapol X-080 (protein grade),

Miracloth

Difco Laboratories, Detroit, Michigan, U.S.A.

Bacto-Agar, Bacto-Peptone, Bacto-Tryptone

Fisher Scientific Company, Fair Lawn, NJ, U.S.A.

calcium chloride dihydrate, deoxycholic acid (sodium salt) **(SDOC)**, dextrose, formaldehyde solution (37%(w/w)), hydrochloric acid, manganous chloride, methanol (HPLC grade), paraformaldehyde, silver nitrate, sodium acetate, sodium carbonate, sodium chloride, sodium fluoride, sodium hypochlorite (purified grade), sodium nitrite, sodium phosphate dibasic, sodium phosphate monobasic, sucrose, triethanolamine, water (HPLC grade)

Millipore Corporation, Bedford, Massachusetts, U.S.A.

Immobilon PVDF Transfer Membrane

Pharmacia LKB Biotechnology AB, Uppsala, Sweden

Con A Sepharose, Mono P HR 5/20, Mono Q HR 5/5, Pharmalyte 4-6.5, Pharmalyte 3-10, Polybuffer 74, Polybuffer 96, Sephadex G-150 Superfine

Pierce Chemical Company, Rockford, Illinois, U.S.A.

acetic acid (pHix buffer grade)

Sigma Chemical Company, St. Louis, MO., U.S.A.

1-cyclohexyl-3-(2-morpho-linoethyl) carbodiimide metho-p-toluenesulfonate **(CMEC)**, iminodiacetic acid **(IDA)**, 5-fluoro-2'-deoxyuridine **(FUdR)**, methyl ∝-D-mannopyranoside **(mannoside)**, 4-methylumbelliferyl phosphate, ∝-naphthyl phosphate (disodium salt), pararosaniline, sodium deoxycholate, sweet potato

acid phosphatase, L(+)-tartaric acid, thioglycolic acid (sodium salt), trichloroacetic acid **(TCA)**, Triton X-100

Spectrum Medical Industries, Inc., Los Angeles, CA., U.S.A.

Spectrapor membrane tubing (m.w. cutoff: 6-8 kd) United States Biochemical Corporation, Cleveland, Ohio, U.S.A.

(cyclohexylaminopropane)-sulfonic acid **(CAPS)**, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid **(HEPES)**, N-2-hydroxyethylpiperazine-N-2-propanesulfonic acid **(HEPPS)**, imidazole

II. Acid phosphatase histochemistry

1. Embryonic worm histochemistry

Embryos were permeabilized and fixed by the method of Edgar and McGhee (1986). However, all buffers containing phosphate were replaced with distilled water. Exchanging water for the phosphate buffers did not affect embryo morphology. Embryo permeabilization was kindly and skillfully performed by M.Chung to whom I am greatly indebted. Permeabilized embryos were incubated at room temperature with the staining solution described below. The best results were obtained when the concentration of α-naphthyl phosphate was increased to 15 mM. After incubation for either 15 min or 90-120 min, the enzymatic reaction was stopped by extensive rinses with M9 buffer (Wood, 1988). Specimens were observed through a Zeiss

Universal microscope with Nomarski differential-interference contrast optics. Kodak Ektachrome 400 was used for color photography.

2. Adult worm histochemistry

(a) Whole mounts

A mixed population of worms was washed by repeated centrifugation and resuspension in distilled water. Five microlitres of 37%(w/w) formaldehyde were added to each 45 microlitre aliquot of packed worms. Worms were viewed under a dissecting microscope and randomly cut along their width with a scalpel blade. 35 microlitres of "subbing solution" (0.1%(w/v) gelatin, 0.01%(w/v) chrome alum) was then added to the aliquot of cut worms. Worms were transferred onto a "subbed slide" (slides dipped in subbing solution and dried at 60°C in a vacuum oven for 10 min), covered with a coverslip, and dipped into liquid nitrogen. The coverslip was pried from the slide and the slide was quickly submerged in cold 4%(w/v) paraformaldehyde for 10 min. The slide was then rinsed in cold 50 mM HEPES pH 7.0 for 10 min and subsequently positioned onto a second slide bordered by a gasket containing a reservoir of staining solution. This permits the progress of the staining to be observed through the dissecting microscope. The ACPase staining was complete in approximately 5 min at which time the second gasket slide was removed. Worms on the subbed slide were again immersed in cold 50 mM HEPES pH 7.0, mounted with a drop

of 50%(v/v) glycerol and covered with a coverslip. Nuclei could be stained at the same time by incubating the slide for 1 hr in cold 8 microgram/ml DAPI, 0.5%(w/v) phenoxypropanol, 20 mM Tris·HCl pH 8.0 prior to mounting.

Specimens were observed through a Zeiss inverted microscope equipped with Nomarski differential-interference contrast, polarization, and epifluorescence optics. DAPI stained nuclei were observed using a Neofluor objective with Zeiss filter combination 487701.

(b) Frozen sections

Frozen sections were prepared by the method of Edgar and McGhee (Edgar and McGhee, 1986) and stained with the same staining solution described for the detection of ACPase activity in native gels. Fourteen micrometer thick sections were incubated with the staining solution for 5 min and subsequently rinsed with M9 buffer.

III. Gel electrophoresis

1. Isoelectric focusing

(a) Gel preparation

Isoelectric focusing gels were prepared by the method of McGhee and Cottrell (1986) with the following modifications: in the place of the Pharmalyte mixture cited in the original preparation, 0.6 ml Pharmalyte 3-10 in "wide range" gels and 0.6 ml Pharmalyte 4-6.5 in "narrow range" gels were used.

(b) Sample preparation

Pelleted worms (volume approximately 30-50 microlitres), fresh or frozen, were resuspended in 200 microlitres of 5 mM Tris·HCl pH 7.2, 0.1 mM EDTA. The worms were sonicated twice for 30 s at 50 W power using a Braun-Sonic 2000 sonicator with microprobe. Ten microlitres of 1 M calcium chloride and 20 microlitres of 25%(v/v) Triton X-100 (roughly 2.5%(v/v) in the final extract) were then added to the sonicate. Following a quick mixing, the mixture was incubated at room temperature for 30 min and then centrifuged for 10 min at 10,000 rpm. The supernatant was decanted free of the pellet and as much as 10 microlitres of extract (routinely 5 microlitre) was loaded onto each "lane" of the gel. In the case of the narrow range gels, the sample was loaded 2 cm from the leading edge of the positive pole "wick." On wide range gels, sample was loaded in the centre of the gel.

To test the ability of these isoelectric focusing gels to resolve ACPase isoelectric variants, samples were treated with the modifying reagent CMEC. Before loading onto isoelectric focusing gels, crude samples were incubated with an equal volume of 80 mM CMEC for 5 hr on ice. The induced charge-shift ACPase isoenzymes were best resolved on narrow range gels.

(c) Isoelectric focusing running conditions

Isoelectric focusing gels were run under the

conditions of McGhee and Cottrell (1986) with the exception of the running voltage and run time which were 2000 V (150 mA, 150 W, 2.0 °C) and 80 min respectively. The pH gradient formed across the gel was determined by excising 0.5 cm gel slices along the width of the gel, soaking the slices in 0.5 ml 50 mM KCl, and measuring their pH after 5 min of incubation. pH values were plotted versus the distance in cm of the gel slices on the gel. From this plot, the distance a protein migrated on a gel could be used to determine its pI.

2. Native polyacrylamide electrophoresis

The following preparation was based on the gel system described by Newby and Chrambach (Newby et al., 1978; Newby and Chrambach, 1979).

(a) Gel preparation

The best results were obtained with a gel system utilizing both "stacking" and "resolving" zones. The "stacking gel" was comprised of the following: 3%(w/v) acrylamide (29 acrylamide:1 bis(w/w)), 55 mM imidazole·HCl pH 7.5, 20%(v/v) glycerol, 0.5%(v/v) Genapol X-080, 0.25%(w/v) SDOC, 0.025%(w/v) APS, and 0.5 microlitre/ml TEMED. The "resolving gel" consisted of 5%(w/v) acrylamide (29 acrylamide:1 bis(w/w)), 55 mM imidazole·HCl pH 7.5, 20%(v/v) glycerol, 0.5%(v/v) Genapol X-080, 0.25%(w/v) SDOC, 0.05% APS, 1 microlitre/ml TEMED. Once polymerized, gels were pre-run overnight in 55 mM

imidazole·HCl pH 7.5, 0.25(w/v) SDOC, 1 mM thioglycolic acid at 50 V (unrestricted amperage) at room temperature. Running buffer consisted of 2.52 g HEPPS, 0.83 g imidazole, 2.5 g SDOC, 0.15 g thioglycolic acid in 1 litre distilled water total volume.

(b) Native gel running conditions

Prior to electrophoresis, all samples were either dialyzed against running buffer (two changes of 500 ml, overnight at 4 °C) or buffers were changed by gel filtration. Glycerol was added to all samples to give a final 20%(v/v) concentration. Bromophenol was added as a tracking dye. 0.75 mm X 16 cm X 18 cm native gels were prepared with one large preparative well into which sample was loaded. The gels were run at 250 V for 7-8 hours (at 10 °C). After electrophoresis, a strip from each of the two sides of the gel was cut away and subsequently divided into 1 cm lengths. Each of the 1 cm long pieces were dropped into 0.5 ml of ACPase activity stain. Those pieces, which stained red, indicated the relative position of the ACPase enzyme in the gel. After making the appropriate excision to recover the rest of the ACPase, the remainder of the gel was immersed in ACPase activity stain to ensure that the bulk of the enzyme activity had been recovered.

To elute enzyme activity from the gel into solution, 0.25 ml, per cm gel length, of 10 mM HEPPS, 12 mM imidazole, 0.25%(w/v) SDOC, 0.5%(v/v) Genapol X-080, 2 mM

DTT was added to the gel segment. The gel slice was incubated in the elution buffer overnight (at 4 °C) with gentle mixing. After the initial volume of buffer was collected, a second volume of elution buffer was added. The gel slice was gently mixed with the buffer for 12 hr in the cold before the solution was decanted off and combined with the initial elution fraction. The fraction of ACPase activity that could be recovered by this method varied widely between 25 and 65% of the original activity.

3. SDS polyacrylamide electrophoresis

SDS slab gel electrophoresis was performed according to the method of Laemmli (1970).

IV. Gel stains

1. Periodic acid-Schiff's (PAS) stain for glycoproteins

Schiff's reagent was prepared by the method of Segrest and Jackson (1972). Ten grams pararosaniline·HCl was dissolved into 2 l distilled water to which 200 ml of 1 N HCl and 17 g of sodium metabisulfite were added. This solution was stirred overnight at 4°C. Five grams of decolorizing carbon (charcoal) was then mixed into the solution and the mixture was filtered through Whatman No.1 filter paper. Carbon was again added and the mixture was filtered twice more. This solution was stored at 4°C in the dark.

Gels were stained following overnight fixation in 7.5%(v/v) glacial acetic acid, 15%(v/v) ethanol. After

two rinses with distilled water, gels were soaked in 0.7%(w/v) periodic acid for 2-3 hrs. Once the periodic acid solution was discarded, the gel was soaked in 0.2%(w/v) sodium metabisulfite. After 30 minutes this solution was discarded and the gel was soaked in fresh metabisulfite solution for an additional 2-3 hours. Finally, the metabisulfite solution was discarded and the gel was soaked in Schiff's reagent for 12-18 hours (overnight). The entire procedure was performed at room temperature.

2. Silver stain for proteins

Nanogram quantities of protein in SDS gels were visualized by the following "silver staining" protocol (T.Garber, personal communication). Prior to staining, gels were fixed in 7.5%(v/v) glacial acetic acid, 15%(v/v) ethanol for at least 2 hr. The fix was discarded and the gel was briefly rinsed with distilled water. The gel was then soaked in 500 ml of 2.5 micrograms/ml DTT for 30 min. After the DTT treatment, the solution was discarded and the gel was soaked in 500 ml of 1 mg/ml silver nitrate for 30 min. Excess silver nitrate was removed by briefly rinsing the gel twice with distilled water. The gel was then rinsed twice with "developer"; (3%(w/v) sodium carbonate, 0.018%(v/v) formaldehyde. Finally, 500 ml of fresh developer was added to visualize protein bands. The staining reaction was quenched by adding 7.5%(v/v) glacial acetic acid, 15%(v/v) ethanol.

3. Coomassie blue stain for proteins

Microgram quantities of protein were detected by staining SDS gels with 7.5%(v/v) glacial acetic acid, 15%(v/v) ethanol, 0.1%(w/v) Coomassie blue for at least 2 hr. Gels were destained with 7.5%(v/v) glacial acetic acid, 15%(v/v) ethanol.

4. Acid phosphatase activity stain

ACPase activity in native gels was detected by its hydrolysis of α-naphthyl phosphate in the following staining solution. To 5 ml 4%(w/v) pararosaniline, 2.4 M HCl, 5 ml freshly prepared 4%(w/v) sodium nitrite was added and the solution was gently mixed for 10 min. This "diazotized" pararosaniline solution was added to 81.75 ml 0.2 M sodium acetate/acetic acid buffer, pH 4.7. The pH was corrected with 8.25 ml 1 M NaOH. Finally, 100 mg alpha-naphthyl phosphate was added to the solution. Gels were immersed in this solution until bands were visible. Gels were routinely stained for 5-10 min (at 23°C) but could be stained for as long as a day.

V. Worm culture

1. Small scale growth

Media and growth conditions for small cultures were the same as those described by Brenner (1974). The original stock of wild-type C. elegans, strain Bristol (N2) as well as the Bergerac strain, were obtained from the Caenorhabditis Genetics Stock Center, Columbia, MO.,

U.S.A.

2. Axenic culture

Media and conditions for axenic cultures were those described by Vanfleteren (1978).

3. Large culture

The egg tray method (Yarbrough and Hecht, 1984; McGhee, 1987) was found to be the most reliable way of growing moderate amounts of worms (50 to 150g). All phosphate buffers were replaced with distilled water.

4. Isolation of specific stages

Embryos and most stages of larvae were isolated by standard methods (reviewed by Sulston and Hodgkin, 1988). Dauer larvae were obtained from starved, large scale, cultures. Worms isolated from exhausted cultures were treated with 1.0%(w/v) SDS for 15 min. A homogeneous population of dauers was obtained. Sucrose flotation (see Sulston and Hodgkin, 1988) separated viable dauer larvae from debris.

To obtain synchronous cultures of larvae, dauers were reintroduced onto egg trays laden with food. Once the dauer population had developed to healthy adults their embryos were harvested. These embryos were essentially synchronous and when allowed to grow, gave staged larval populations. Larvae representing each stage were isolated. Embryos and larvae were frozen and stored at -20°C.

5. Oocyte isolation

Oocytes were isolated by the method of Kennedy et al. (B.Kennedy, K.Ito, J.McGhee, *[2]). The **fer-1**(hc1) mutant was replaced with the **fer-1**(B232) mutant which gave a higher percentage of oocytes over embryos; **fer-1**(B232) proved to be less "leaky" mutation. Oocytes were frozen in liquid nitrogen and stored at -70 °C.

6. FUdR-blocked embryos

FUdR-blocked embryos were prepared by a method originally described by L.Edgar (personal communication). Small NGM (nematode growth media) plates containing 5 micrograms/ml FUdR and 40 micrograms/ml thymidine were seeded with bacteria and incubated at 37 °C for three days prior to the introduction of dauer larvae. The FUdR plates were seeded with 100 microlitres of packed dauers and incubated at 16 °C for 2-3 days. Once the population had matured, the FUdR-blocked embryos were harvested by first gently scraping the surface of the agar plate and then by treating them with alkaline hypochlorite as with normal embryos (see Sulston and Hodgkin, 1988).

VI. Acid phosphatase purification protocol

The steps in a typical purification protocol for the C. elegans ACPase were as follows:

(1) Crude Extract. Fifty grams of frozen worms were washed with 35 mM Tris·HCl at pH 8.0, centrifuged in a Beckman JA-10 rotor for 5 min at 2,500 rpm at room

temperature, and the pellet was resuspended in 5 volumes of 35 mM Tris·HCl at pH 8.0, 2 mM DTT. Aliquots of thirty five ml each in 50 ml conical centrifuge tubes were sonicated on ice, twice, for 45 seconds, each time using the large probe on a Braun-Sonic 2000 sonicator set at 280 Watts. The sonicate was decanted into 500 ml centrifuge bottles and centrifuged for 1 hr at 10,000 rpm in the Beckman JA-10 rotor chilled to 4°C. The supernatant was recovered and the pellet was discarded.

(2) Supernatant Clarification. Enough 1 M calcium chloride was added to the crude supernatant to give a final concentration of 50 mM calcium. The extract was incubated on ice for 30 min before centrifugation in the JA-10 rotor at 10,000 rpm for 1 hr at 4°C. The supernatant was filtered through Miracloth and then titrated to pH 4.3 by the dropwise addition of glacial acetic acid. The acidified solution was again centrifuged in the JA-10 rotor at 10,000 rpm for 1 hr at 4°C. The clarified supernatant was collected and the pellet was discarded.

