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The Characterisation of Alkaline
Phosphatase Activity in Rat Testes

Frederick S. Nagy

A Thesis

in

The Department

of

Chemistry

Presented in Partial Fulfillment of the Requirements
for the Degree of Master of Science at
Concordia University
Montreal, Quebec, Canada

October 1987

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ISBN 0-315-44848-2

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ABSTRACT

THE CHARACTERIZATION OF ALKALINE PHOSPHATASE ACTIVITY IN
RAT TESTES.

FREDERICK S. NAGY

The objective of this thesis has been the characterisation and quantitation of alkaline phosphatase from mature and immature rat testes. Various testes extracts were assayed for activity and were characterised by their heat sensitivity, extent of inhibition by various amino acids, gel electrophoresis and neuraminidase treatment. The testicular enzyme behaves similarly to the liver-bone-kidney type alkaline phosphatase. There are no significant differences in the properties of the enzyme from immature (13-day-old) and mature animals.

Results show that the process of spermatogenesis does not include major changes in the type or quantity of alkaline phosphatase present in the testes, nor are there major differences in the type or quantity of alkaline phosphatase noted when extracted by the Butanol-Tris as compared with the Aqueous-Tris methods.

To my mother and father for their inspiration,
To my wife and daughter for their patience
and understanding, and to Phil Chartrand for
that final push.

I would like to thank my research supervisor, Dr. Mary-Judith Kornblatt for her guidance and direction and particularly her patience. A heartfelt thanks to Dr. R.T. Rye for making it possible for me to finish this thesis. I would also like to thank my research committee for their advice and help, and my fellow students for stimulating conversations. Finally, a note of thanks to Annemarie Good for the typing of this work.

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SECTION 1 - INTRODUCTION

(1.1) STATEMENT OF OBJECTIVES

The purpose of this study was threefold. First, we wanted to partially characterize the alkaline phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1) of rat testes. Secondly, we wanted to see if there were any developmental changes in alkaline phosphatase type or quantity during the development of rat testes from infant to adult. And thirdly, we wanted to compare the results obtained by using two different extraction procedures, both of which have been used by others in working with various animal tissues.

ALKALINE PHOSPHATASE

(2.1) DISTRIBUTION IN NATURE

Alkaline phosphatase is a ubiquitous enzyme, very widely distributed in nature.

Many different species of bacteria synthesize alkaline phosphatase under suitable growth conditions, including *E. Coli*. This type of alkaline phosphatase has been extensively studied.

Some classes of lower order animals do not have the ability to synthesize this enzyme, but most do. Annelids, molluscs, arthropods, insects, etc., all have this ability (1). Multicellular plants do not contain any appreciable amounts, while algae, in contrast, are a particularly rich source.

All vertebrates show alkaline phosphatase activity. Certain adult tissues are alkaline phosphatase positive in all species. These tissues include the intestinal mucosa, the skeleton, and the kidneys.

In addition, some tissues such as the brain and skeletal muscle, show little alkaline phosphatase activity in adult animals, but go through an intensely positive stage during organo-genesis (2). I shall talk more about this aspect, that is, developmental changes, in a subsequent section.

As a generalisation, we can say that, at least in mammals, alkaline phosphatase is most abundant in those tissues concerned with molecular transport; i.e. as in secretory organs and in developing tissues, whereas it is mostly absent from muscle, mature connective tissues, cartilage and red blood cells (3).

(2.2) STRUCTURE

The amino acid composition of several different alkaline phosphatases is presented in Table 1.

It is interesting to note that there is a great similarity in amino acid composition between two quite dissimilar mammalian tissues such as swine kidney and human placenta. Even when the comparison is made between E. Coli alkaline phosphatase and a mammalian alkaline phosphatase the similarity is still very recognisable (4).

Recently, a large group of workers has succeeded in determining the complete amino acid sequence of E. Coli alkaline phosphatase (5). See Figure 1. Moreover, the amino acid sequence around the enzymatically active site has been worked out for a number of species. This is presented in Table 2. Again there is a striking similarity in sequence to be observed even with very dissimilar species (4).

The intact protein contains approximately twenty percent carbohydrate including sialic acid, neutral hexoses, glucosamine and galactosamine. The amounts and types vary from species to species, and even from tissue to tissue within a species. Some, such as E. Coli do not contain any carbohydrate at all.

TABLE 1

% Amino Acid Composition of alkaline phosphatase from various sources

(4)

Amino Acid	E. Coli	Swine Kidney	Human Placenta
Lysine	5.7	5.5	4.6
Histidine	1.9	4.2	2.9
Arginine	2.9	5.7	6.1
Aspartic acid	11.7	9.8	10.3
Threonine	10.7	6.6	6.4
Serine	4.1	5.4	4.9
Glutamic acid	10.6	9.5	10.5
Proline	4.4	5.0	5.3
Glycine	10.0	8.9	9.3
Alanine	14.5	10.1	11.6
Cystine	0.8	0.0	1.3
Valine	5.0	7.6	6.6
Methionine	1.6	3.1	2.3
Isoleucine	3.0	4.6	3.6
Leucine	8.6	7.3	8.2
Phe-Alanine	1.9	2.7	2.4
Tyrosine	2.3	4.0	3.5
Tryptophan	0.8	0.0	0.0
Total Residues/ molecule	858	1144	1063
Assumed mol. weight	89000	156000	125000

FIGURE 1

Amino acid sequence for E. Coli
alkaline phosphatase (5)