(3) HAP Batch Purification. Dry HAP was added directly to the clarified extract (0.1 g HAP per ml supernatant). After 30 min of gentle mixing at room temperature, the HAP was pelleted by centrifugation in the JA-10 rotor at 2,000 rpm for 5 min at 4°C. The supernatant was discarded and the HAP pellet was gently resuspended in one volume equivalent (equivalent to the volume of the discarded supernatant) of 50 mM bis-Tris·HCl

pH 6.0, 50 mM ammonium sulphate, 1mM DTT. Without delay, the resuspended HAP was centrifuged at 2,000 rpm for 5 min at 4°C. After discarding the supernatant, the pellet of HAP was resuspended in one equivalent volume of 50 mM bis-Tris·HCl pH 6.0, 0.5%(v/v) Genapol X-080. This mixture was gently shaken for 30 min at room temperature and the HAP was pelleted again by centrifugation as before. The supernatant was decanted off and centrifuged again in the JA-10 rotor at 7,500 rpm for 5 min to remove any remaining HAP. Both manganese chloride and calcium chloride were added to the supernatant to give final concentrations of 1 mM of each.

(4) Con-A Sepharose Chromatography. The total supernatant volume from the HAP batch purification was loaded onto a Con-A Sepharose column (35 ml bed volume) at a flow rate of 1.0 ml per min. One column volume of 50 mM bis-Tris·HCl pH 6.0, 1 mM manganese chloride, 1 mM calcium chloride, 0.5%(v/v) Genapol X-080 was passed through the column following the sample loading. ACPase activity was eluted from the column by the stepwise addition of 50 mM bis-Tris·HCl pH 6.0, 1 mM manganese chloride, 1 mM calcium chloride, 0.5%(v/v) Genapol X-080, 50 mM methyl-mannoside. 2.0 ml fractions were collected. Activity rich fractions were pooled and dialyzed overnight at 4°C against two volumes of 500 ml 25 mM triethanolamine·IDA pH 8.3, 0.5%(v/v) Genapol X-080, 10 mM TMAC, 2 mM DTT.

(5) Chromatofocusing. After dialysis, the glycoprotein fraction was loaded onto a prepacked Mono P

HR 5/20 column pre-equilibrated with the same buffer used for dialysis. Following sample loading, 6 ml of 25 mM triethanolamine·iminodiacetic acid (IDA) pH 8.3, 0.5%(v/v) Genapol X-080, 10 mM TMAC, 1 mM DTT were passed through the column. In a stepwise manner, 47 ml of 3%(v/v) Polybuffer 96, 7%(v/v) Polybuffer 74, IDA, pH 5.0, 10 mM TMAC, 1 mM DTT was passed through the column. The column was run at 4°C with a flow rate of 0.5 ml/min; 1 ml fractions were collected. Activity rich fractions were pooled and an equal volume of 20 mM HEPPS, 25 mM imidazole, 0.5%(w/v) SDOC, 0.5%(v/v) Genapol X-080, 2 mM DTT (the final pH comes to 7.5) was added to the sample.

(6) Sephadex G-150 Chromatography. The ACPase sample was loaded directly onto a Centriprep 30 concentrator and centrifuged twice in a Sorvall RT6000 centrifuge in a H-1000 swinging bucket rotor at 1,500 rpm at 4°C until the total fraction volume had been reduced to roughly 1 ml (centrifugation time as according to the manufacturer, depending on original volume). The concentrated fraction was then loaded onto a 40 X 1.6 cm Sephadex G-150 Superfine column (void volume (Vo): 26 ml (determined using Blue Dextran-2000), total bed volume (Vt): 90 ml (determined using bromophenol blue)) pre-equilibrated with 10 mM HEPPS, 12 mM imidazole, 0.25%(w/v) SDOC, 0.5%(v/v) Genapol X-080, 2 mM DTT running buffer. The column was run at room temperature under one column height pressure (flow rate: 0.02 ml/min) and 0.5 ml fractions were collected. Activity rich fractions were pooled.

(7) Mono Q Chromatography. The Sephadex G-150 fraction was loaded directly onto a Centricon microconcentrator and centrifuged in a JA-20 rotor at 3,000 rpm. After a 30 min spin, the microconcentrator was refilled with sample and centrifuged for another 30 min. When the retentate had been reduced to 1 ml, the sample was centrifuged for a final 30 min at which time the concentrate was recovered (as described by the manufacturer). An equal volume of 100 mM Tris·HCl pH 8.3, 0.5%(v/v) Genapol, 1 mM DTT was added to the concentrate and the sample was loaded onto a Mono Q column. The column was run at 0.5 ml/min in the presence of 50 mM Tris·HCl pH 8.3, 0.5%(v/v) Genapol X-080, 1 mM DTT and bound protein was eluted by the stepwise addition of 1 M NaCl in running buffer. 0.5 ml fractions were collected and active fractions were pooled. The pooled ACPase sample was re-applied to the Mono Q column once it had been re-equilibrated with running buffer. The column ran under the previous conditions and activity fractions were pooled.

Protein amounts were determined by a dye-binding assay (Bradford, 1976) using bovine serum albumin as the standard. The protein concentration in the final purified fraction was determined relative to various amounts of BSA on a Coomassie blue stained SDS-gel.

VII. Acid phosphatase enzyme kinetics

ACPase activity was measured in 40 mM sodium

acetate/acetic acid buffer pH 4.7, 0.5%(v/v) Genapol X-080 containing 1.0 mM α-naphthyl phosphate. The assay gave a linear response during the period of time (5 to 10 min) most measurements were made (at high enzyme concentrations measurements were made only during the first 2 minutes of the assay). Hydrolysis of α-naphthyl phosphate was monitored by observing increases of absorbance at 322 nm. Assays were conducted at room temperature (23°C). A Beckman DU-9B Spectrophotometer was used for all assays. One unit of activity was defined as the amount of ACPase required to hydrolyzes 1 μmole of α-napthyl phosphate per min.

1 mM α-naphthyl phosphate was incubated in assay buffer to which 0.8 units sweet potato acid phosphatase (1 unit defined as the amount of enzyme required to hydrolyze 1 μmole p-nitrophenyl phosphate per min at pH 4.8 at 37°C; this enzyme also hydrolyzed α-naphthyl phosphate but at a reduced rate) was added. After 1 hr, when the absorbance had reached a plateau, the change in absorbance was read. In this manner, the change in the extinction coefficient upon complete hydrolysis of α-naphthyl phosphate was estimated to be 2390/(M·cm) (pH 4.7, 0.5%(v/v) Genapol X-080). This value was consistent with the value for the extinction coefficient of α-naphthol determined by others (McGhee, 1987).

VIII. Amino acid analysis and N-terminal sequencing

Amino acid analysis and N-terminal sequencing was

performed on protein immobilized on Immobilon P (see Matsudaira, 1987) by the Protein Microchemistry Centre, University of Victoria. Sequence analysis was performed using an Applied Biosystems 470A gas phase sequencer with on-line PTH analyzer. 200 pmol of ACPase yielded no N-terminal sequence, suggesting the protein was N-terminally blocked. The same immobilized protein sample was used to obtain the amino acid composition.

CHAPTER THREE: RESULTS

On the basis of its expression, the C. elegans ACPase can be considered a bonafide marker of gut specific gene expression. The C. elegans ACPase activity is clearly restricted to the gut in adult worms. Moreover, beginning late in embryogenesis, the expression of the ACPase in all stages of C. elegans development occurs exclusively in the gut.

The protein corresponding to the gut ACPase activity has been identified and purified to apparent homogeneity. The purified protein was characterized in order to: (1) delineate the biological function of this enzyme; (2) determine the number of structural genes required for its synthesis; and, (3) to define conditions for future genetic selection screens. Our main objective, however, was to obtain amino-terminal sequence from the purified protein and, based on its protein sequence, construct an oligonucleotide probe for gene cloning. The details of these studies are presented below.

I. Acid phosphatase expression

1. Spatial expression in the adult

Using basic methods of histochemistry (Lojda et al., 1979), samples of sonicated worms were assayed in vitro for the presence of the following potential gut hydrolases: sulfatases; alkaline and acid phosphatases; endopeptidases; and aminopeptidases. One hydrolytic

activity, an ACPase activity, was particularly intense. The ACPase activity shown in Figure 5a was determined to be specific to the C. elegans intestine by a number of criteria. First, the activity was present in axenically grown worms and in non-feeding embryos (eg., fig.2a). Therefore, ACPase activity was inherent to the worm and not due to consumed bacteria. Moreover, the ACPase has been mutated genetically and mapped to a worm chromosome (linkage group II; J.McGhee and D.Ferrari, unpublished results), further eliminating the possibility that the observed activity was of bacterial origin. In transverse and longitudinal sections of adult worms, ACPase activity was clearly restricted to the gut (not shown). In controls without substrate, background staining in non-gut tissues equalled that observed when substrate was included in the staining solution.

Close examination of the ACPase staining pattern (fig.5a, fig.6a) shows that the activity was expressed only along the edge of the gut lumen and not in the cell body. In some whole mounts, the staining was restricted to the edge of the gut lumen in the area of the microvilli and not throughout the lumen. In most specimens, however, staining was too intense to delimit the exact region of expression.

Figures 5a and 5b show an additional aspect of the ACPase expression; namely, the anterior region of the gut is devoid of ACPase activity. By staining with the nuclear dye DAPI, six cells of the gut were identified

Figure 1. C. elegans embryos stained 60-90 min for acid phosphatase activity. 1(a) Pregastrulation embryos and early postgastrulation embryos, 0-2.0 hrs after fertilization, stained for ACPase expression; red areas denote ACPase staining. Stain is ubiquitously expressed. Bar = 75 micrometres. **(b)** "Football stage" embryos, 4.0-5.5 hrs after fertilization. Staining is limited to the embryonic gut cells. Bar = 75 micrometres. **(c)** "Lima bean stage" embryo, 5.5-6.3 hrs after fertilization. Stain is restricted to the embryonic gut cells. Bar = 75 micrometres. **(d)** "Comma stage" embryo, 6.3-6.8 hrs after fertilization. Staining is still limited to the gut. Bar = 75 micrometres. All embryos shown were permeabilized by the capable hands of M.Chung.

Figure 2. C. elegans embryos stained 15 min for acid phosphatase activity. 2(a) "Early vermiform stage" embryo, 8.0-10.5 hrs after fertilization. ACPase expression is confined to the edge of the gut lumen. g = gut; ph = pharynx. Bar = 75 micrometres. **(b)** "Late vermiform stage" embryos, 10.5 hrs until just before hatching. Bar = 50 micrometres.

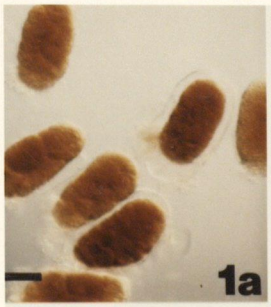
Figure 3. FUdR arrested embryo stained 15 min for phosphatase activity. Embryos were grown in the presence of FUdR thereby arresting their morphology at an early stage in embryogenesis. ACPase staining is localized to the presumptive gut cells. Bar = 75 micrometres.

Figure 4. Collection of C. elegans embryos stained 15 min for phosphatase activity. Embryos at various developmental stages were stained for ACPase activity. Only staining in vermiform stage embryos could be detected. pr = pregastrulation stage; pg = postgastrulation stage; lb = lima bean stage; v = vermiform stage. Bar = 100 micrometres.

Figure 5. Young adult worm stained for acid phosphatase activity. 5(a) Whole mount of a C. elegans adult hermaphrodite. ACPase staining is restricted to the edge of the lumen of the gut. No staining can be seen in the anterior region of the gut. g = gut; go = gonad; ag = anterior gut; ph = pharynx. Bar = 50 micrometres. **5(b)** The same worm was stained with the nuclear dye DAPI. Nuclei are seen as blue spots. The cells of the gut that do not stain for ACPase activity are clearly defined by their nuclei.

Figure 6. The int1 and int2 cells of the anterior region of the gut do not stain for acid phosphatase activity. 6(a) The anterior region of the gut is completely devoid of ACPase activity as are other non-gut tissues. **6(b)** The four nuclei of the int1 cells (one nucleus is not in the plane of the picture) as well as the two cells of the int2 cells can be readily identified by their lack of ACPase staining. int1 = the four int1 cells; int2 = the two int2

cells; g = gut; go = gonad; ph = pharynx. Bar = 150 micrometres.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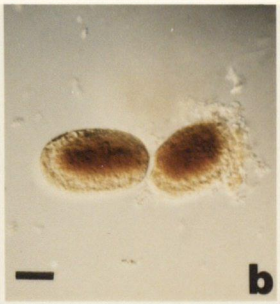
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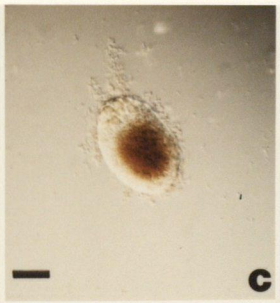
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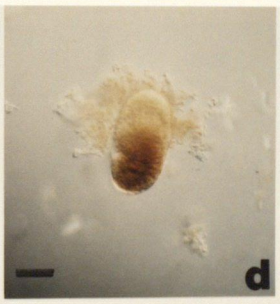
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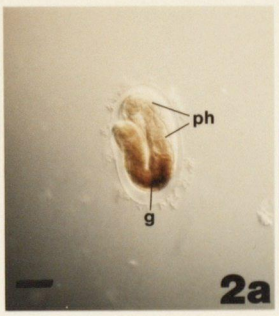
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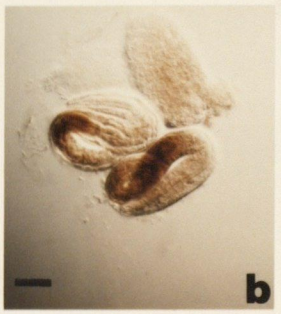
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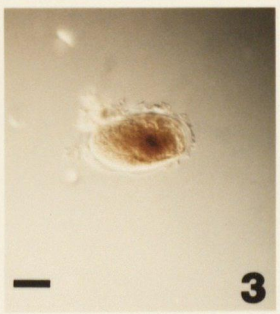
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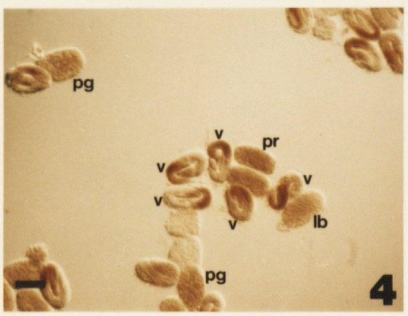
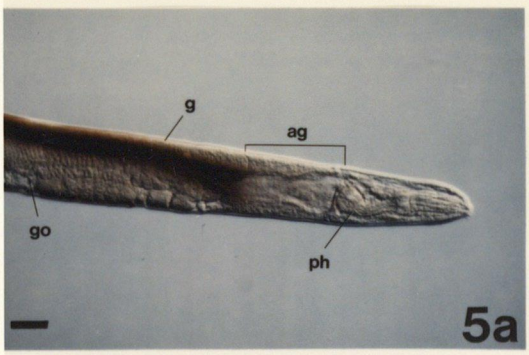
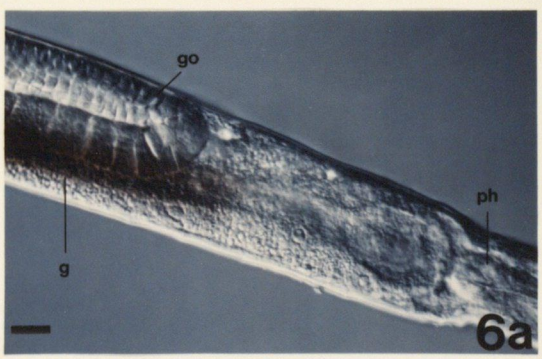
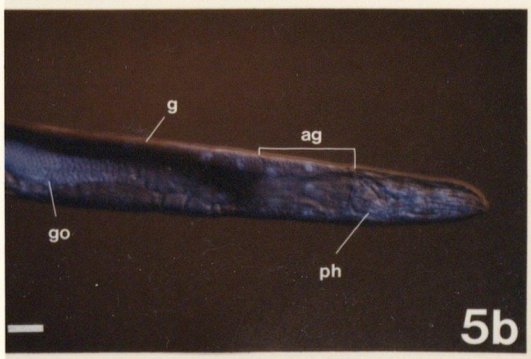
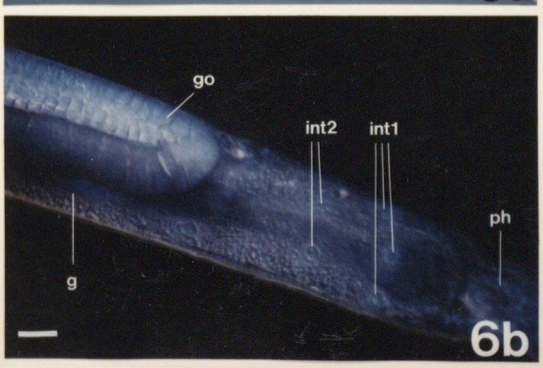

pg
v
pr
v
v
v
v
lb
pg
4


g
ag
go
ph
5a


go
ph
g
6a


g
ag
go
ph
5b


go
int2
int1
ph
g
6b

that did not stain for ACPase activity (fig.5b, fig.6b): the anterior ring of four cells, which comprise the int1 cells, and the two int2 cells that lie posterior to them. The int1 cells attach directly to the pharyngo-intestinal valve. Both C. elegans strains N2 and Bergerac displayed this same pattern of expression (not shown).

2. Temporal expression

Embryos at various stages of development could be divided into two groups; those that stained heavily for ACPase after 15 min (fig.1a,b,c and d), and those that stained for ACPase activity only after 60 to 90 min (fig.2a,b,3,4). Figure 4 shows a collection of embryos representing most stages of development after just 15 min of staining. After this length of time, only vermiform stage embryos displayed ACPase activity. The vermiform stage (approximately 8 hrs after fertilization) is morphologically distinct as it is the first stage in development in which an embryo exhibits vermiform, or worm-like, characteristics. Vermiform stage embryos stained almost instantly upon addition of the staining solution. The staining in vermiform embryos was clearly gut-specific and was restricted to the edge of the gut (fig.2a,b). As in adult worms, the anterior region of the gut in vermiform embryos lacked ACPase activity.

Figure 3 shows an embryo whose development has been blocked by the thymidine analogue FUdR. DNA replication arrests during early postgastrulation in embryos cultured

in the presence of FUdR (K.Millen and J.McGhee, manuscript in preparation). However, gene activity progresses normally and many embryonic gene products accumulate. In these embryos, ACPase activity was detectable after only 15 min of staining. ACPase activity was restricted to those cells that have been identified as presumptive gut cells (K.Millen and J.McGhee, personal communication).

Figures 1a,b,c,d show those embryos that, only after a full 60 to 90 min of staining, express ACPase activity. Pregastrulation and early postgastrulation embryos displayed ubiquitous but weak expression (Fig.1a). Slightly older embryos (fig.1b,c,d) exhibited a weak, localized staining in the developing intestine.

3. Developmental profile of acid phosphatase activities on isoelectric focusing gels

Isoelectric focusing gels stained for ACPase activity displayed (both in crude extracts and after purification) a single band at pI=4.7 (fig.7), which was designated pho-1 (not to be confused with the **pho-1** gene; ie., pho-1 will be referred to as the product of the **pho-1** gene). Pho-1 activity, in as little as 15 ng of crude extract, or the equivalent of one fifth of an adult worm, was detectable on this gel system (data not shown).

From the stained gel in figure 8, it is apparent that pho-1 activity per worm gradually increases during C. elegans development. This observation was confirmed by

Figure 7. Acid phosphatase activity in crude and in purified acid phosphatase preparations on isoelectric focusing gels. Equal quantities of enzyme activity (2 X 10^{-3} units), from crude and purified ACPase, were run on a "wide range" gel (see Materials and Methods). The major ACPase band (pho-1) in crude homogenates corresponds to that ACPase activity which has been purified to apparent homogeneity. The top end of the gel is the acidic end; the range of pH on the gel was determined to be pH 2.5 to pH 9.0.

Figure 7

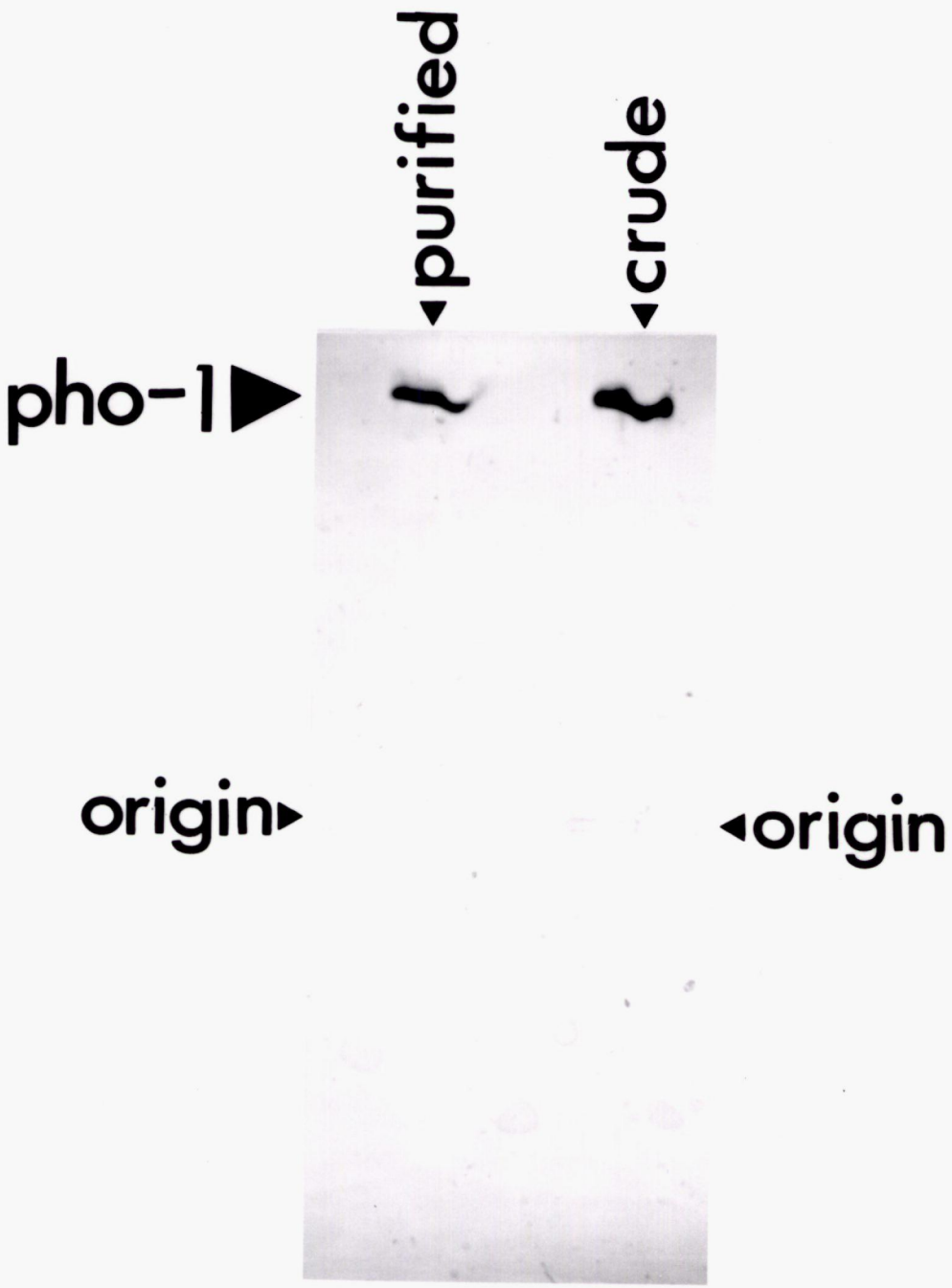
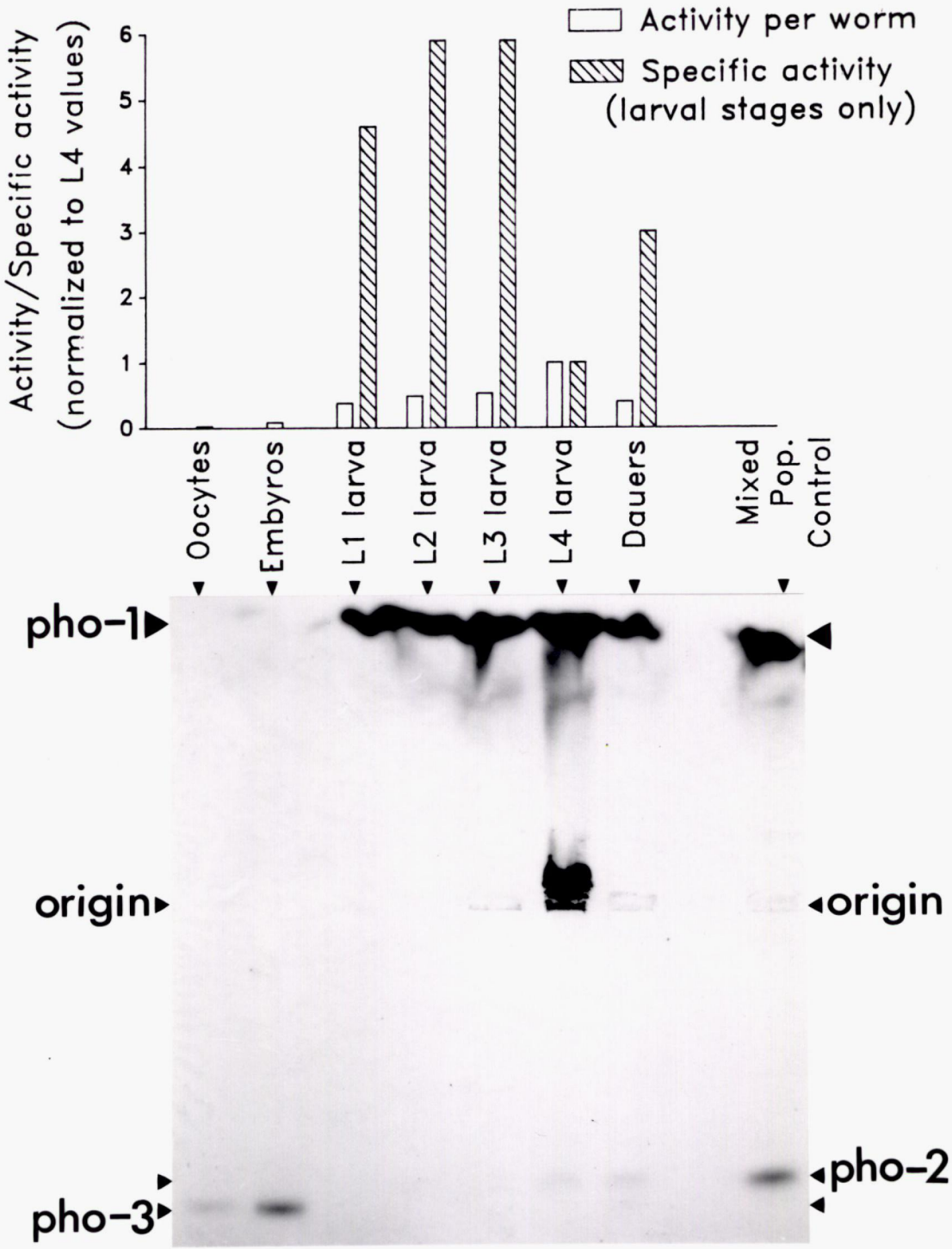


Figure 8. Developmental expression of C. elegans acid phosphatases as seen on an isoelectric focusing gel. Extracts were made from representative stages of C. elegans development: oocytes (pre-fertilization); embryos (0-11.25 hr after fertilization); L1 larvae (11.5-18.25 hr after fertilization); L2 larvae (18.5-25.75 hr after fertilization); L3 larvae (26-35.25 hr after fertilization); L4 larvae (35.5 hr after fertilization); and a mixed population of worms, mainly comprised of older worms (days after fertilization). Before the extracts were made, worms were titred such that the equivalent of 2,500 individual worms was loaded and run per lane (error in the titre ranged from 2,500±470 to ±930 for oocytes and the mixed population, respectively). The gel was left to stain overnight in ACPase stain and the gel was photographed through a green filter. Three distinct activity bands were present. The range of pH across the gel was from pH 2.5 to pH 9.0.

The graphical presentation of the ACPase activity in these extracts was determined by spectrophotometric assay. All values were normalized against L4 values. Specific activity and activity per worm were determined for the predominant species of ACPase, (pho-1).

Figure 8


Activity per worm
Specific activity
(larval stages only)
Activity/Specific activity
(normalized to L4 values)
0
1
2
3
4
5
6
Oocytes
Embyros
L1 larva
L2 larva
L3 larva
L4 larva
Dauers
Mixed Pop.
Control
pho-1
origin
pho-3
origin
pho-2

spectrophotometric assay (see fig.8); embryos were found to have the least pho-1 activity and L4 larvae the most. The specific activity, that is, the ACPase activity normalized to the total protein mass, remained relatively constant during larval growth (see fig.8). However, there was a drop in specific activity from the third larval stage to the fourth. Pho-1 activity per worm in dauer larvae was roughly equivalent to that in L2 and L3 larvae (40%, 49% and 53% of L4 activity respectively). The specific activities of ACPase in dauers and in L3s were comparable.

After prolonged staining, other weakly expressed phosphatases could be detected on this gel system as well. In all larval stages, these weak activities account for less than 5% of total activity (see fig.8; this is a qualitative estimate since, on these gels, enzyme activity is not linearly related to stain intensity). The weakly expressed phosphatases are not due to contaminating bacteria, since they do not correspond to any observed E. coli ACPase band (data not shown). As shown in figure 8, oocytes lack pho-1 activity, though a basic-pI ACPase (designated pho-3) is detectable. (The activity bands were designated according to pI; pho-1 was the most acidic, pho-3 the least). Oocyte pho-3 activity was weak compared to the total ACPase activity observed in other developmental stages (eg., only 3% of L4 levels). The first developmental stage in which pho-1 activity could be detected was in embryo extracts (fig.8). The

total ACPase activity in embryos, however, was split between pho-1 and pho-3 activities. Together these activities were still only 9% of the total activity expressed in L4 larvae. L1 larvae displayed a large increase (over 4 fold) in activity per worm over that in embryos. All detectable activity in L1 larvae came from pho-1 activity; pho-3 was not expressed (fig.8). Another basic-pI ACPase activity (designated pho-2), was detectable at low levels in L4s, dauers, and in the mixed population (which mainly consists of adult worms). Pho-2 was distinguishable from pho-3 since in dauer larvae both bands could be resolved (though both activities were on the fringe of detectability). Nonetheless, pho-2 and pho-3 could still be isozymes of the same protein.

4. Resolution of pho-1 charge-shift mutants on isoelectric focusing gels

The ability of the "narrow range" isoelectric focusing gel to detect isoelectric variants was shown, first by use of CMEC (see Materials and Methods) and then ultimately by the isolation of a ACPase charge-shift mutant (**pho-1**(ca101); J.McGhee and D.Ferrari, op. cit.). Figure 9 shows the characteristic band patterns associated with **pho-1**(ca101) homozygous mutant animals, **pho-1**(ca101) heterozygous animals, and wild type animals.

Note that heterozygous worms display a three-band pattern characteristic of a homodimer. The middle band in Figure 9 was weaker than would be expected from a single

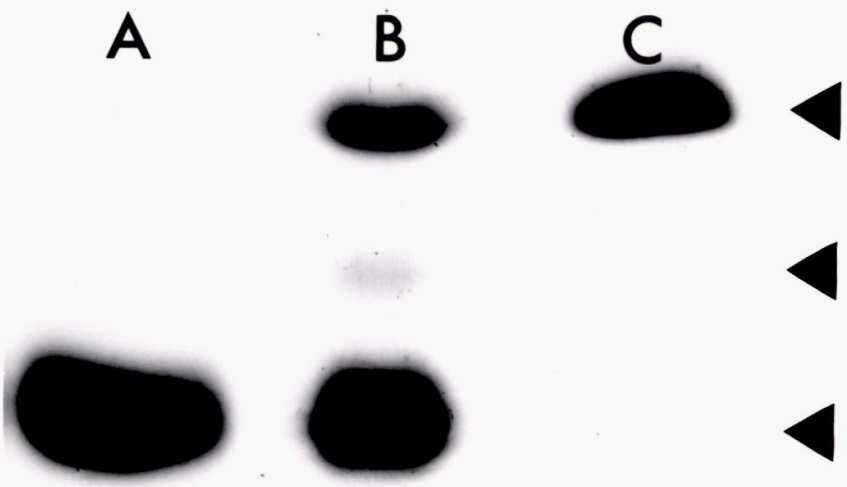


Figure 9. Phenotypes of the pho-1 charge-shift mutation as seen on an isoelectric focusing gel. Extracts were run on a "narrow range" I.E.F. gel and stained for acid phosphatase activity. The top of the figure marks the acidic end of the gel. **Lane A.** The acid phosphatase band pattern for animals homozygous for the charge-shift allele, **pho-1**(ca101). **Lane B.** The phosphatase band pattern for heterozygotes, **pho-1**$^{+}$/**pho-1**(ca101). **Lane C.** The wild type activity band.

hermaphrodite. However, the heterozygote extract was made from an actively proliferating population of hermaphrodites. As predicted by Mendelian genetics, in ensuing generations, progeny of a heterozygotous hermaphrodite will disproportionate towards homozygous genotypes.