10 20
Thr-Pro-Glu-Met-Pro-Val-Leu-Glu-Asn-Arg-Ala-Ala-Gln-Gly-Asn-Ile-Thr-Ala-Pro-Gly-
30 40
Gly-Ala-Arg-Arg-Leu-Thr-Gly-Asp-Gln-Thr-Ala-Ala-Leu-Arg-Asn-Ser-Leu-Ser-Asp-Lys-
50 60
Pro-Ala-Lys-Asn-Ile-Ile-Leu-Leu-Ile-Gly-Asp-Gly-Met-Gly-Asp-Ser-Glu-Ile-Thr-Ala-
70 80
Ala-Arg-Asn-Tyr-Ala-Glu-Gly-Ala-Gly-Gly-Phe-Phe-Lys-Gly-Ile-Asp-Ala-Leu-Pro-Leu-
90 100
Thr-Gly-Gln-Tyr-Thr-His-Tyr-Ala-Leu-Asn-Lys-Lys-Thr-Gly-Lys-Pro-Asp-Tyr-Val-Thr-
110 120
Asp-Ser-Ala-Ala-Ser-Ala-Thr-Ala-Trp-Ser-Thr-Gly-Val-Lys-Thr-Tyr-Asn-Gly-Ala-Leu-
130 140
Gly-Val-Asp-Ile-His-Glu-Lys-Asp-His-Pro-Thr-Ile-Leu-Glu-Met-Ala-Lys-Ala-Ala-Gly-
150 160
Leu-Ala-Thr-Gly-Asn-Val-Ser-Thr-Ala-Glu-Leu-Gln-Asp-Ala-Thr-Pro-Ala-Ala-Leu-Val-
170 180
Ala-His-Val-Thr-Ser-Arg-Lys-Cys-Tyr-Gly-Pro-Ser-Ala-Thr-Ser-Gln-Lys-Cys-Pro-Gly-
190 200
Asn-Ala-Leu-Glu-Lys-Gly-Gly-Lys-Gly-Ser-Ile-Thr-Glu-Gln-Leu-Leu-Asn-Ala-Arg-Ala-
210 220
Asp-Val-Thr-Leu-Gly-Gly-Gly-Ala-Lys-Thr-Phe-Ala-Glu-Thr-Ala-Thr-Ala-Gly-Glu-Trp-
230 240
Gln-Gly-Lys-Thr-Leu-Arg-Glu-Gln-Ala-Gln-Ala-Arg-Gly-Tyr-Gln-Leu-Val-Ser-Asp-Ala-
250 260
Ala-Ser-Leu-Asn-Ser-Val-Thr-Glu-Ala-Asn-Gln-Gln-Lys-Pro-Leu-Leu-Gly-Leu-Phe-Ala-
270 280
Asp-Gly-Asn-Met-Pro-Val-Arg-Trp-Leu-Gly-Pro-Lys-Ala-Thr-Tyr-His-Gly-Asn-Ile-Asp-
290 300
Lys-Pro-Ala-Val-Thr-Cys-Thr-Pro-Asn-Pro-Gln-Arg-Asn-Asp-Ser-Val-Pro-Thr-Leu-Ala-
310 320
Gln-Met-Thr-Asp-Lys-Ala-Ile-Glu-Leu-Leu-Ser-Lys-Asn-Glu-Lys-Gly-Phe-Phe-Leu-Gln-
330 340
Val-Glu-Gly-Ala-Ser-Ile-Asp-Lys-Gln-Asp-His-Ala-Ala-Asn-Pro-Cys-Gly-Gln-Ile-Gly-
350 360
Glu-Thr-Val-Asp-Leu-Asp-Glu-Ala-Val-Gln-Arg-Ala-Leu-Glu-Phe-Ala-Lys-Lys-Glu-Gly-
370 380
Asn-Thr-Leu-Val-Ile-Val-Thr-Ala-Asp-His-Ala-His-Ala-Ser-Gln-Ile-Val-Ala-Pro-Asp-
390 400
Thr-Lys-Ala-Pro-Gly-Leu-Thr-Gln-Ala-Leu-Asn-Thr-Lys-Asp-Gly-Ala-Val-Met-Val-Met-
410 420
Ser-Tyr-Gly-Asn-Ser-Glu-Glu-Asp-Ser-Gln-Glu-His-Thr-Gly-Ser-Gln-Leu-Arg-Ile-Ala-
430 440
Ala-Tyr-Gly-Pro-His-Ala-Ala-Asn-Val-Val-Gly-Leu-Thr-Asp-Gln-Thr-Asp-Leu-Phe-Tyr-
449
Thr-Met-Lys-Ala-Ala-Leu-Gly-Leu-Lys

TABLE 2

Amino acid sequence around the active site of alkaline phosphatase.
(4)

<u>Organism</u>	<u>Sequence</u>
E. Coli	-Tyr-Val-Thr-Asp-Ser-Ala-Ala-Ser-Ala
S. Marcescens	(Thr. or Ser)-Asp-Ser-Ala-
Calf Intestine	-Asp-Ser-Ala-
Human Placenta	Val-Thr-Asx-Ser-Ala-Ala-Ser-Ala

(2.3) METAL CONTENT

There is considerable and conclusive evidence that zinc is an integral component of alkaline phosphatase and that it is absolutely essential for enzymatic activity. Zinc has been consistently found in pure enzymes from a variety of sources (6). E. Coli alkaline phosphatase contains four zinc atoms (7).

It has been postulated that zinc may have a role in the binding of substrate and that it may act to provide the enzyme with the proper conformation, or to enhance conformational stability (8). Simpson and Vallee (9) showed that two of the four zinc atoms of the E. Coli alkaline phosphatase are rapidly removed by chelating agents and enzymatic activity is lost. The other two atoms are only removed slowly over a period of twenty-four hours. Conversely, apoenzyme to which only two gram-atoms of zinc has been added is nearly as effective enzymatically as the native enzyme supporting the view that only two zinc atoms related to functionality are located in an area accessible to the surrounding environment, i.e. on the surface of the molecule, whereas the other two might be buried in the folds of the molecule and may serve in stabilizing its structure.

Various authors have reported an activator effect of Magnesium on alkaline phosphatase although the magnitude of this effect varies with the source of the enzyme (10, 11). As an example, of five tissues of the rat investigated by Pickering, only the intestinal extracts showed greatly increased alkaline phosphatase activity with added Mg²⁺ (12). In the case of the E. Coli enzyme, magnesium alone does not confer catalytic activity, but it does influence it if zinc is present. If the enzyme contains only two gram-atoms of zinc, magnesium enhances activity 5-fold. In the case of 4-Zn phosphatase the addition of Mg increases activity 1.4-fold (13).

A study of the effect of various divalent metal ions on alkaline phosphatase apoenzyme has been published by Simon & Sutherland (14). This is shown in Table 3. Zinc was most effective in restoring the activity of EDTA-treated alkaline phosphatase from rat livers, while a combination of Zn and Mg was needed to restore full activity. Other divalent cations also had some activating effect. These findings influenced the composition of our alkaline phosphatase assays.

TABLE 3

Metal activation of membrane-bound, EDTA-treated, alkaline phosphatase from rat livers (14)

<u>Preparation</u>	<u>Addition, (umol/l)</u>	<u>Relative activity (% control)</u>
Control	None	100
EDTA-Treated	None	3
	+2	
EDTA-Treated	Zn (10)	24
	+2	
"	Zn (25)	51
	+2	
"	Zn (50)	29
	+2	
"	Zn (100)	15
	+2	
"	Mg (5000)	20
	+2	
"	Mg (7500)	24
	+2	
"	Mg (10000)	23
	+2	
"	Mg (25000)	22
	+2	
"	Zn (25) + Mg (7500)	88
	+2 +2	
"	Zn (25) + Mg (10000)	100
	+2 +2	
"	Zn (25) + Mg (25000)	81
	+2	
"	Zn (25) + Cu (10000)	5
	+2 +2	
"	Zn (25) + Mn (10000)	11
	+2 +2	
"	Zn (25) + Ca (10000)	56
	+2 +2	
"	Zn (25) + Co (10000)	66
	+2	

(2.4) SUBUNIT STRUCTURE

Several authors have demonstrated that alkaline phosphatase is made up of subunits. Using sucrose-density gradient centrifugation, Ghosh & Fishman (15) reported two molecular weight variants of 70,000 and 240,000 daltons derived from human placenta. The higher molecular weight variant converted to the lower molecular weight variant by storage. Harkness (16) used highspeed equilibrium centrifugation to calculate a molecular weight of 125,000 from the same enzyme source. Nakasaki (17) reported values of 110,000 and 220,000 for enzyme from rat livers by means of Sephadex G-200 gel filtration. This data indicates that alkaline phosphatase exists as a monomer, dimer, and tetramer.