II. Acid phosphatase purification

Because of its properties, the gut ACPase was difficult to purify. The gut ACPase required detergent to maintain activity in the absence of lipid (see below) and was sensitive to oxidation. In the absence of DTT, additional activity bands were detected on I.E.F. gels (presumably corresponding to more basic, oxidized forms of pho-1). Moreover, the detergent used (Genapol X-080) compounded the problem of oxidation as it readily auto-oxidizes in the light at room temperature (manufacturer).

The ACPase was also associated with large molecular weight aggregates at several points in the purification (see below). During the course of the purification the apparent molecular weight of the ACPase changed from over 50,000 kd in the crude extract, to 320 kd after Con-A Sepharose chromatography in the presence of non-ionic detergent, to a final 130 kd in the final steps of the purification. In the high molecular weight form, the ACPase could not be resolved from the other proteins in the aggregate. Both standard chromatography and affinity chromatography (with immobilized p-aminobenzyl phosphonic

acid or immobilized tartrate) were ineffective in fractionating this material. More details of unsuccessful trials to purify the ACPase will not be described; only the steps required to purify the ACPase are described below. Table 1 shows a purification table summarizing those steps. Chromatographic purification of the C. elegans ACPase is shown in Figure 10.

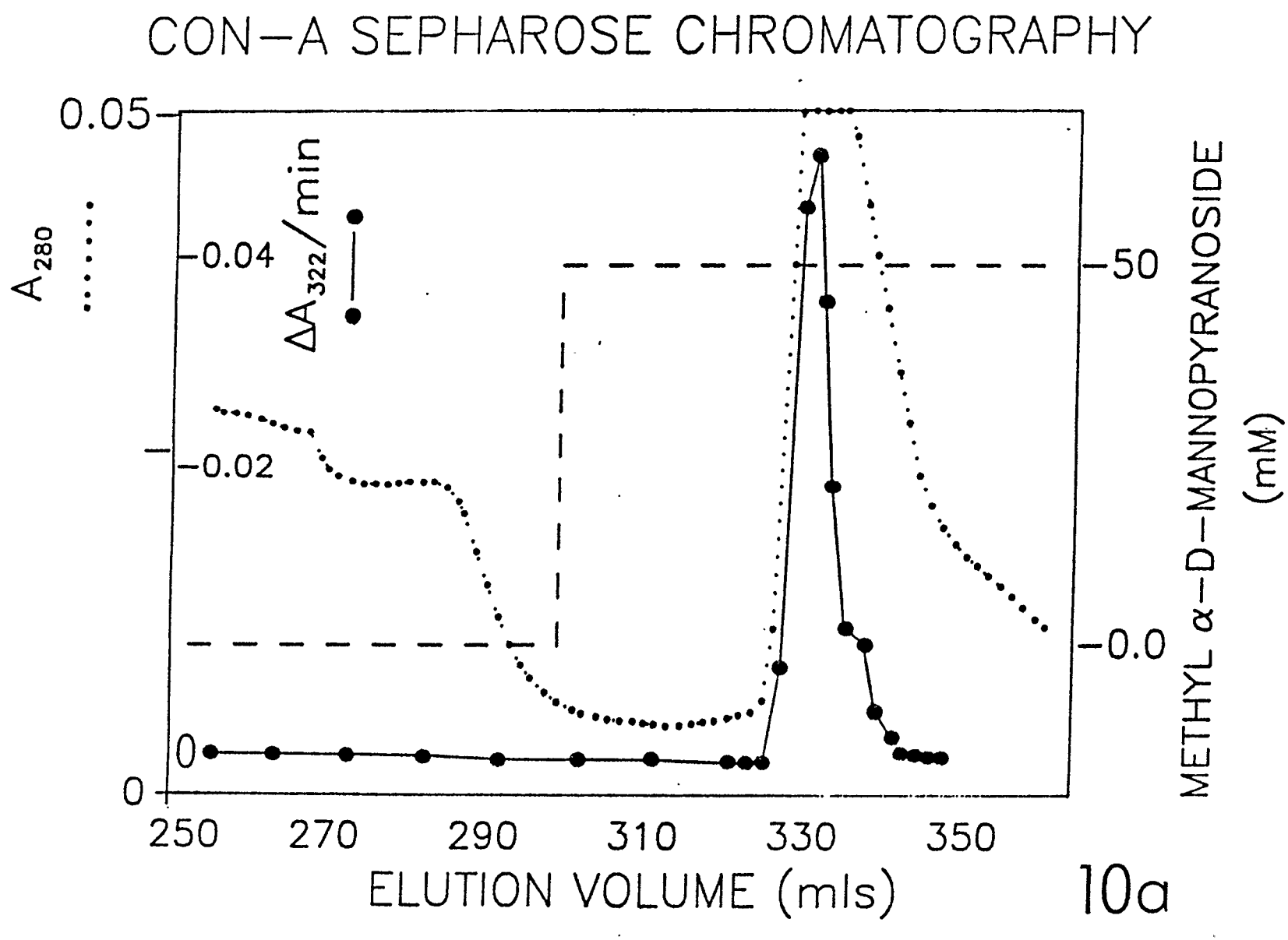
The purification began with tissue disruption by sonication. Disruption by Stansted cell disruptor proved to be less successful; the extract produced gave inconsistent results later in the purification. Recovery of soluble ACPase activity was enhanced by using a high pH sonication buffer (35 mM Tris HCl pH 8.0). Significant activity loss occurred in buffers at pH 8.5 or greater. Sonication of the sample was best performed 35 mls at a time in 50 ml conical tubes using a large probe at full power (280 Watts). The smaller volumes gave more efficient disruption. The crude extract obtained from sonication, and subsequent centrifugation, was a turbid emulsion containing most of the ACPase activity; no appreciable activity could be detected in the material pelleted by centrifugation. This extract was too lipid-rich for ammonium sulfate fractionation (a problem encountered by others, eg. Yarbrough and Hecht, 1984). The lipid also seemed to interfere with chromatographic purification of the ACPase in the crude extract. Attempts to remove the lipid, by ethanol precipitation, completely abolished all ACPase activity. Ethanol precipitation had

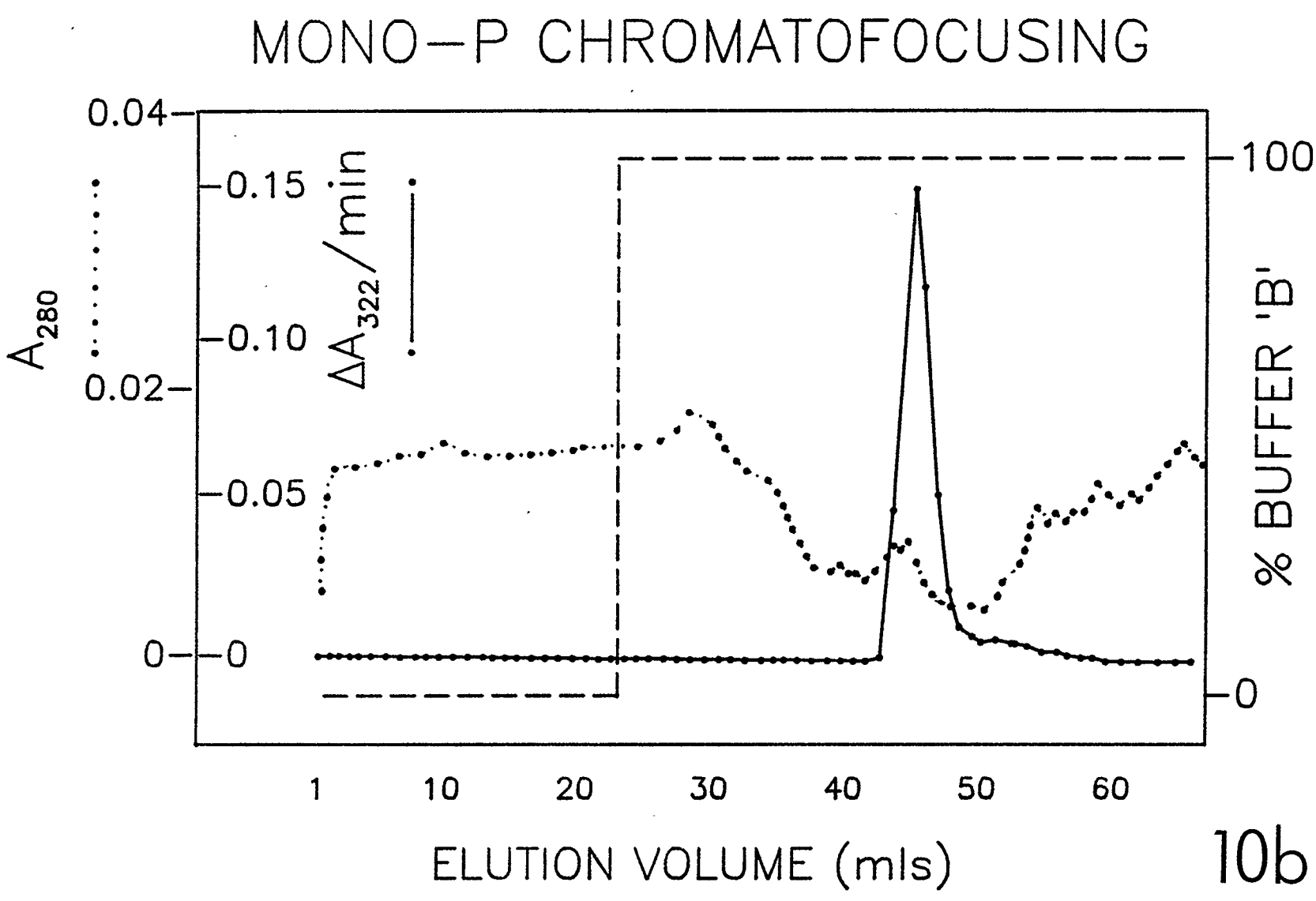
TABLE 1: SUMMARY OF TYPICAL PURIFICATION OF *C. elegans* ACID PHOSPHATASE

Fraction	Volume (ml)	Total Protein (mg)	Total Activity (units[1])	Specific Activity (u/mg)	Fold Purif.
Crude extract	250	1375	92.0	0.067	1
Clarified supernat.	250	31	58.6	1.9	28
HAP eluent	250	4.0	20.9	5.2	78
Con-A Sepharose	16	0.48	15.1	31.5	470
Mono-P chromato.	6	45 (μg)	16.7	371	5,540
Sephadex G-150	4	40 (μg)	6.2	155	2,313
Mono-Q (combined)	2	8 (μg)	3.1	388	**5,780**

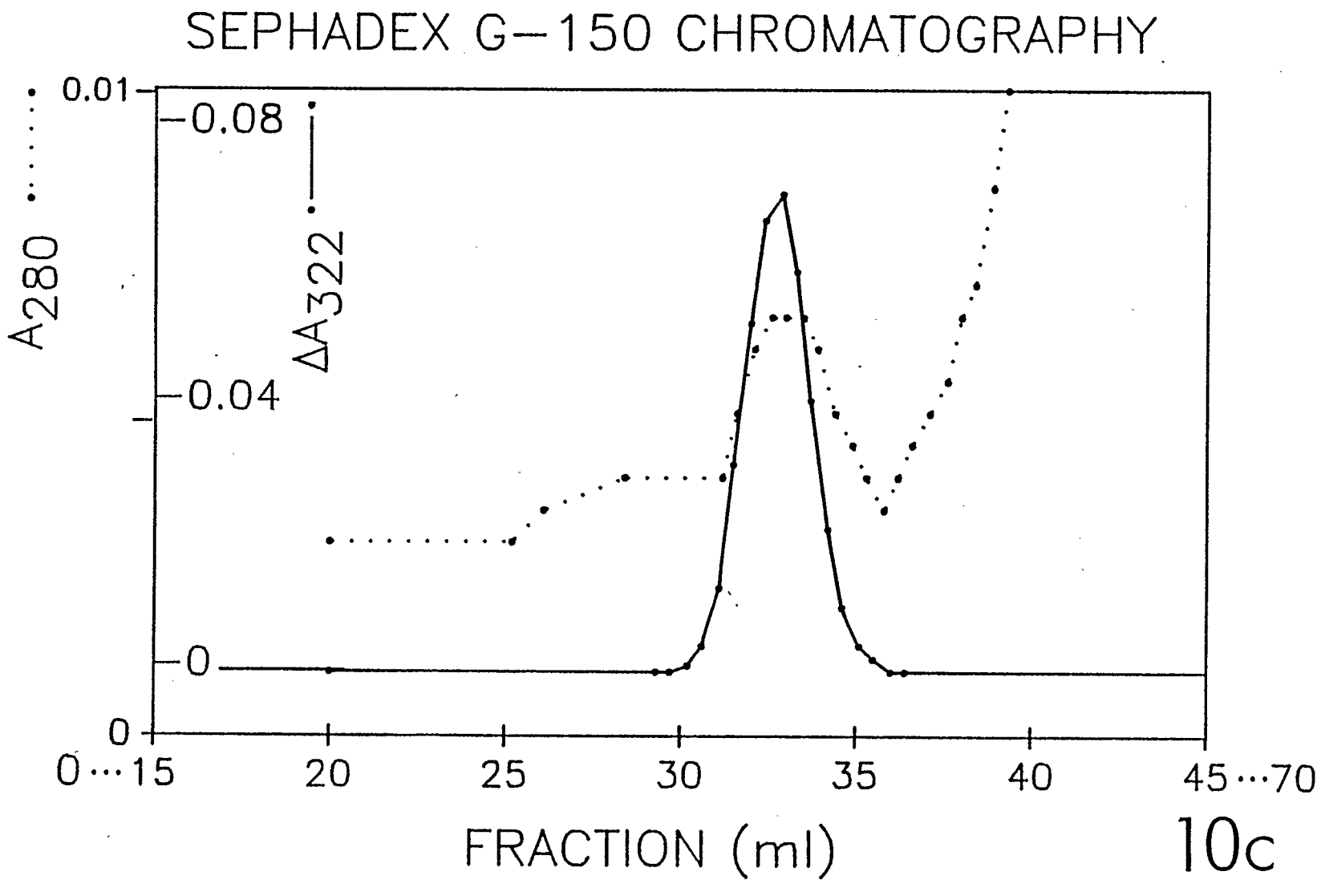
[1]Enzyme units defined as micromoles of α-naphthyl phosphate hydrolyzed per minute (see Materials and Methods). The complete purification has been duplicated and is generally representative of both trials; the results of numerous incomplete purification trials are also consistent with the above table.

Figure 10. Chromatographic purification of the C. elegans acid phosphatase. 10(a) Con-A Sepharose chromatography. The eluent from the HAP step was applied directly to a 1.6 X 20 cm Con-A Sepharose column at 4°C. The column was then washed with 20 ml 50 mM bis·Tris·HCl pH 6.0, 0.5% Genapol X-080, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and enzyme activity was eluted by the stepwise addition of 50 mM methyl mannoside in running buffer. Flow rate = 1.0 ml/min; fraction size = 2 ml. Enzyme activity was assayed using 10 microlitres of each fraction, with α-naphthyl phosphate as the substrate in 50 mM acetic acid/acetate pH 4.7, 0.5% Genapol X-080. Changes in absorbance at 322 nm indicate enzymatic hydrolysis of α-naphthyl phosphate. Protein concentration (A_{280}), was determined as indicated. **(b)** Chromatofocusing on the Mono-P column. After the sample from the Con-A column had been dialyzed against Mono-P running buffer (25 mM triethanolamine·IDA pH 8.3, 0.5% Genapol X-080, 2 mM DTT, 10 mM TMAC), the sample was applied to the Mono-P column and eluted upon addition of the Polybuffer (buffer "b"; see Materials and Methods). Flow rate = 0.5 ml/min; fraction size = 1.0 ml; 10 microlitres of each fraction was assayed for ACPase activity. **(c)** Sephadex G-150 chromatography. After the Mono-P eluent had been concentrated in the presence of sodium deoxycholate, the sample was loaded directly onto a pre-equilibrated 16 X 40 cm Sephadex G-150 column (for column specifications, ie. void volume etc., see Materials and Methods) at room temperature. Flow rate = 0.02 to 0.05 ml/min; fraction size = 0.5 ml; 10 microlitres of each fraction was assayed for activity. **(d)** Calibration curve for the Sephadex G-150 column determined through least squares regression. The protein standards used (in kilodaltons) are as follows: equine alcohol dehydrogenase (160); bovine serum albumin (BSA) dimer (134); ovalbumin dimer (90); BSA monomer (67); ovalbumin monomer (44.5); carbonic anhydrase (29); α-lactalbumin (14.2).