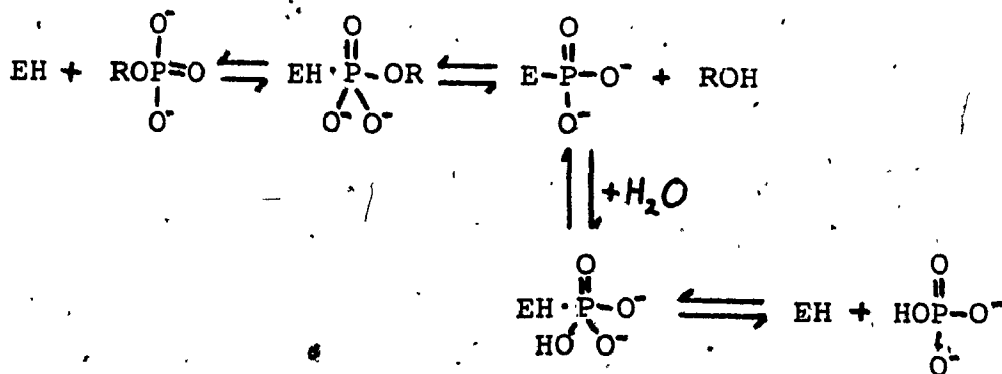
This is supported by data (18) from studies on E. Coli. This enzyme is reported to be a dimer of molecular weight of 86,000. Moreover, isolated alkaline phosphatase dimers, under the right conditions do associate to form tetramers which are enzymatically active. This association is accompanied by a change in conformation (19).

(2.5) ACTION OF ALKALINE PHOSPHATASE

Alkaline phosphatase acts as a hydrolase, cleaving the P-O bond. It can also act as a phosphotransferase whereby the phosphoryl group is transferred to an acceptor molecule without the appearance of inorganic phosphate.

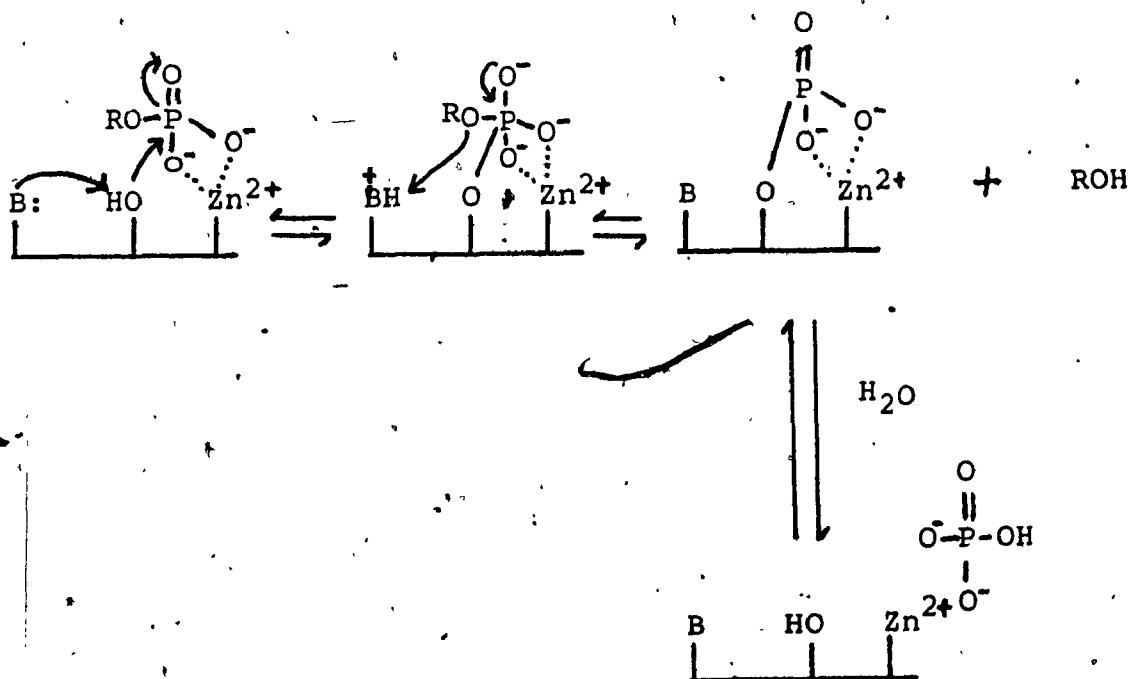
In addition to the P-O-C bond, the following ones are also split by alkaline phosphatase: P-F, P-O-P, and P-N. Concerning the structural requirements of the acceptor molecules concerned in these transfer reactions, there is a necessity for R-OH where R is H or an alkyl substituent.

The outline of the reaction is as follows:



The first reaction results in the Michaelis complex wherein the substrate is bound to the enzyme noncovalently. This is followed by the alcohol being cleaved while the phosphate group becomes covalently bound to the enzyme. This phosphoryl-enzyme is converted subsequently to a noncovalent complex by the addition of water from which, finally, orthophosphate is released with the regeneration of free enzyme (4).

Proposed mechanism for the action of alkaline phosphatase:—



This model states that one zinc atom on the enzyme binds and neutralizes the negatively charged phosphate in the approaching substrate, thus exposing the phosphorous atom to nucleophilic attack. A nearby imidazole group on the enzyme assists in the transfer of a proton from an active serine to the leaving group. This is the proton which is donated to the leaving group during the breakdown of the intermediate (20).

(2.6) HYPOTHESIS OF ALKALINE PHOSPHATASE SYNTHESIS

The synthesis of the polypeptide backbones of glyco proteins, including alkaline phosphatase occurs on polyribosomes within the cell. Sugars are added after the separation of the polypeptide from the ribosomes. There is evidence that some sugars are, at least in part, incorporated in the rough-surfaced endoplasmic reticulum. Recently, it has been found that oligosaccharides may be pre-assembled as polyisoprenol lipid-oligosaccharide complexes with subsequent transfer to the growing glycoprotein.

Other terminal sugars, such as sialic acid and others are incorporated one by one after the glycoprotein has left the endoplasmic reticulum and has entered the Golgi apparatus (15,21). At about this time, two (or four) identical polypeptide chains dimerize and interact with 2 to 4 g-atoms of Zinc to form the active enzyme. Where and when this happens is not known for mammalian systems, but Schlesinger (22) has shown, by using C¹⁴-amino acid incorporation studies, that in E. Coli, the subunits pass through the cell membrane and dimerize and interact with Zn⁺² in the periplasmic space.

Final localization of alkaline phosphatase in the cell after it leaves the Golgi apparatus has been studied by electron microscopic cytochemistry and differential centrifugation (23,24,25,26). The conclusion drawn from these studies is that, in the majority of mammalian cell types studied, most of the alkaline phosphatase is localised on the plasma membrane. However, there are also much smaller amounts of activity with intracellular sites, such as the Golgi apparatus and the nucleus. For E. Coli, the majority of the alkaline phosphatase is located in the periplasmic space (27,28).

(2.7) PHYSIOLOGICAL FUNCTION OF ALKALINE PHOSPHATASE

There is no agreement, as yet, among researchers on this point. Following are some suggested functions: hydrolysis of phosphate esters (29), supply of ortho-phosphate (30), supply of non-phosphate moiety (31), synthesis of phosphate esters ("transferase") (32), and others, some more logical than others.

Walsh (33) states, rather conclusively, that for E. Coli, the function of alkaline phosphatase is "... to provide inorganic phosphate for the cell and to render the remainder of the substrate molecule more lipophilic for its passage across the bacterial cytoplasmic membrane into the cell. "

(2.8) ISOZYMES

As is the case with many other enzymes, alkaline phosphatase occurs as a number of slightly different isozymes, and different ones occur in different tissues, the best characterised being those obtained from placenta (34), intestine, and a so-called liver-bone-kidney type (7).

Several lines of evidence are available for the assumption that there are a minimum of three separate structural loci which are concerned with determining the protein portion of the different alkaline phosphatase isozymes, one which codes for the placental enzyme, another for the intestinal form and at least one locus for the liver-bone-kidney type (35). For example, the mutant gene which causes the rare recessive disease hypophosphatasia results in a gross deficiency of liver, bone, and kidney alkaline phosphatase, but does not affect the intestinal or placental enzymes (36). As well, purified placental and liver enzymes differ in their amino acid composition and in their amino-terminal amino acid sequences. They are also shown to have very different peptide maps (37).

Hypophosphatasia is accompanied by the appearance of large quantities of phosphoryl-ethanolamine in the urine. This suggests that this substance builds up because of the lack of phosphatase and as a corollary, that phosphorylethanolamine might be one of the naturally-occurring substrates for alkaline phosphatase (36).

(2.9) DIFFERENTIATION OF ISOZYMES

Even though it may not be true that in vitro alkaline phosphatase behaves the same way as in vivo, it has been shown to be possible to differentiate the various alkaline phosphatase isozymes isolated from different tissues of the body by the employment of various techniques. These include their sensitivity to heat inactivation, the use of a combination of specific inhibitors, as well as electrophoretic techniques (38,39,40).

(2.10) AMINO ACIDS

The amino acids L-phenylalanine and L-homoarginine are of great value in the differentiation of these isozymes since they have been shown to be organ-specific inhibitors (7).

The results of a number of workers are presented below. Their work has demonstrated that, in humans and rats, the intestinal and placental forms of the enzyme are sensitive to L-phenylalanine inhibition (10,42,41).

<u>Tissue</u>	<u>% inhibition</u>
intestine	77 %
liver	8 %
bone	10 %
placenta	79 %

Fishman (43) who has done a lot of the ground-breaking work in this area, has also tried D-phenylalanine, but this isomer had no inhibitory effect on any human extracts. There seems, therefore, to be a type of double specificity; one originating with the tissue source of the alkaline phosphatase and the other due only to the isomeric form of phenylalanine.

L-Homoarginine, however specifically inhibits the liver-bone-kidney isozyme (44).

Byers (45) studied the enzyme-inhibitor complex. By using a rapid mixing and quenching technique that measured phosphoryl-enzyme, he showed that L-phenylalanine bound exclusively and instantaneously to the phosphoryl phosphatase to form a stable compound that could not be readily dephosphorylated. In the absence of L-phenylalanine, the steady-state concentration of phosphoryl-phosphatase was approximately zero. But at a pH = 9.0 and L-phenylalanine concentration of 5 mM, over 80% of the total enzyme was present as phosphoryl phosphatase.

These results suggest that there is a unique difference in the nature (or conformation) of the catalytic site of the intestinal and placental enzyme compared to the sites of alkaline phosphatase prepared from other tissues. Various workers have shown that rat tissue alkaline phosphatases behave very similarly to human tissue extracts when incubated with L-phenylalanine; that is, intestinal enzyme is inhibited the greatest, while kidney, bone and liver extracts were affected much less, with kidney being the least affected (11,43,46).

Further work by Fishman (47), and Lin (48) showed that L-Homoarginine was a strong inhibitor of human bone and liver alkaline phosphatase (73 % of original activity being lost) while having virtually no effect on the placental or intestinal isozymes (45).

<u>Tissue</u>	<u>%_Inhibition</u>
Intestine	3 %
liver	78 %
bone	80 %
placenta	5 %

Others showed that these results were true with a number of mammals including the rat (11), mouse and dog. See also Table 4.

TABLE 4

Inhibition of human alkaline phosphatases (10,48,49)

	<u>Liver</u>	<u>Bone</u>	<u>Intestine</u>	<u>Placenta</u>
Inhibition by L-Phe. in % a)	0 - 10	0 - 10	75 - 80	75 - 80
Inhibition by L-Har. in % b)	78	78	5	5
Heat inactivation in % c)	50 - 70	90 - 100	50 - 60	0 - 5

a) 0.0005 M L-Phenylalanine in the assay.

b) 8 mM L-Homoarginine in the assay

c) Heated for 10 minutes at 56 degrees C.

(2.11) HEAT INACTIVATION

Aqueous extracts of various human tissues were heated at 55 degrees C by Moss (50) who used it to distinguish isozymes. The treatment time required to lose 50% of initial activity was recorded. Results indicated an appreciable difference in heat-lability among the different isozymes. The bone enzyme was the most heat-labile, losing 50% of activity after eight minutes. By contrast, liver, kidney and intestinal extracts were much less heat sensitive, losing 50% of activity in eighteen, twelve and fifteen minutes respectively.

Numerous investigators have examined various rat tissue extracts (11,46,49,51). The bone, kidney and liver isozyme is greatly inactivated by heat treatment at 56 degrees C for 15-30 minutes (85 - 100% deactivation); whereas the intestinal form is much less affected (35 % deactivation).

It is of interest to note that, whereas in all mammalian species investigated, except the human, the placental isozyme behaves like the liver-bone-kidney variety upon heat treatment, the human placental isozyme is unique in having an incredible resistance to heat denaturation.- 56 degrees C for 30 minutes resulted in only 0 - 2% loss of activity (51).

(2.12) ELECTROPHORESIS

Polyacrylamide gel electrophoresis in alkaline phosphatase characterisation was pioneered by, among others, Kaplan (52) and Allen (53), who showed that it could be used to distinguish between liver, bone, placental and intestinal alkaline phosphatase in human sera. Since then, it has been widely used for the separation of different phosphatases from a variety of sources (49,54).

Interpretation of electrophoretic results is not however without possible pitfalls. Even with the most careful treatments one can get unexpected results. These may even prove to be repeatable. It may be difficult to decide whether one is dealing with true structural differences or with changes that occurred during purification procedures (55). A minor electrophoretic band which one might be tempted to call an artifact could well result from molecular heterogeneity, while bands with appreciable activity may represent molecular aggregates which, with time, may break down to subunits showing greater mobility and a net increase in activity (56).

As an example, various authors (57,58) have reported that alkaline phosphatases from human tissues show bands with enzymatic activity near the origin.

Moss (59) hypothesized that alkaline phosphatase can exist in the form of a heavy lipoprotein complex, which was responsible for this slow-moving band. Walker (57) agreed with him. Kaplan (52) reported that in most but not all of his experiments, he had a narrow band of intense activity very close to the origin for which, at first, he could apply no reason. Later work showed it could be ascribed to a complex formed with lipoproteins. See figure 2.