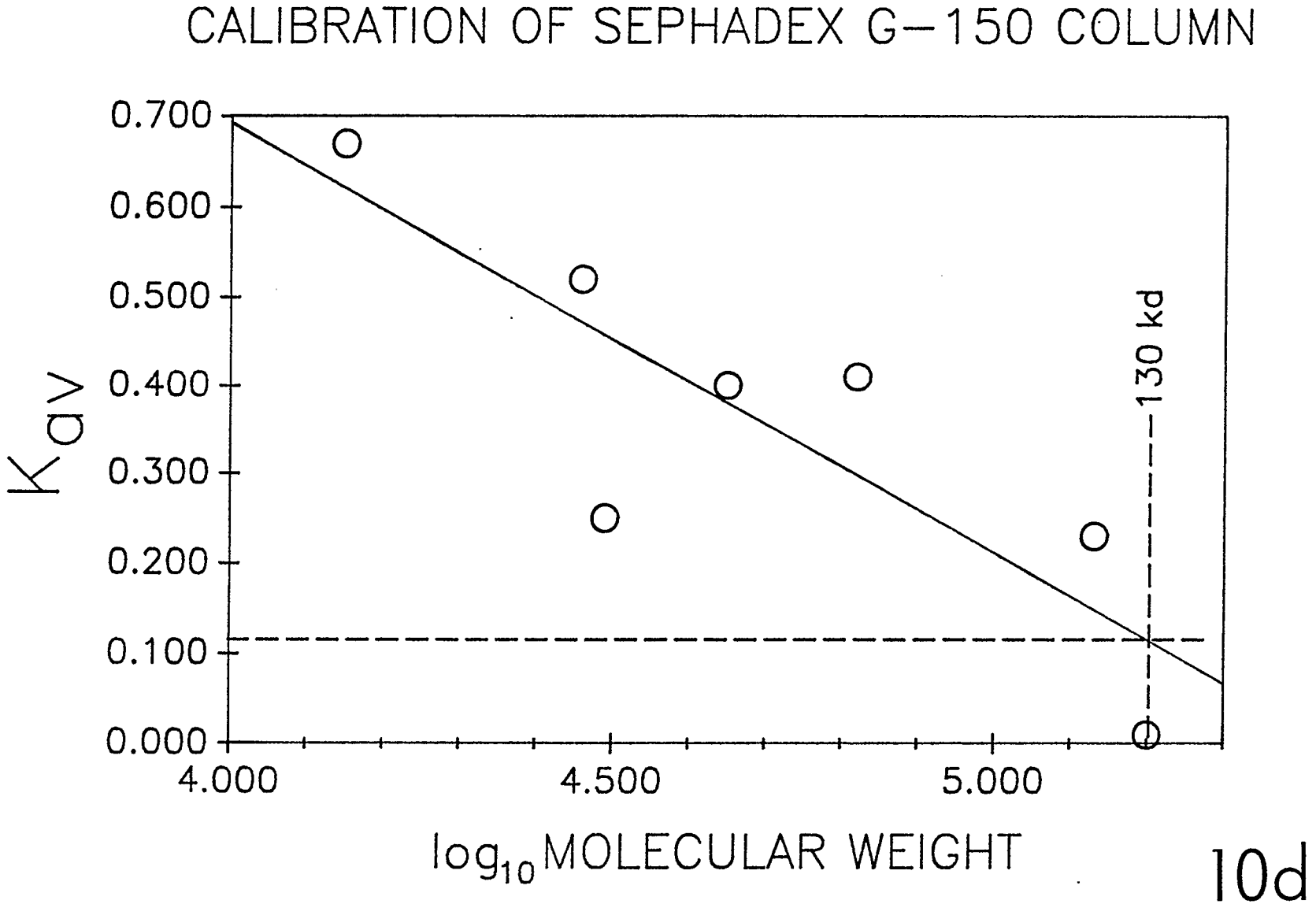

CON–A SEPHAROSE CHROMATOGRAPHY
0.05
0.04
0.02
0
A_{280}
ΔA_{322}/min
50
0.0
METHYL α–D–MANNOPYRANOSIDE
(mM)
250
270
290
310
330
350
ELUTION VOLUME (mls)
10a


MONO–P CHROMATOFOCUSING
0.04
0.02
0
A_{280}
0.15
0.10
0.05
0
ΔA_{322}/min
100
0
% BUFFER 'B'
1
10
20
30
40
50
60
ELUTION VOLUME (mls)

10b


SEPHADEX G-150 CHROMATOGRAPHY
0.01
0.08
0.04
0
A280
ΔA322
0
0…15
20
25
30
35
40
45…70
FRACTION (ml)

10c


CALIBRATION OF SEPHADEX G-150 COLUMN
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
K_{av}
130 kd
4.000
4.500
5.000
$\log_{10}$ MOLECULAR WEIGHT

10d

been successful in the purification of ACPase from yeast (Boer and Steyn-Parve, 1966).

To clarify the extract, a combination of two steps was employed. First, much of the turbidity of the solution was removed by the addition of calcium chloride to the extract. Second, acidification of the extract removed any remaining particulate matter and removed proteins more pH labile than the ACPase. The resulting clear extract could be left overnight at 4°C without any appreciable loss in ACPase activity. Little or no ACPase activity could be detected in either the pellet from the calcium chloride precipitation or from the acidification.

ACPase activity from the clarified extract eluted in the void volume of a Bio-gel A50m gel filtration column (data not shown). In other words, at this point in the purification, the ACPase seemed to be entraped in a large molecular weight aggregate (≥50,000 kd).

Hydroxyapatite (HAP) was found to be one of the few matrices that would bind the ACPase in the clarified extract. HAP binds so tightly to the ACPase that neither high salt (1.5 M NaCl) nor pH changes could dislodge active enzyme from it. However, non-ionic detergents (Triton X-100, Genapol X-080) could recover upwards of 50% of the bound ACPase activity. This was the basis of the HAP batch purification step. Dry HAP solid was added directly to the clarified extract, mixed and pelleted by centrifugation. The supernatant was decanted off and the HAP pellet was washed with a mild salt solution (50 mM

bis Tris, 50 mM ammonium sulfate, 1 mM DTT, pH 6.0). The added salt improved the net recovery of ACPase activity. The binding of the ACPase to HAP was independent of pH, so a slightly acidic buffer wash was chosen in order to maintain ACPase stability (50 mM bis Tris, 0.5%(v/v) Genapol X-080, pH 6.0). Despite its overall effectiveness, this step had to be completed quickly if great losses in enzyme activity were to be avoided. After the HAP batch purification, the concentration of protein in the sample was extremely low (8.0 to 17.5 micrograms/ml). It is likely that this adversely affected the stability of the ACPase.

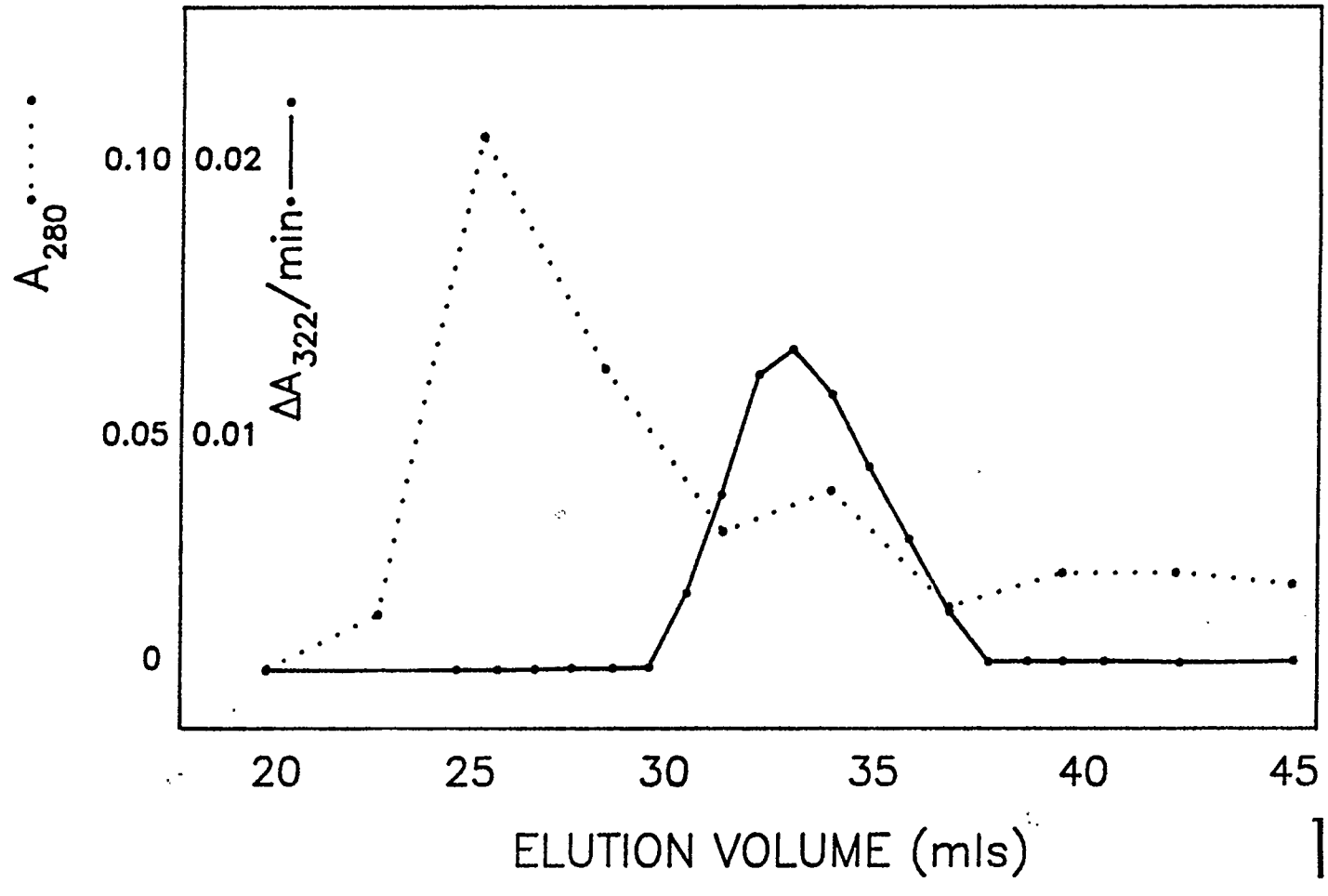
The next purification step, Con-A Sepharose chromatography, was useful as a concentrating step. ACPase activity was eluted from this column by a stepwise addition of 50 mM methyl mannopyranoside. The running buffer used for this column (50 mM bis Tris pH 6.0, 0.5%(v/v) Genapol X-080, 1 mM $CaCl_2$, 1 mM $MnCl_2$) was slightly acidic in order to maintain ACPase activity and optimize the Con-A lectin activity. The presence of trace amounts of calcium and manganese also optimize Con-A binding activity (manufacturer). Despite the importance of DTT in maintaining ACPase activity, DTT drastically reduced the capacity of Con-A Sepharose for glycoproteins. (Con-A activity may be sensitive to reducing agents since it is an active homodimer linked by disulfide bridging.) The addition of DTT was therefore omitted in all buffers used for Con-A Sepharose chromatography. It was present,

however, in all other buffers. Although the capacity of Con-A Sepharose is high (8.5 mg porcine thyroglobulin per ml gel; manufacturer), an inordinately large column (bed volume = 35 ml) was required to bind the roughly 1 mg of glycoproteins in the HAP eluted fraction. Since the applied volume was large, a large column was needed to insure adsorption of all glycoproteins in the sample. (Adsorption is less favored when the concentration of protein free in solution is far less the concentration of matrix bound protein (see Scopes, 1987)). Following this purification step, enzyme recovery was generally high (60.7% to 87.3% of the original activity) and the sample was concentrated, on average, 20 fold.

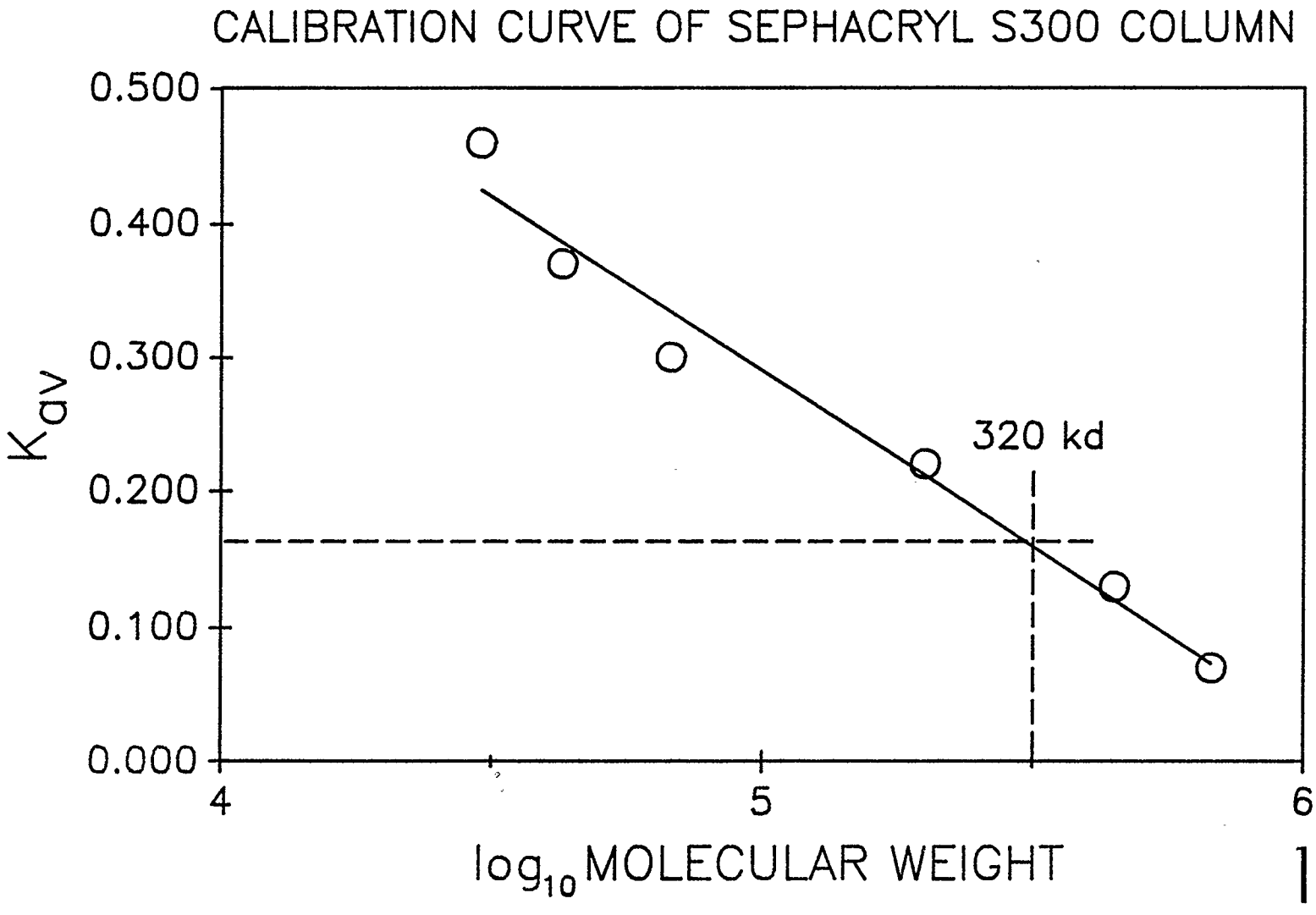
As determined on a Sephacryl S300 filtration column, the apparent molecular weight of the ACPase after the Con-A Sepharose purification step was approximately 320 kd (see fig.11). This molecular weight estimate is also consistent with the elution profile of ACPase activity on a Superose 6B filtration column. Non-ionic detergents seemed to have partially liberated the ACPase from the large molecular weight aggregate with which it was previously associated. The ACPase was completely soluble at this point in the purification. All ACPase activity remained in solution after the Con-A Sepharose fraction had been centrifuged at 100,000 X g for 1 hr. Note that in this form, the ACPase could be successfully purified by standard chromatographic techniques, which before this point it could not.

Figure 11. Sephacryl S300 column chromatography of the Con-A Sepharose purified acid phosphatase. 11(a) Sephacryl S300 Gel Filtration. One ml of Con-A Sepharose purified ACPase was applied to a 1.6 X 35 cm Sephacryl S300 column (void volume = 72 ml, as determined by the elution volume of blue dextran 2,000) that had been pre-equilibrated with the running buffer, 50 mM bis·Tris·HCl pH 6.0, 0.5% Genapol X-080, 0.2 mM DTT at 4 °C. Flow rate = 0.1 ml/min; recovery of enzyme activity = 95.7%. Protein concentration (A_{280}) and ACPase activity (A_{322}) are as indicated. **(b)** Calibration curve for the Sephacryl S300 column. The molecular weight of the protein standards used are (in kd) as follows: thyroglobulin (669); apoferritin (443); β-amylase (200); BSA (67); ovalbumin (43); carbonic anhydrase (30).