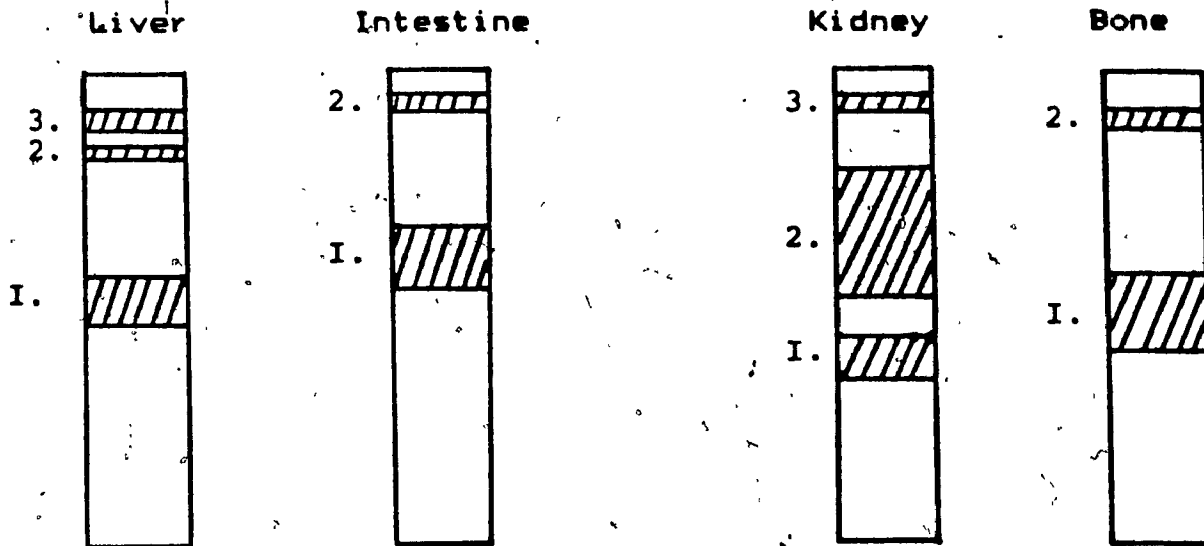
In the experiment performed by Moss (50), the author ascribed the slow-moving minor bands to either enzyme aggregates, environmental factors, or attachment of the alkaline phosphatase to some other protein fractions; in this case probably a lipoprotein. Freezing and thawing of this section, followed by extraction and re-electrophoresis fractionated the slow band into a portion with the original mobility and a faster moving component, probably due to the dissociation of the enzyme-lipoprotein complex.

Cornish, as well as Eaton and Moss, stated that despite intensive purification procedures, some alkaline phosphatases failed to produce sharply delineated bands (60,61).

After having stated all of the above, it is nevertheless true that researchers have demonstrated that polyacrylamide gel electrophoresis is a valuable tool in identifying the various isozymes of alkaline phosphatase.

FIGURE 2.

Electrophoresis patterns of alkaline phosphatase
obtained from human organ sources (50)



Relative Intensities

<u>Tissue source</u>	<u>Band Number</u>	<u>Relative Intensity</u>
Bone	1:2	34:1
Intestine	1:2	26:1
Kidney	1:2:3	1:34:6
Liver	1:2:3	42:1:1.4

(2.13) NEURAMINIDASE (ACETYLNEURAMINYL HYDROLASE)

E.C. 3.2.1.18

An additional experiment which may be performed in conjunction with electrophoresis is pretreatment of the enzyme with neuraminidase. This procedure was introduced by Robinson and Pierce (62). This technique, which selectively removes sialic acid (neuraminic acid), residue from glycoproteins will be described in the Methods section.

Neuraminidase has been used in a further attempt to identify alkaline phosphatase isozymes types, by measuring the retardation of their electrophoretic mobility after treatment, (removal of sialic acid decreases the net charge on the glycoprotein).

Working with human tissue extracts and using 5 % gels, Smith (54) stated that, after neuraminidase treatment the intestinal extract showed the least retardation of mobility. See Figure 3.

Pickering (11) also used neuraminidase during his work with rat tissues. He reported little or no effect on the electrophoretic mobility of the intestinal band, whereas the mobility of the kidney band was reduced by 30 %.

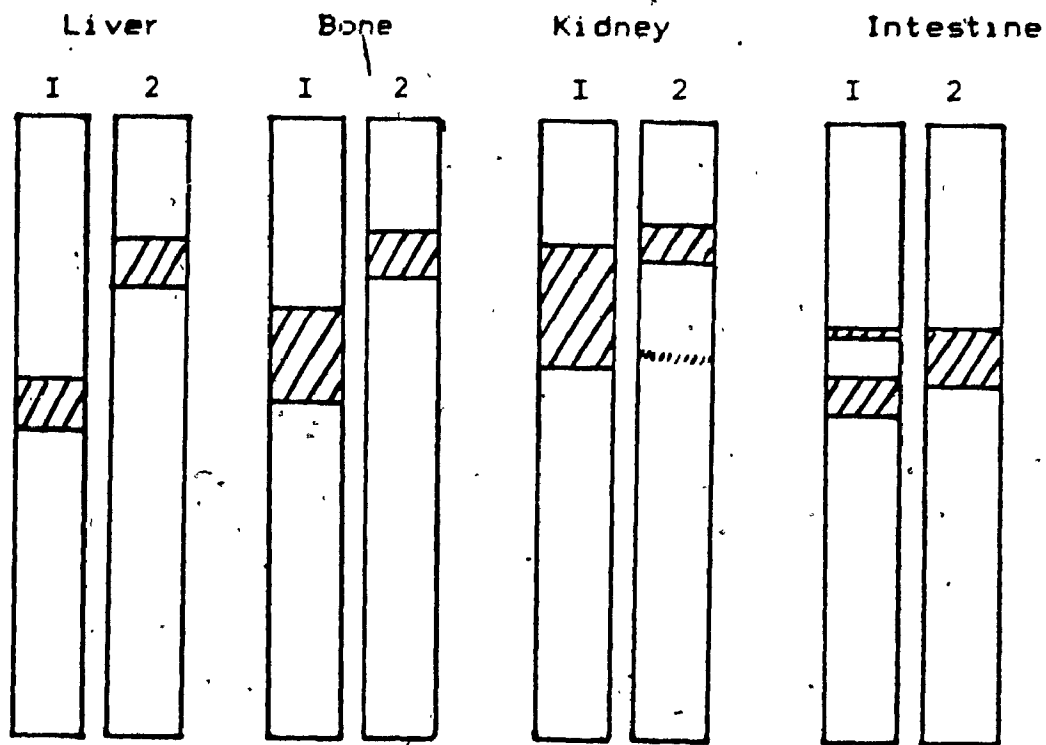
Liver gave two bands of activity, one having the same mobility as that of the bands found from other tissues and a faster-moving one. After neuraminidase treatment, it appeared that the slower-moving band's position remained unchanged, whereas the fast-moving band was replaced by a band with a mobility of only 65 % of that of the slow band. Finally, bone extract was retarded the most: 60 %.

These results correspond to the findings from human tissues in most respects.

Additionally, one can note that Saini (63) has stated that adult rat intestinal alkaline phosphatase does not contain sialic acid. Robinson (62) Komoda (64) have both stated that the same is true for adult human intestinal alkaline phosphatase, but that fetal intestinal alkaline phosphatase does contain sialic acid. This is an example of a developmental change in an enzyme, about which more will be said later.

FIGURE 3

Alkaline phosphatase electrophoresis patterns: effects of Neuraminidase treatment (54)



1: Before neuraminidase treatment

2: After neuraminidase treatment

SPERMATOGENESIS

(3.1) STRUCTURE OF THE TESTES

The testes of mammals is composed of interstitial tissue and seminiferous tubules. The interstitial tissue is composed of Leydig Cells, blood vessels, lymphatic channels, and numerous macrophages (65).