SEPHACRYL S300 GEL FILTRATION:
ELUTION PROFILE OF CON–A SEPHAROSE PURIFIED
ACID PHOSPHATASE



11a


CALIBRATION CURVE OF SEPHACRYL S300 COLUMN
0.500
0.400
0.300
0.200
0.100
0.000
K_{av}
320 kd
4
5
6
$\log_{10}$ MOLECULAR WEIGHT

11b

After an overnight dialysis, the ACPase sample was loaded onto a Mono P column. Losses in enzyme activity were generally small as a result of the dialysis. It was important that all buffers used in the Mono P chromatofocusing purification step were extensively degassed. Especially in the pH range used (pH 8 to pH 5), dissolved carbon dioxide could greatly skew the established pH gradient across the column and reduce peak resolution.

In order to maximize the resolution of the next column, sample concentration was a necessary step. In ultrafiltration, the large detergent micelles did not pass through the membrane and the concentrated sample became viscous with detergent. The increase in detergent concentration reduced enzyme activity. Non-ionic detergents, such as Genapol X-080, have large aggregation numbers and form large molecular weight micelles (aggregation no.= 88, micelle Mr = 49 kd; manufacturer). However, the addition of sodium deoxycholate, a detergent with a low aggregation number (4-10, micelle Mr = 1.7-4.2 kd; manufacturer), effectively dispersed the non-ionic detergent micelle. No detectable loss in ACPase activity occurred during ultrafiltration.

In the presence of sodium deoxycholate, ACPase activity eluted in the included volume of a Sephadex G-150 Superfine column (see fig.10c). This column was useful both as a purification method and as a means by which Polybuffer ampholines from the chromatofocusing column

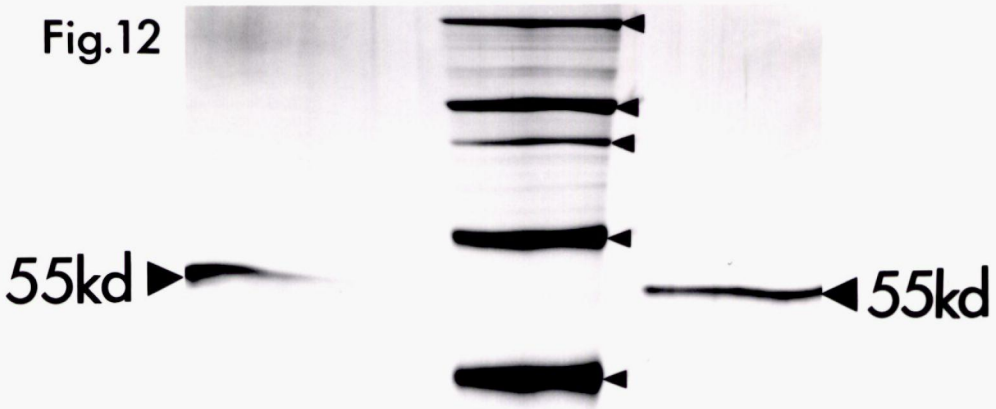
could be removed. Unfortunately, this column flowed slowly and tended to compress. The native molecular weight of the ACPase as determined by Sephadex G-150 chromatography was found to be 130 kd (see fig.10d; assuming the ACPase is a globular protein, ie. the ACPase Stroke's radius is proportionate to its molecular weight). This value agrees well with the monomeric Mr as determined by denaturing gel electrophoresis (see below). Moreover, this value is consistent with the evidence from I.E.F. gels indicating the ACPase is homodimeric.

The large amount (roughly 5 mg) of sodium deoxycholate (an anionic detergent) in the sample after the sizing column was a potential problem for the next step in the purification, Mono-Q anion exchange chromatography. Concentrating the sample by ultrafiltration removed much of the SDOC. An equal volume of 2 X Mono-Q running buffer was then added to increase the pH of the sample. Dialysis of the sample and losses of enzyme activity were thereby avoided. After two passes through the Mono-Q column, the ACPase was seemingly pure (see fig.12). The enzyme eluted in the void volume of this column indicating that the ACPase did not interact with the Mono-Q charge groups. As was previously shown, the enzyme binds tightly to the Mono-P anion exchange column at the same pH. However, the Mono-P is a much weaker anion exchanger than the Mono-Q (manufacturer). The property of the ACPase that allows it to bind the Mono-P but not the Mono-Q may account for the success of

Figure 12. Purity and identity of the final acid phosphatase preparation. A silver stained 9% SDS-polyacylamide gel of purified ACPase. **Lane A.** Two dimensional gel electrophoresis analysis of the final ACPase preparation (0.25 microgram). In the first dimension the ACPase was run on a native gel. In the second dimension, the gel slice corresponding to bulk of the ACPase activity was transferred onto the denaturing gel shown. **Lane B.** Molecular weight markers (0.30 microgram each), from top to bottom: myosin heavy chain (205 kd); β-galactosidase (116 kd); phosphorylase b (94.4 kd); bovine serum albumin (66.2 kd); ovalbumin (43 kd). **Lane C.** Final ACPase preparation (0.25 microgram).

Figure 13. PAS staining of the acid phosphatase. Two 9% SDS-polyacrylamide gels, (a) and (b), run in parallel. Lanes A. and D., lanes B. and E., lanes C. and F. are complementary. **Gel (a), lane A.** PAS stained standards low molecular weight standards (5 micrograms each). Only ovotransferrin, bovine serum albumin (BSA), and ovalbumin are detectable. **Lane B.** PAS stained C. elegans ACPase (1 microgram). **Lane C.** PAS stained high molecular weight standards (5 micrograms each). Only BSA and ovalbumin are detectable. **Gel (b), lane D.** Coomassie blue stained low molecular weight standards (from top to bottom, 1 microgram each: carbonic anhydrase (30 kd); ovalbumin; BSA; ovotransferrin (76-78 kd)). **Lane E.** Coomassie blue stained ACPase (1 microgram). **Lane F.** Coomassie blue stained high molecular weight standards (from top to bottom, 1 microgram each: myosin heavy chain; β-galactosidase; phosphorylase b; BSA; ovalbumin). The R_f values for the ACPase and ovalbumin are indicated on both gels.

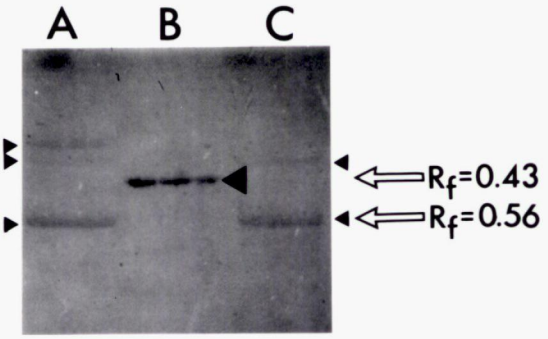
Fig.12



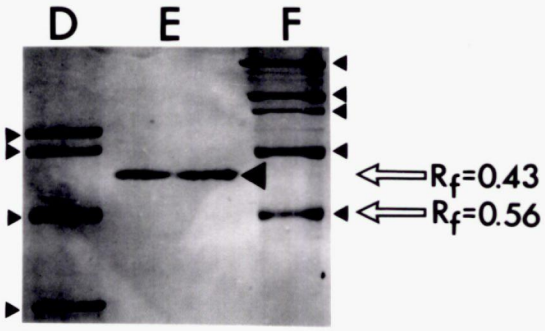
A B C

Fig.13

(a)



(b)



the Mono-Q purification step.

On occasion, the ACPase did bind the Mono-Q column instead of pass through in the column void volume. When this occurred, the sample was eluted from the column with a step gradient of 0.5 M NaCl. The sample was subsequently de-salted using Centricon ultrafiltration and taken up in 50 mM Tris·HCl pH 8.3, 0.5% Genapol X-080, and 0.25% sodium deoxycholate. Then the sample was loaded as before onto the Mono-Q column. This time, the ACPase activity would invariably come through in the void volume; the protein would be pure. Sodium deoxycholate is apparently required in some concentration in order to solubilize the ACPase from the other proteins in the impure fraction.

As shown in table 1, the final preparation has been purified approximately 6,000 fold with a net recovery of 3.4% activity. The largest single loss (46.1% within step recovery) sustained was during Sephadex G-150 chromatography. The largest fold purification was obtained by the initial clarification of the extract (28 fold, within step, purification).

When silver stained, 0.25 microgram of the final product, on a 9% SDS-polyacrylamide gel, gave a single band corresponding to a molecular weight of 55 kilodaltons (see fig.12). This 55 kd protein was determined to be the ACPase by the following criterion. Purified enzyme was loaded and run on a native gel (see Materials and Methods). The portion of the gel containing the ACPase

activity was first identified by activity staining and then excised. The excised gel slice was then loaded and run on a 9% SDS-polyacrylamide gel. Only one 55 kd band was visible when the 0.25 microgram of ACPase was silver stained for protein (see fig.12). A gel slice from a portion of the native gel which had no ACPase activity failed to produce any band on a silver stained SDS-gel (not shown). BSA and the ACPase migrate to distinct positions on the native gel. The ACPase and the 55 kd protein should have been resolved into two bands had they been two different proteins.

III. Enzyme properties

1. Physical properties of the gut acid phosphatase

Figure 13 shows two gels run in parallel; one stained for glycoproteins with PAS reagent and the other stained for proteins with Coomassie blue. Note that only those standards that are glycosylated (BSA, ovalbumin, ovotransferrin) stain with the PAS reagent. The gut ACPase appears to be a glycoprotein since it stained red with the PAS reagent. One microgram of ACPase was easily detectable whereas 5 microgram of each of the standards was required for their detection. PAS reagent colors some glycoproteins more intensely than others depending on the type of the oligosaccharide and on overall carbohydrate content of the protein (Segrest and Jackson, 1972). The ACPase may be highly glycosylated or its sugar moieties may substantially differ from those in the standards. The

fact that the ACPase stains with PAS reagent is consistent with its binding to the lectin Con-A in the purification procedure.

In the absence of lipid, the ACPase seems to require the presence of detergent for its activity. Purified ACPase was loaded onto a Con-A Sepharose column in the presence of detergent, and several column volumes of running buffer without detergent were passed through the column. No activity was recovered after 0.5 M methyl mannopyranoside was passed through the column. Enzyme activity could not be reconstituted by the subsequent addition of detergent. Full activity was recovered when detergent had not been removed from the running buffer.

2. Attempted N-terminal sequencing of the gut acid phosphatase

Because a limited amount of the 55 kd protein was isolated, a different strategy was employed for amino acid sequence analysis of the ACPase. Although impure, the 55 kd protein could be obtained in greater amounts earlier in the purification procedure. When the 55 kd protein was sufficiently pure (after the chromatofocusing step) that it could be resolved from the other proteins on an SDS-gel, the PVDF (polyvinylidene difluoride) gel transfer and sequencing method of Matsudaira (1987) was used. Although this provided a relatively large amount of pure protein for N-terminal sequencing (200 pmol), the protein was N-terminally blocked and did not sequence.

After the failed attempt at N-terminal sequencing, the same sample was taken out of the sequenator and subjected to hydrolysis for standard amino acid analysis. Enough protein sample was present to obtain a reliable amino acid composition. If the protein had not been blocked, enough protein was present to obtain a clear N-terminal sequence. The amino acid composition of the 55 kd protein is shown in Table 2. Cystine, cysteine and tryptophan residues were not protected and therefore do not appear in the table. Glutamic acid and glutamine as well as aspartic acid and asparagine hydrolyze to give the same product and therefore cannot be individually distinguished.

The ACPase may be naturally N-terminally blocked or inadvertently blocked during the purification procedure. Another protein that co-purified with the ACPase did produce N-terminal sequence suggesting the ACPase was not blocked during the purification. However, a protein may be N-terminally blocked in ways other than by post-translational modification. The nature of the first few amino acids in the vicinity of the amino terminus can influence whether a protein is sequencable. These amino acids can undergo secondary reactions which prevent N-terminal amino acid sequencing reactions.

3. Enzyme kinetics

The pH optimum of the gut ACPase was, as might be expected from I.E.F. gels and histochemistry, in the acid

TABLE 2: AMINO ACID COMPOSITION OF THE C. elegans GUT ACID PHOSPHATASE AND ACID PHOSPHATASES OF OTHER SPECIES

amino acid	C. elegans Gut ACPase mol %	E. coli ACPase[1] mol %	Human Type 5 ACPase[2] mol %	Yeast PHO5 ACPase[3] mol %
Asx	9.9	4.5	9.6	15.4
Glx	10.9	9.0	8.0	8.3
Ser	3.9	6.3	6.2	7.5
Gly	6.7	8.9	7.4	6.2
His	1.5	2.7	3.4	1.7
Arg	2.6	6.3	5.9	2.8
Thr	6.2	4.5	5.6	8.1
Ala	6.6	11.6	8.3	6.2
Pro	6.0	7.1	5.6	2.8
Tyr	9.4	1.8	4.3	6.9
Val	8.7	7.1	6.8	6.0
Met	2.2	1.8	1.9	2.1
Iso	6.8	6.3	3.7	4.5
Leu	10.7	12.5	10.8	7.5
Phe	6.1	1.8	5.2	5.8
Lys	2.2	5.4	4.3	5.1
Cys	-	1.8	0.9	2.1
Trp	-	0.9	1.9	1.5

[1]Touati, E., and Danchin, A. (1987). **Biochimie 69**, 215-221.
[2]Arima, K., Oshima, T., Kubota, I., Nakamura, N., Mizunaga, T., and Toh-e, A. (1983). **Nucl. Acids Res. 11**, 1657-1672.
[3]Ketcham, C.M., Roberts, R.M., Simmen, R.C.M., and Nick, H.S. (1989). **J. Biol. Chem. 264**, 557-563.

range. Figure 14 shows the ACPase pH dependency curve from pH 3.0 to 7.0. The pH optimum of the ACPase was not 4.7, the pH at which enzyme activity was assayed for the purification, but rather 3.5 (see fig.14). Since different buffers have different ionic strengths, a high molarity of salt (100 mM NaCl, and 250 mM NaCl) was included in all buffers in order to reduce the effect of ionic strength on enzyme activity. C. elegans ACPase activity could not be detected in buffers greater than pH 5.5. Thus, this ACPase activity definitely has an acidic optimum, and is therefore, an ACPase (E.C. 3.1.3.2).

Some of the kinetic parameters that characterize the C. elegans ACPase were determined from the plot shown in figure 15. The K_m was determined to be 0.319 mM for the substrate ∝-napthyl phosphate (50 mM Acetic acid/Acetate pH 3.5, 0.5% Genapol; 23 C). The maximum rate of hydrolysis (V_{max}) of the C. elegans ACPase (under the assay conditions used: 0.08 microgram of enzyme in 1 ml assay buffer) was determined to be 0.0689 micromole ∝-napthyl phosphate per min. Given both the value of V_{max} and the mass of enzyme assayed (0.08 microgram diluted into 1 ml), the turnover number (k_{cat}; $k_{cat} = V_{max}/E_t$) for the ACPase was determined to be 780 (molecules substrate/active site of enzyme)$\cdot s^{-1}$.

Table 3 shows some of the inhibitors of C. elegans ACPase activity. Ortho-vanadate was the most potent inhibitor of ACPase activity, only 1 mM was required to affect total inhibition. The ACPase was also sensitive to

Figure 14. Acid phosphatase pH activity curve. The rate of hydrolysis of ∝-naphthyl phosphate by the gut ACPase was measured as a function of pH. The buffers used are listed in the figure. All assay mixtures contained 1 mM ∝-naphthyl phosphate, 0.5% Genapol X-080, 20 microlitres of the final enzyme preparation, and all assays were conducted at 23 °C.

Figure 15. Eadie-Hofstee plot of acid phosphatase activities. The values shown for V_{max} and V_{max}/K_m were determined by interpolation using the least-squares regression line estimate. Twenty microlitres of the final enzyme preparation in 1 ml of assay buffer (pH 3.5, 0.5% Genapol X-080) was assayed for activity as a function of substrate concentration. The value of V_{max} is 0.0689 units; the value of K_m (for ∝-naphthyl phosphate) = 0.319 mM.