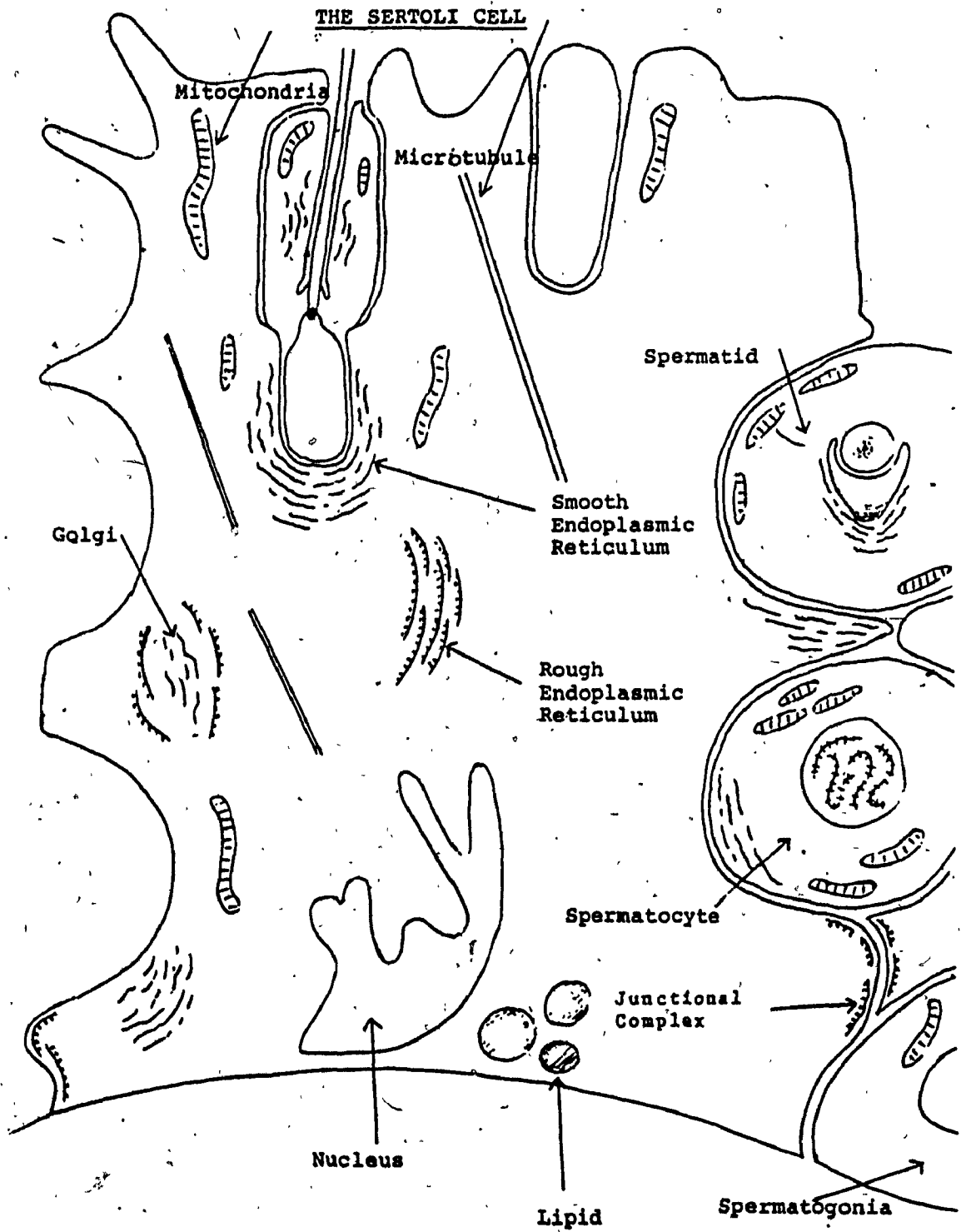
It is in the seminiferous tubules that the gametes develop in several layers representing successive generations. These evolve in a cyclical manner in close symbiosis with Sertoli cells which are in contact at all times with the gamete during its development (66).

See Figure 4 (67).

The seminiferous tubules are enclosed by a complex limiting membrane, also called the basement membrane. The whole set of seminiferous tubules with the interstitial tissues are enclosed by the tunica albuginea (68).

In mature testes, the seminiferous tubules are composed of Sertoli cells (24 - 32 %), spermatogonia and some primary spermatocytes (3 - 9 %), pachytene spermatocytes (15 - 24 %), and spermatids (35 - 58 %) (69).

FIGURE 4



(3.2) THE PROCESS OF SPERMATOGENESIS

Spermatogenesis is just one example of a biological process called differentiation whereby a base stock of primary cells go through a series of biochemical and structural changes to finally give rise to cells which are very different from the starting stock. Another example is the development of red blood cells from stem cells located in bone marrow.

The established concept is that the population of base cells divide; some enter the spermatogenic pathway of differentiation and some remain as base cells to replenish the supply. This process has been extensively studied and its stages are well known (70,71).

The duration of this process in the rat takes approximately forty days. In the first stage the base stock of cells, or gonocytes, start proliferating at about four days of age and divide to form Type A spermatogonia. In six-day old animals the Type A spermatogonia begin to produce intermediate spermatogonia while maintaining their own population. In turn the intermediate cell type gives rise to the final "gonial" stage: type B spermatogonia.

In the second stage, Type B spermatogonia differentiate to form primary spermatocytes.

Various subphases of this meiotic stage have been identified and named Prophase --> leptotene --> pachytene --> zygotene . The pachytene stages are reached around age 14 - 18 days old. The cells now divide to form secondary spermatocytes, having twenty-three pairs of chromosomes. These now go through the second phase of meiosis. The cells at this point contain 23 unpaired chromosomes. The spermatocyte has by now moved away from the basement membrane where spermatogonia continue to divide.

The third stage of spermatogenesis is the development of spermatids from secondary spermatocytes. This occurs during days 18 - 21 of the cycle, and ends in the production of spermatozoa. During this time the spermatids go through a complex series of biochemical and histological changes to assume the final form of the spermatozoa (72).

Of course, in the adult animal, all of these phases are present simultaneously in the seminiferous tubules, with the spermatogonia at the basement membrane, and the progressively more differentiated cells at higher levels of the Sertoli cell.

(3.3) HORMONAL CONTROL OF SPERMATOGENESIS

There is evidence for the involvement of the gonadotrophic hormone in stimulating the growth of the seminiferous tubules, as well as the growth of the testes (73). The exact role of the pituitary gonadotrophins is not at all clearly understood but the theory is that Follicle-Stimulating Hormone (FSH) maintains the germinal epithelium and Leutinizing Hormone (LH) facilitates the completion of spermatogenesis by stimulating the interstitial Leydig cells to produce testosterone (74,75).

It is generally accepted that the main source of testosterone hormone is the Leydig cells (76).

These androgens act directly on the tubules (77), but it is not known if testosterone acts directly on the germinal cells or other cells in the testes. Dorrington (78) demonstrated that spermatocytes and spermatids take up testosterone and metabolize it to dihydrotestosterone, whereas the tubules metabolize it to androstanediol and androstenediol.

As far as spermatogenesis is concerned, studies indicate that the completion of the meiotic division is under the control of testosterone and that the final stages of spermatid maturation are dependent on FSH (79,80).

(3.4) BIOCHEMISTRY OF SPERMATOGENESIS - SOME FEATURES

There is a variety of evidence that enzyme levels change during spermatogenesis. Some enzyme levels increase while others decrease during this process. Similarly, sulfogalactoglycerolipid levels in the testes of twenty-two day old rats increase ten-fold over the levels found in 7 - 15 day-old rats (81,82).

Experiments performed by Millette and Bellve (83) demonstrated that a variety of new surface antigens appear in the surface membrane of pachytene primary spermatocytes which were not there previously, but which persist through to mature sperm cells.

Working with the mouse, antibodies were prepared to i) purified pachytene primary spermatocytes, ii) purified round spermatids, iii) mix of cells from isolated seminiferous tubules, and iv) spermatozoa. Using antibody absorption, immunofluorescence, and cytotoxicity assays, they were able to show these four antibody preparation showed no or very little affinity for various somatic cells, nor did they label any spermatogonia or spermatocytes up to the leptotene/zygotene stage.