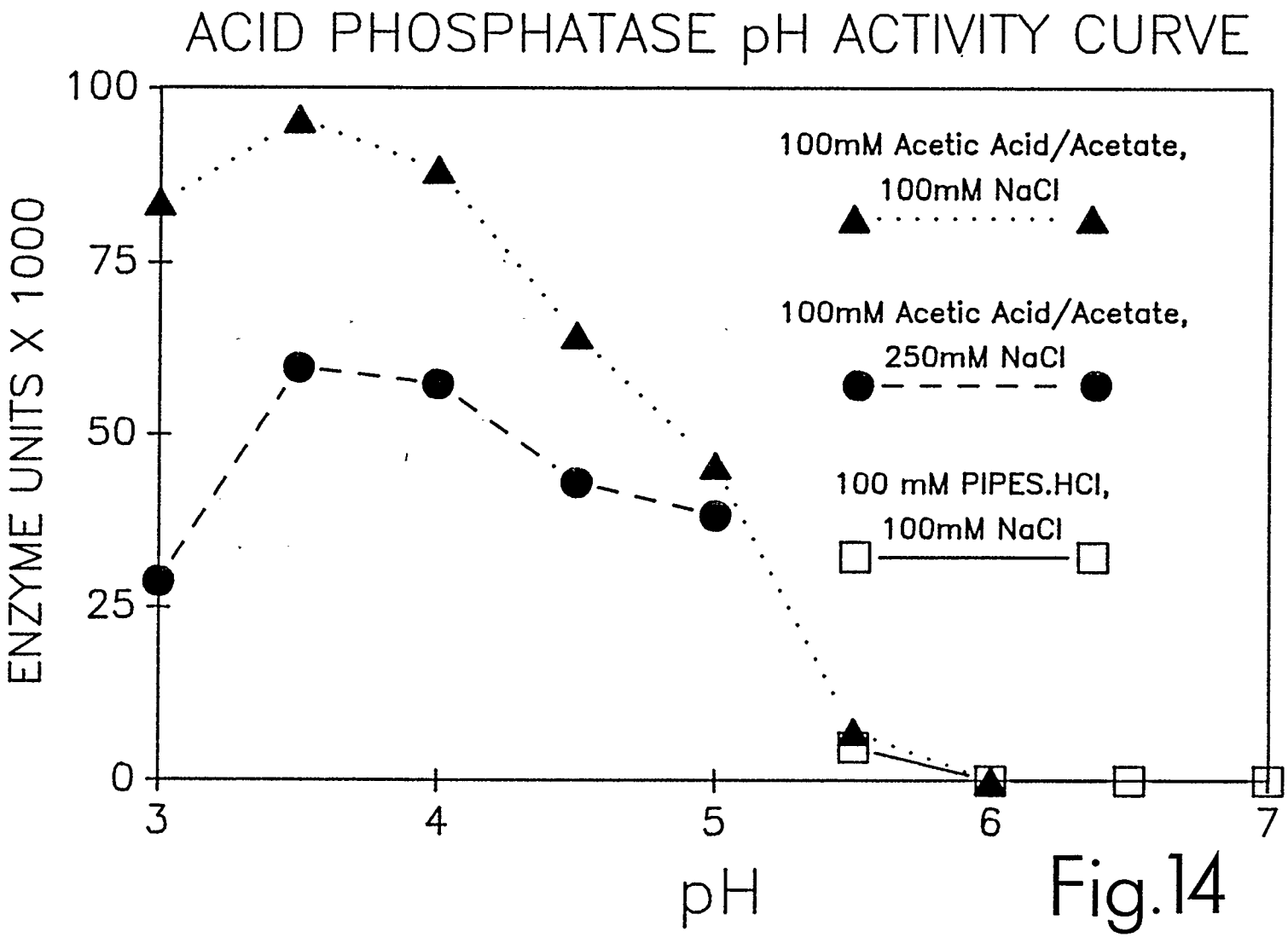

ACID PHOSPHATASE pH ACTIVITY CURVE
ENZYME UNITS X 1000
100
75
50
25
0
3
4
5
6
7
pH
100mM Acetic Acid/Acetate,
100mM NaCl
100mM Acetic Acid/Acetate,
250mM NaCl
100 mM PIPES.HCl,
100mM NaCl

Fig.14

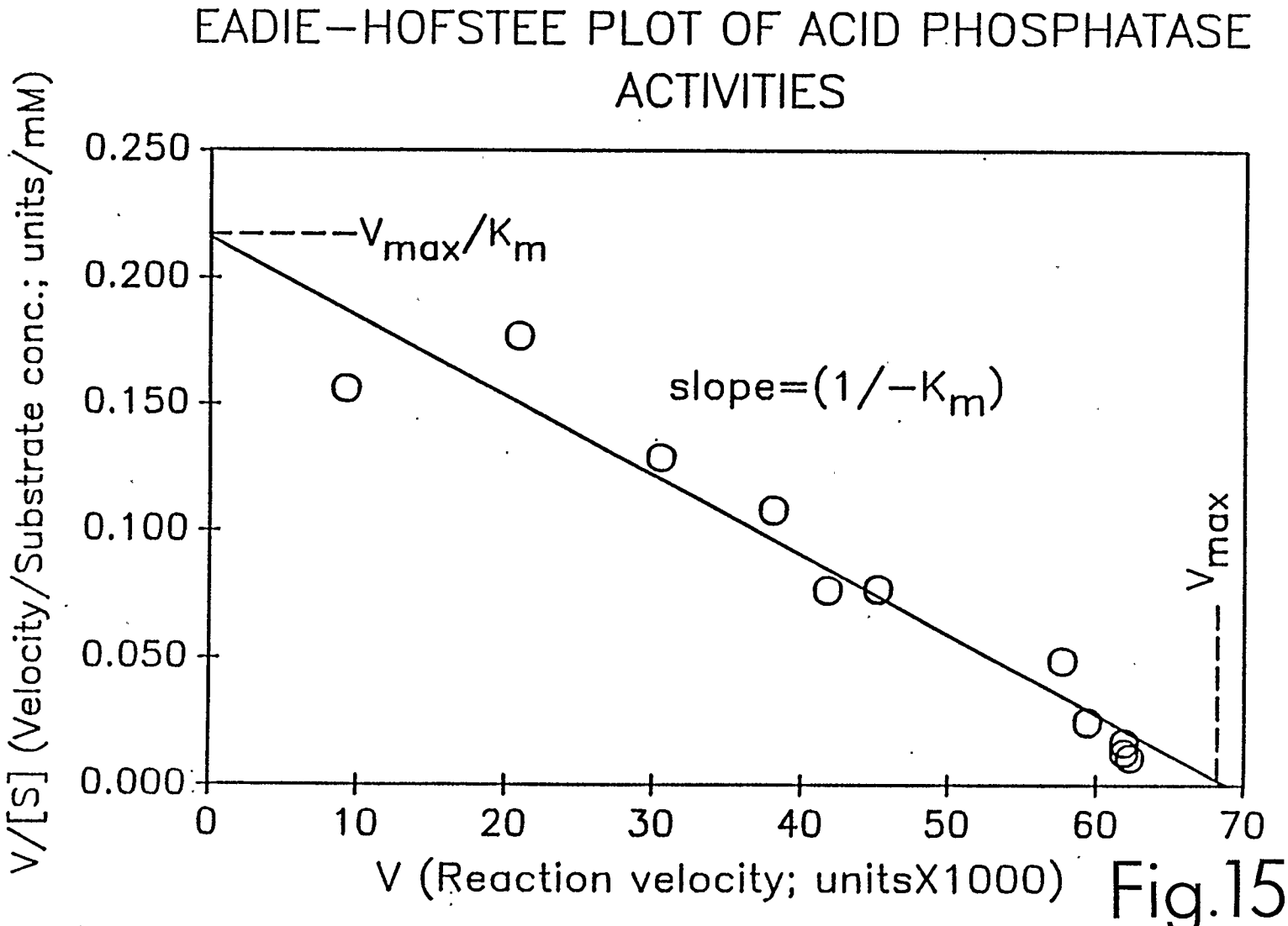

EADIE–HOFSTEE PLOT OF ACID PHOSPHATASE ACTIVITIES
V/[S] (Velocity/Substrate conc.; units/mM)
0.250
0.200
0.150
0.100
0.050
0.000
V_{max}/K_m
slope=(1/$-K_m$)
V_{max}
0
10
20
30
40
50
60
70
V (Reaction velocity; unitsX1000)

Fig.15

Table 3: Inhibitors of the C. elegans acid phosphatase

Inhibitor	Concentration (mM)	Relative Activity %
Control	-	100
Levamisole	0.1	111
	1.0	97.7
	10.0	3.9
Sodium fluoride	0.1	13.3
	1.0	3.1
	10.0	1.6
Sodium orthophosphate	1.0	50.0
Sodium orthovanadate	1.0	0.0
L-(+)-Tartrate	0.1	18.0
	1.0	5.5
	10.0	2.3

L-(+)-tartrate, NaF, and to a lesser degree, sodium phosphate. Levamisole proved to be an potent inhibitor but only at higher concentrations (10 mM).

CHAPTER FOUR: DISCUSSION

I. *C. elegans* GUT ACID PHOSPHATASE EXPRESSION

I have described a major ACPase activity that is localized to the adult gut of *C. elegans* as judged by histochemical staining. This activity is expressed at the edge of the gut lumen in the region of the microvilli. All but the six anterior-most cells of the gut express the ACPase. These sets of cells, the int1 and int2 cells, do not undergo nuclear divisions at the beginning of L1 lethargus (see White, 1988); all gut cells but int1 and int2 are binuclear. The anterior ring of four cells that comprise the int1 cells are also specialized in having shorter microvilli than the rest of the gut (Sulston *et al.*, 1983). However, these cells still have microvilli; ruling out one possible reason why these cells do not stain.

The major expression of the gut localized ACPase starts during the vermiform stage in embryogenesis, corresponding to the 16 E cell stage of gut development. Gut localized staining can be detected prior to the vermiform stage but this activity is at least 20 fold less intense (as judged by the length of time required to detect activity staining in younger embryos). A ubiquitous ACPase activity is present in even younger embryos but this activity disappears soon after gastrulation. The ubiquitous activity is weak, at least 20 fold less intense (as judged by the staining activity)

than the ACPase activity in vermiform embryos.

The onset of the major ACPase activity in embryos may be the controlled at the level of transcription (which seems consistent with the known gut cell lineage; see below) or at the level of translation. The definitive experiment in which distinguish between these two levels of control would be to culture embryos in the presence of the RNA polymerase inhibitor alpha-amanitin. If ACPase activity is transcriptionally controlled, then alpha-amanitin treated, transcriptionally blocked embryos would not stain for ACPase activity.

In embryos treated with FUdR, gut ACPase activity is nearly as intense as in vermiform stage embryos. In FUdR embryos, the presumptive gut cells are located in an abnormal position, lying on the top surface of the embryo instead of in the interior (K.Millen and J.McGhee, personal communication). ACPase staining is restricted only to these presumptive gut cells. Therefore, gut ACPase expression occurs even though normal cell contacts, between gut and non-gut tissues, have not been made. This suggests ACPase expression is determined by factors intrinsic to the gut cell lineage and not be cell-cell interaction. Gut specificity could have been conferred onto the gut ACPase by other non-gut cells prior to the point of morphological arrest. However, evidence from other gut markers (Laufer et al., 1980; Edgar and McGhee, 1986) and from additional differentiation markers (Cowan and McIntosh, 1985), suggests early gut development occurs

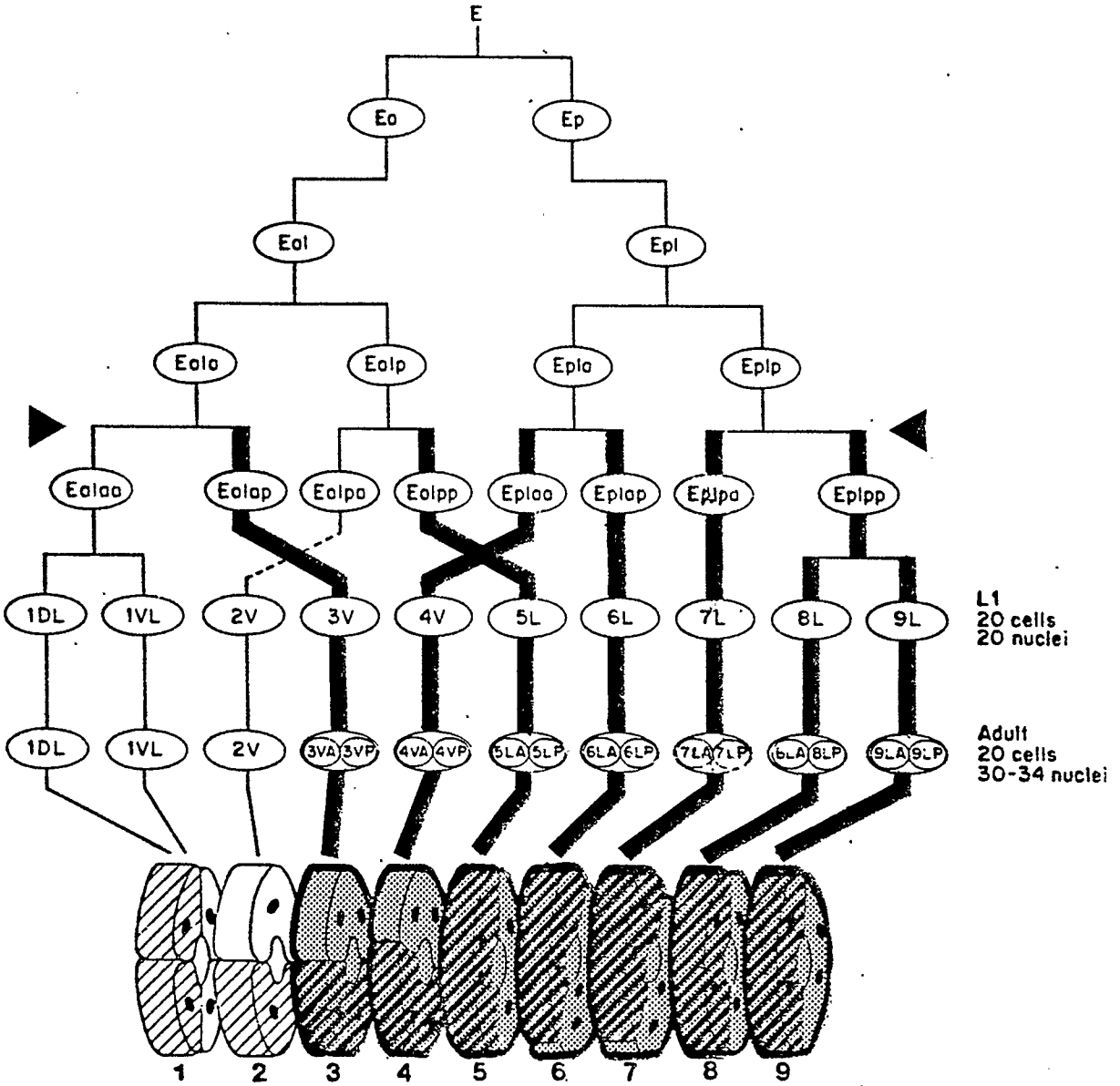
independently of other tissues. To definitively prove whether the gut ACPase is a lineage autonomous marker, ACPase activity must be demonstrated in an isolated E cell cultured in the absence of any other embryonic cell (see Edgar and McGhee, 1986).

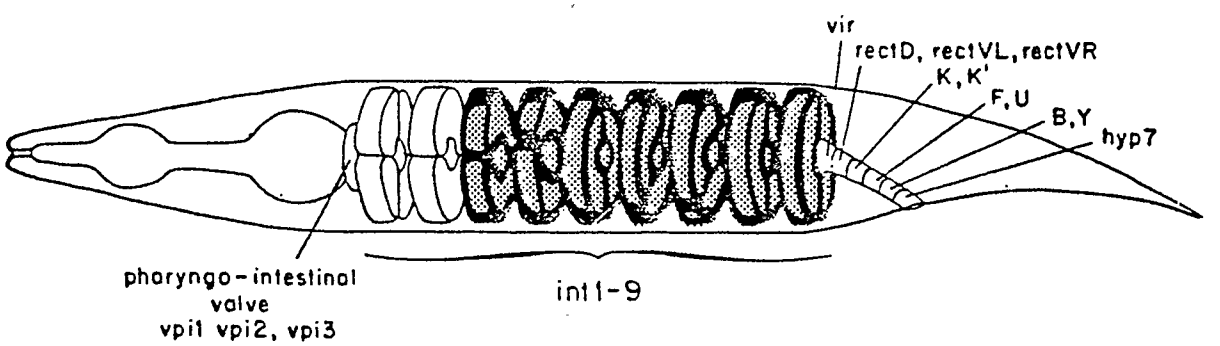
Similarities in the origin of the int1 and int2 cells, as indicated by the known E-cell lineage, correlate with the absence of ACPase activity in these cells (see fig.16). The int1 and int2 cells are both established at the 16 E cell stage and both are the anterior daughters of neighboring cells. Although it may seem likely, the decision not to express the ACPase in these cells is not necessarily determined autonomously by the E-cell lineage. Non-gut tissues such as the pharynx could confer the decision not to express ACPase in the int1 and int2 cells. If the pharynx is involved in the determination of int1 and int2 cell fate, then ablation of the pharyngeal progenitor cells should lead to the expression of ACPase activity in these gut cells.