They showed strong binding for pachytene spermatocytes, round spermatids and residual bodies. The implication is that some new components are synthesized and inserted into the cell membrane at the pachytene spermatocyte stage and that these are unique to spermatozoan development.

Letts et al. (84) studied the appearance and disappearance of a variety of glycoprotein glycosyltransferases and demonstrated that synthesis is active in spermatocytes and early spermatids, but becomes negligible in late spermatids and spermatozoa. They claimed, on the basis of histological evidence, that this decrease correlates with the degeneration of the Golgi apparatus.

It is interesting to theorize that since the Golgi apparatus in these later stages is degenerate, no glycoproteins can be completed since the Golgi apparatus is the location, as shown previously, where a variety of carbohydrates are attached to polypeptide backbones.

Spermatogonia, the least differentiated of the germinal cells, have low levels of several enzymes which appear in larger amounts in developing spermatocytes.

- Lactate dehydrogenase (E.C.1.1.1.27) designated as LDH-X is a unique isozyme thus far only found in the testes.

It first appears in the developing testes of the rat at approximately twenty days old - when pachytene spermatocytes are being formed in the seminiferous tubules (85).

- Similar results were found for Carnitine Acetyltransferase, with very low levels of activity found in fourteen day old rats increasing progressively until the thirty-fifth day, and corresponding with the appearance of spermatids (85,86).

- Levels of testicular adenylyl cyclase progressively increased during the development of rats from 25-60 days of age (85).

- A unique type of cyclic nucleotide phosphodiesterase "f" (PDE) begins to appear in testes at 40 days of age, and achieves adult levels at 50 days old. In testes from immature rats (20 days old or younger), only the "c" type of PDE is present (85).

- Testicular hyaluronidase begins to appear at around thirty-three days of age and increases 400-fold reaching its peak in 60 day old animals. This coincides with the beginning of the development of the pro-acrosomal cap of spermatids (85,87).

- Among lysosomal enzymes, Males (88) has shown that B-glucuronidase activity is detectable only in spermatogonia (as well as Sertoli cells). Its specific activity is high in the immature rat, but with the appearance of spermatocytes, activity markedly declines. Acid phosphatase is detectable in all germinal cells, but its specific activity is highest in spermatids.

- Isozyme I of B-galactosidase was highest in 4-day-old rats when only gonocytes and Sertoli cells are present, whereas B-galactosidase II was not detected through the spermatocyte stage but had a high specific activity in spermatids.

- The activities of Isozymes I and II of N-acetyl-B-glucosaminidase are high in spermatogonia and spermatocytes, then decline with the appearance of spermatids (89).

All of this data shows that in actively differentiating cells, in this case germinal cells, various enzyme levels can vary enormously with the age of the animals. Since alkaline phosphatase is a glycoprotein and since the Golgi apparatus degenerates in late spermatids and spermatozoa, we decided to see if alkaline phosphatase varies in activity as do so many of these other enzymes. We expected to see an initial increase in activity with age, and then a leveling off or drop in activity.

Given that there are testes-specific enzymes, we wanted to explore this possibility as well for alkaline phosphatase.

(3.5) ALKALINE PHOSPHATASE IN SPERMATOGENESIS
HISTOCHEMICAL EVIDENCE

i) Accessory sex organs

The seminal vesicles and prostate of the adult rat, as well as the epididymis of mice show high alkaline phosphatase activity, and this activity is highly dependent on the continued presence of testosterone (90,91,92).

ii) Testes

The evidence here is often contradictory. All researchers agree that alkaline phosphatase activity is found in both interstitial tissues and seminiferous tubules.

Dempsey (93) reported strong activity in spermatids, and basement membrane, little in interstitial cells of rat testes. Hypophysectomy abolished all activity.

Others reported strong activity in basement membrane and some activity in the cytoplasm of developing germ cells (94,95). Conversely, McAlpine (96) reported little activity in the seminiferous epithelium with strong activity in primordial germ cells.

There has been, however, no quantitative studies done on alkaline phosphatase activity in rat testes. We wished to provide this data.

(4) EXTRACTION METHODS

Our third purpose for this work was to compare and contrast the activities as well as the inhibition characteristics and electrophoretic properties of testicular alkaline phosphatase obtained by a butanol extraction and an aqueous extraction method.

Ahmed (97) compared the enzyme activities of extracts of human placental alkaline phosphatase obtained by aqueous and butanol extraction. He found that the butanol extract showed 80 % more activity. After having partially purified the enzyme, he proceeded to a paper electrophoresis step which demonstrated that the alkaline phosphatase migrated as a single broad band. He is the only author I have found who has compared these two extraction methods, whereas there are many references, of course, to either one or the other extraction method and various results obtained through them.

SECTION II - MATERIALS AND METHODS

(1) - MATERIALS

Acrylamide and bis-acrylamide were purchased from BDH Chemicals Canada. p-Nitrophenyl phosphate, B-naphthyl phosphate, Fast Violet B, Triton X-100, L-Homoarginine, L-Phenylalanine, sucrose, and bovine serum albumin came from Sigma Chemical Co. U.S.A.. Neuraminidase was purchased from Boehringer Mannheim Canada. Acrylamide and bis-acrylamide were re-crystallized before use. All other chemicals were of reagent grade.

(2) - METHODS

(2.1) LOWRY PROTEIN ASSAY

Protein was assayed by the method of Lowry et al (98).

(2.2) ALKALINE PHOSPHATASE ASSAY

The method used was an adaptation of the method of Hausamen (99).

p-Nitrophenyl phosphate, the substrate, is split by alkaline phosphatase to free p-nitrophenol, which, in alkaline solution has a strong absorbance at a wavelength of 400 nm, at which wavelength the substrate has little or no absorption.

Unless otherwise stated, the reaction mixture (volume of 1.4 ml) contained 50 mM glycine buffer of pH 10.5, 0.5 mM MgCl₂, and 5.5 mM p-nitrophenyl phosphate. To this was added 0.1 ml of the testicular extract or fraction, for a total volume of 1.5 mls, and immediately well mixed. The initial rate of change of optical density was recorded at 400 nm in a spectrophotometer equipped with a Haake circulating waterbath maintained at 37 degrees C for approximately five minutes. Each assay was performed in, at least, duplicate and was read against a blank containing the complete reaction mixture except for the substrate. Activities are reported as μM of p-nitrophenol produced per minute at 37 degrees C per gram of tissue.

Specific activities are expressed as micromoles of p-nitrophenol released per minute, per milligram of protein using a measured molar absorbancy of 1.90×10^4 for p-nitrophenol in 0.02 N NaOH, with 0.02N NaOH for a blank.

See also Frajola (100) and Torriani (101).