Tissue specificity can be conferred onto a marker by many tissues or sets of tissues through complex cell-cell interactions. The developmental regulation of a lineage autonomous marker, however, will not (by definition) exhibit such complexity. Thus, it is promising that pho-1, which is definitely tissue specific, seems also to be expressed lineage autonomously. If pho-1 expression is E-lineage autonomous, it will be more valuable for comparisons with the gut esterase, which has been proven

Figure 16. Expression of pho-1 activity in the C. elegans intestinal lineage. The left side of the C. elegans intestinal lineage is shown with the designations of each embryonic cell; (cross-hatching denotes the left gut cells in the worm). The gut cells in relation to other tissues and cells in C. elegans are shown in the lower schematic. ACPase staining cells are shaded and the lineage relationships of these cells are shown by the dark bars. The gut cells that do not stain for ACPase activity in the mature adult are descendants of the anterior daughters of the Eala and Ealp cells, respectively. The developmental stage at which the major ACPase activity first appears (indicated by an arrow) is the 16E cell stage. This figure was adapted from Wood et at., 1985.

INTESTINAL LINEAGE





to be E-lineage autonomous (Edgar and McGhee, 1986). If the factors that determine E-lineage specificity act directly on autonomous markers, then upstream conservation between the ACPase and the esterase genes will identify cis-acting control elements on which these factors act.

Only one predominant ACPase activity from whole adult worm extracts could be detected on isoelectric focusing (I.E.F.) gels. This ACPase, pho-1, corresponds to the gut localized ACPase activity. The earliest stage in development at which pho-1 activity can be detected on I.E.F. gels is coincident with the major onset of gut-localized ACPase activity in embryos. That is, pho-1 activity during development matches the expression of the gut-localized ACPase during development.

The weak ubiquitous staining pattern seen in early embryos corresponds to a basic-pI ACPase, pho-3, detectable on I.E.F. gels only in oocytes and embryo extracts. This may represent a maternal ACPase activity. Pho-2, another basic-pI ACPase activity. can be detected on I.E.F. gels in extracts from L4 and dauer larvae as well as the mixed population (which mainly includes adult worms). This activity is detectable only in stages with mature gonads (see Kimble and Ward, 1988); this activity may represent a gonad specific ACPase that is not present in oocytes.

There may be other species of α-naphthyl phosphatases present in C. elegans. Additional phosphatases could conceivably be denatured by the conditions of the

extraction or the electrophoresis. Many proteins can be inactivated by detergent extraction or are not stable at their pI. However, it seems likely that the range of ACPase activities observed on I.E.F. gels truly reflects that seen *in vivo*. The same staining conditions used *in vivo* were adopted for the *in vitro* staining.

During larval development, pho-1 activity per worm and worm growth (Byerly *et al.*, 1976) appear to be coupled. The relationship between pho-1 activity and worm growth is also reflected in the relatively constant specific activity of pho-1 through the L1, L2 and L3 larval stages. At L4 lethargus, however, pho-1 specific activity decreases. This is probably the result of increases in gonad protein synthesis, which occurs at this stage in development (see Kimble and Ward, 1988). Pho-1 activity in dauer larvae is similar to that in L2 and L3 larvae. Given that the ACPase is expressed in the region of the gut microvilli, this was somewhat a surprise. The dauer larva is a specialized developmental stage for the long term survival of worms in times of stress and food shortage (reviewed by Riddle, 1988). The intestinal lumen in the dauer larva is shrunken and the microvilli are small (Popham and Webster, 1979). The reduced size of the microvilli has no bearing on gut ACPase activity.

II. *C. elegans* ACID PHOSPHATASE (STRUCTURE/FUNCTION)

1. Kinetic properties

(a) Km

The Eadie-Hofstee plot, which was used to determine the K_m (0.319 mM) and V_{max} (0.0689 units), was linear for the conditions of the assay, namely, at pH 3.5 and in the presence of a detergent. Least squares regression was used to derive the best fit line for the plot. As the pH optimum of ACPase activity was determined to be pH 3.5, all assays performed in order to determine kinetic parameters were conducted at this pH.

(b) Substrate specificity

The specificity of an enzyme for a given substrate is given by the apparent second order rate constant defined by k_{cat}/K_m. The gut ACPase k_{cat}/K_m for α-naphthyl phosphate was determined to be 2.5 X 10^5 $s^{-1}M^{-1}$ (50 mM Acetic acid/Acetate pH 3.5, 0.5% Genapol X-080). This value is roughly 100 fold less than the diffusion-controlled limit for a second order rate constant (acetylcholine esterase has a k_{cat}/K_m of 1.6 X 10^8 $s^{-1}M^{-1}$, which is close to the fastest enzymatic reaction possible limited only by diffusion and not the subsequent chemical step; see Rawn, 1983). Nonetheless, the specificity of the C. elegans ACPase for α-naphthyl phosphate, a synthetic substrate, appears to be reasonably high compared to other enzymes. Elastase, for instance, catalyzes the hydrolysis of Ac-pro-ala-pro-ala-NH_2 with a k_{cat}/K_m of 2,200 $s^{-1}M^{-1}$.

(c) Inhibitors

Inhibition by L-(+)-tartrate, NaF, and o-vanadate is a characteristic of a wide number of acid phosphatases (Hollander, 1971). In this respect, the C. elegans ACPase is very much like other acid phosphatases. The C. elegans ACPase is also inhibited by the alkaline phosphatase inhibitor (McComb et al., 1979), levamisole. However, relatively high concentrations of levamisole are necessary to inhibit the C. elegans gut ACPase (10 mM versus 0.015 mM for 50% inhibition for rat kidney alkaline phosphatase; McComb et al., 1979). As expected, phosphate, a product of the ACPase enzymatic activity, was an inhibitor.

2. Physical properties

I have purified, to apparent homogeneity, the ACPase designated pho-1. This enzyme is a 110 kd homodimeric (130 kd as determined by Sephadex G-150 chromatography) glycoprotein that, in the absence of lipid, requires the presence of detergent to maintain activity. Therefore, the gut ACPase appears to be a membrane-bound enzyme. This is generally consistent with the expression of ACPase activity in the gut along the edge of the lumen. The overall purification of the ACPase was roughly 6,000 fold and the recovery of ACPase activity was 3.4%. Homology between pho-1 and other acid phosphatases would be best determined through amino acid sequence comparison. Without pho-1 N-terminal sequence, however, comparisons between different amino acid compositions must suffice.

Apart from the fact that leucine appears to be most prevalent amino acid in the C. elegans (Asp, Asn, Glu and Gln were not individually determined), E. coli and human ACPases, no similarity is apparent between the amino acid compositions of these proteins (see Table 2). The yeast **PHO5** ACPase is even more different because its most prevalent amino acid is threonine (8.1%). The differences in the amino acid compositions of these ACPases emphasizes the lack of similarity between ACPases in general.

3. Function of the gut acid phosphatase

The general physical properties of the gut ACPase, its low pH activity optimum, and its inhibition by NaF and tartrate, are all characteristics of general, high molecular weight acid phosphatases (E.C. 3.1.3.2). Like many acid phosphatases, the specific function of the C. elegans ACPase is not known. Its broad substrate specificity together with its gut localization, suggest that this ACPase hydrolyzes organic phosphomonoesters to provide free phosphate for the animal.

Bolanowski et al. (1983) have described an ACPase activity in C. elegans that they have ascribed to three different acid phosphatases. Two of these acid phosphatases have molecular weights (120 kd and 350 kd) that are equivalent to the molecular weights of two forms of pho-1 present during the course of its purification. Moreover, the two reported phosphatases are both glycoproteins that share the same Km value (1.3 mM;

Bolanowski et al., 1983). Based on these observations it seems likely that both reported acid phosphatases are indeed one species and this species is the ACPase I have described, pho-1. However, since the substrate used by Bolanowski et al. was 4-methylumbelliferyl phosphate, additional ACPase activities may have been identified that could not have been detected with α-naphthyl phosphate.

In their paper, Bolanowski et al. (1983) hint that detergent may be important for ACPase activity. They observe that when detergent was removed from 0.2% NP40 (Nonidet P-40) solubilized ACPase, the subsequent readdition of 0.1% NP40 produced a 40-60% activation of ACPase activity (Bolanoski et al., 1983). In purified ACPase samples, I have shown that the dependence of ACPase activity on detergent is absolute. Once detergent was removed, ACPase activity could not be reconstituted even by the addition of detergent.

On the basis of subcellular fractionation, the C. elegans ACPase activity described by Bolanowski et al. (1983) was judged to be lysosomal. However, on the basis of activity staining, pho-1 does not appear to be lysosomal (if the pho-1 activity is the same activity as they have reported). The expression of the ACPase along the edge of the gut lumen does not correspond to the intracellular location of lysosomes in C. elegans (Clokey and Jacobson, 1986). Moreover, the fact that the six anterior-most cells of the gut do not express ACPase activity is inconsistent with the fact that, just like the

rest of the gut, these cells contain secondary lysosomes (Clokey and Jacobson, 1986). Nonetheless, the large apparent molecular weight of the ACPase (≥50,000 kd) in clarified crude extracts does suggest that the ACPase is complexed in a vesicle such as a lysosome. Only ultrastructural studies of the gut ACPase, by electron microscopy (using the Gomori staining method (1939)), can unambiguously determine the cellular structures with which this protein associates.

III. Genetics of the gut acid phosphatase (pho-1)

The same method of generating and screening isoelectric focusing variants used to map the gut esterase (McGhee and Cottrell, 1986) has been adopted for the gut ACPase (J.McGhee and D.Ferrari, personal communication). Mutations in the gut ACPase were induced with ethylmethanesulfonate (EMS) and "narrow range" I.E.F. gels (see Materials and Methods) were used to screen for subsequent charge change mutants. One mutant allele, **pho-1**(ca101) acted like a normal Mendelian marker and has since been used to locate the ACPase gene (**pho-1**) on the C. elegans linkage map (J.McGhee and D.Ferrari, personal communication).

The **pho-1** structural gene lies on the genetic map between blistered-2 (**bli-2**) and dumpy-10 (**dpy-10**), 0.15 to 0.5 map units (m.u.) and 1.3 m.u. from each, respectively. It is clear from two-dimensional gel analysis of the pure ACPase (fig.12), and from the activity pattern of worms

heterozygous for the ca101 charge-shift allele, that the gut ACPase polypeptide is encoded by only one gene, **pho-1**. This suggests that further genetic analysis of the **pho-1** locus will be straightforward.

Provided that phosphate is present in the media of the worm, the gene encoding the gut ACPase is probably not essential (if the gut ACPase is a general non-specific hydrolase). In liquid culture, worms are commonly grown in M9 media containing 50 mM phosphate buffer (Sulston and Hodgkin, 1988). Phosphate, however, is a potent inhibitor of the gut ACPase. Nonetheless, worms proliferate and are healthy in M9 media with E. coli.

If the gut ACPase is not an essential gene (at least not when phosphate is provided), a ACPase null mutant could be easily obtained and stably maintained. One of the immediate uses of a null mutant would be to prove definitively whether or not the gut ACPase corresponds to the pho-1 band on I.E.F. gels. If the pho-1 band can no longer be detected on I.E.F gels in extracts from null mutants, then pho-1 can only be the gut ACPase. Once the gut ACPase is cloned, a later use of a null mutant might be as a recipient of pho-1 plasmid constructs. Regions necessary for gene expression could thereby be delineated.

IV. Schemes for cloning the C. elegans acid phosphatase

Both the facts that the ACPase is a relatively rare protein and that the ACPase is N-terminally blocked have worked against efforts to clone the C. elegans ACPase gene

via an oligonucleotide probe. Peptides produced by specific proteolytic cleavage have been successfully used to obtain protein sequence for rare proteins (eg., Bodner et al., 1988). However, the greatest promise for cloning the **pho-1** gene is through a molecular genetics approach.

1. "Cosmid rescue" of ca101 isoelectric variants by transformation

A new way in which to clone the **pho-1** gene has become possible by recent advances in DNA transformation (Fire et al., in press) and by the extent to which the genome has been physically mapped in contigs (contiguous cosmids). The **pho-1** gene maps to an area on linkage group II that is extensively covered with contigs (Coulson et al., 1988*[3]). Thus, the **pho-1** gene has probably already been cloned and the cosmid containing the **pho-1** gene needs only to be identified. It has been shown that a null mutant of the gut esterase can be rescued by transformation with a cosmid that includes the wild type gut esterase gene (E.Aamodt, M.Chung, and J.McGhee, personal communication). In a similar manner, it should be possible to rescue a worm homozygous for the ca101 charge-shift allele of the **pho-1** gene by transformation with the cosmid that corresponds to the wild type **pho-1** gene.

The gene nearest to **pho-1** that has been mapped to both the genetic and the physical map is **dpy-10** (A.Levy and J.Kramer*[1]). **Dpy-10** lies roughly 1.3 m.u. from **pho-1**. In kilobases (kb), **dpy-10** is only 430 kb from **pho-1**. This

span is insignificant considering a cosmid can accommodate 45 kb (see Maniatis et al., 1982); **pho-1** is only about 10 cosmids away from **dpy-10.**

Pho-1 is not removed by a tightly linked chromosomal deficiency, mnDf30 (Sigurdson et al., 1984). This deficiency lies between **pho-1** and **dpy-10** on the genetic map. If it can be determined which cosmids span mnDf30, the number of cosmids to be screened in order to find **pho-1** can be reduced. Using each cosmid as a probe, the ends of mnDf30 could be identified by Southern analysis. mnDf30 break-points would be defined by those cosmid probes that display unique hybridization bands. This task, however, might prove more difficult than just injecting all cosmids in the vicinity of **dpy-10** into **pho-1**(ca101) worms and assaying for rescue of the mutation on I.E.F. gels.

2. Isolation of pho-1 cDNA in E. coli phosphatase screen

Recently, a method has been described whereby the ACPase gene from Zymomonas mobilis has been cloned (Pond et al., 1989). In this method, a Z. mobilis chromosomal DNA library was transformed into E. coli TC4. Transformants were grown on Luria agar containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-phosphate, p-toluidine salt. Blue spots identified transformants expressing ACPase activity. Because the endogenous ACPase in E. coli is not synthesized in exponentially dividing cells (Dassa et al., 1982), it

probably does not interfere with the selection screen. Moreover, the E. coli ACPase pH optimum is pH 2.5 which is far below the pH at which the screen was performed.

In a similar manner, the gut ACPase from C. elegans could be cloned. First, C. elegans cDNA would be ligated into the plasmid used by Pond et al. (1989), pLOI193, and transformed into E. coli. Second, transformant colonies would be lifted onto nitrocellulose. Lastly, ACPase expressing transformants would be identified by dipping the colony lift into gut ACPase activity stain. Simpler still, a C. elegans lambda-gt11 cDNA library could be plated out onto E. coli. ACPase containing phage would be identified by staining nitrocellulose plaque lifts with the ACPase activity stain. Cloning **pho-1** by either of these techniques, however, assumes that active gut ACPase will be produced by the prokaryotic host. This may be an unreasonable assumption given that the C. elegans ACPase is a glycosylated protein and this post-translational modification may not be correctly performed by bacteria. The enzyme may be inactive if not glycosylated.

V. SUMMARY

In the experiments and studies described in this thesis, a lineage-specific, possibly lineage-autonomous, marker of development has been established. The immediate value of such a marker is for molecular genetic comparisons between it and another marker (the gut

esterase) expressed in the same lineage. These comparisons require the cloning of the **pho-1** gene.

Although the goal of cloning this gene has not been reached, the biochemistry and developmental expression of this marker have been characterized. This characterization will aid future genetic and molecular studies of this marker. Purified ACPase may be useful in further developmental studies. Antibodies raised against the ACPase may provide a particularly valuable tool in defining exactly when in development translation of the ACPase occurs. The same antibodies may be useful in ultimately cloning the **pho-1** gene through screens involving C. elegans xpression libraries. Furthermore, if all else fails, the **pho-1** gene could still be cloned through traditional biochemical methods. Amino acid sequence has been obtained from minute amounts of N-terminally blocked proteins. The protein is digested with sequence-specific peptidases and the resulting peptides are sequenced. In turn, the peptide sequence is used to construct an oligonucleotide probe for gene cloning.

Apart from its value as a marker with which to compare the gut esterase, the gut ACPase is by itself an interesting developmental system. Unlike the esterase, the gut ACPase is not expressed in all cells of the gut. The basis of this "sub-lineage" decision is not clear and warrants investigation.

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