(2.3) ENZYME ACTIVITY EXTRACTION WITH TRIS PLUS
TRITON X-100

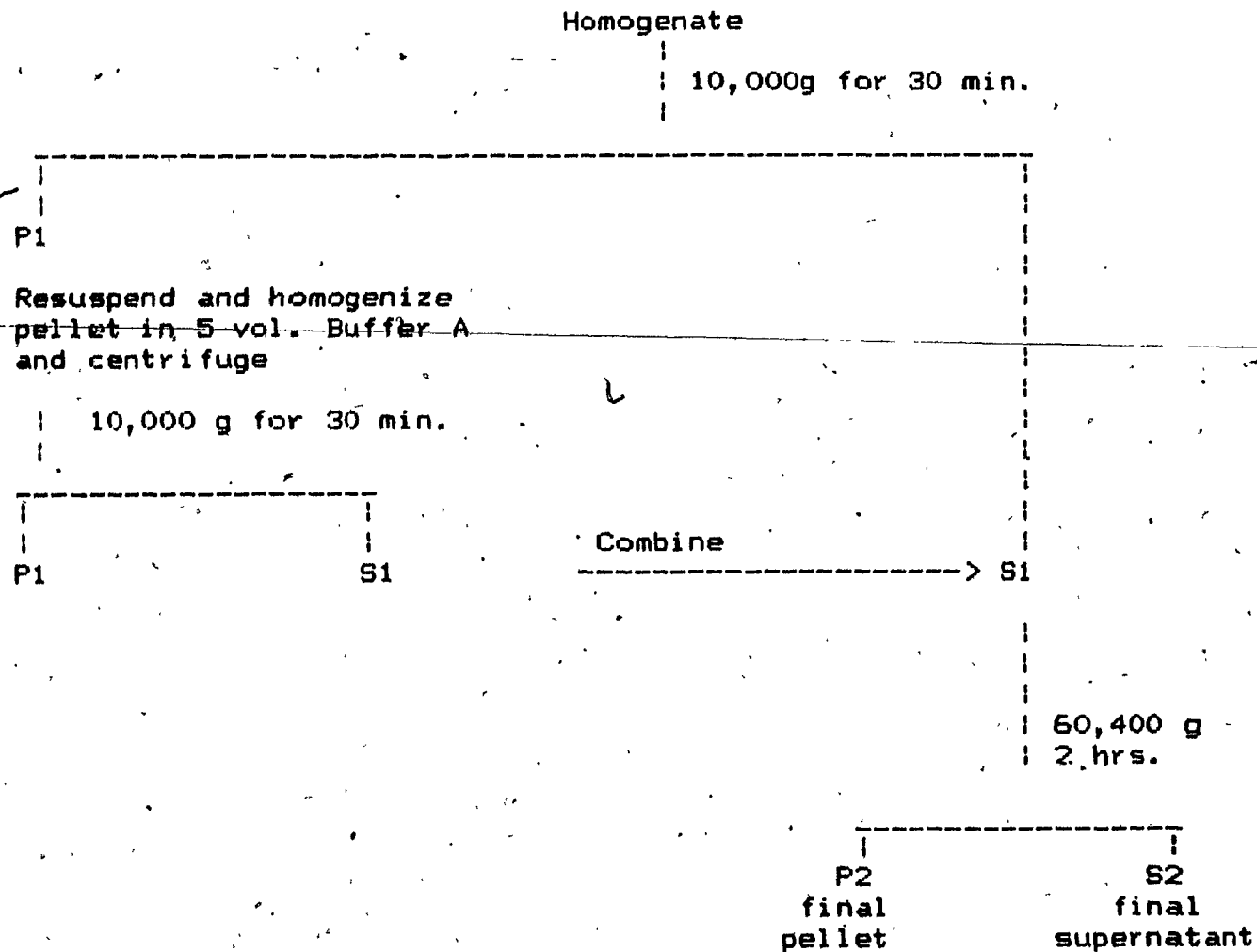
Rats were sacrificed by decapitation and their testes removed. Care was taken to remove their tunicae and as much of the blood vessels present as possible. The testicular tissue was now finely minced using a standard scalpel. This tissue was suspended in ten volumes of Tris buffer, 10 mM, pH 7.4, containing 1 % Triton X-100, 2mM MgCl₂ and 0.025 mM ZnCl₂ (102). This will be referred to as Buffer A.

Homogenization was carried out in a motordriven Potter-Elvehjen homogenizer at 500 RPM and using five strokes of the homogenizer tube. This homogenate was then centrifuged at 10,000 g for thirty minutes. The resulting pellet was rehomogenized in an additional five volumes of Buffer A and recentrifuged at 10,000 g. The pellet was kept. The combined supernatants were again centrifuged at 60,400 g for two hours in a Beckman centrifuge using a Ti-50 rotor at 25,300 rpm. Both the pellet and the supernatant were retained for analysis. All operations from homogenization onwards were done at 4 degrees C. See figure 5.

Pellets were made up to two ml with Buffer A and hand homogenized. The supernatant contained the solubilized enzyme whereas the pellets contained that portion of the alkaline phosphatase activity which was not solubilized by this treatment.

FIGURE 5

Schema for "aqueous" homogenization of rat testes



(2.4) ENZYME ACTIVITY EXTRACTION WITH TRIS-BUTANOL

The method was a variation of the method of Morton (103) Sakiyama (104).

The pre-chopped testicular tissue was homogenized at room temperature in a mixture of three volumes of n-butanol and seven volumes of Tris buffer, 10 mM, pH 7.4, containing 2mM MgCl₂ and 0.025 mM ZnCl₂. This will be called Buffer

B. The homogenate was stirred at 37 degrees C for one hour and overnight at 4 degrees C.

This was then centrifuged at 10,000 g for 30 minutes and the pellet collected. The aqueous phase of the supernatant was centrifuged at 60,400 g for two hours. Both the pellet and the supernatant were kept, as before, for analysis. The pellets were made up to two mls. with Buffer A, no Triton, and hand homogenized.

The tissue from either one adult rat or the pooled tissues from four litters of thirteen-day-old rats, around twenty animals, were used for the extracts prepared by both types of extractions.

All data reported are duplicate determinations.

(2.5) POLYACRYLAMIDE DISK GEL ELECTROPHORESIS

Electrophoresis was performed on samples of the solubilized enzyme in 6.7 % gels that were prepared according to the method of Davis (105). The following changes to his method were made: there was no stacking gel used; Triton X-100 was added to the gels to give a final concentration of 0.1 %. The reservoir buffer contained, in addition to the usual concentrations of Tris and glycine, 0.1 % Triton X-100, 0.01 % MgCl₂ and 0.00136 % ZnCl₂.

Ohkubo (106), working with rat liver extracts, reported a loss of 80 % in alkaline phosphatase activity if zinc was not included in the reservoir buffer. Activity was restored by the addition of zinc.

The solubilized enzyme extract was applied as a solution of two parts of 50 % sucrose to one part of extract to which two drops of Bromphenol Blue was added as indicator. Between 15 and 250 ug of protein were applied to the gel. The current used was 1.5mA/gel; the length of run was two hours.

Various dyes were tried to stain for alkaline phosphatase activity in the gel after electrophoresis was complete, but the best one found was Fast Violet B.

The buffer used was a 60mM Borate buffer of pH 9.7 which contained 5 mM MgSO₄, 0.05 % B-naphthyl phosphate and 0.05 % dye (107,108,109). The dye was added to the buffer just before use, and the solution was filtered. The gels were stained for 30 minutes at room temperature, then distilled water replaced the dye solution for twenty-four hours, after which a final change with distilled water was made. The alkaline phosphatase activity showed up as bands of a reddish-copper colour.

The stained gels were scanned in a recording optical densitometer.

Another method of visualizing the position of the alkaline phosphatase bands was the use of a combination of p-nitrophenylphosphate and CaCl₂ (110,111). The buffer used for this staining method contained 0.2 M tris buffer of pH 9.0, 0.2 % p-nitrophenylphosphate, 0.2 % CaCl₂, 1.5

mM ZnCl₂ and 15 mM MgSO₄. As before, the gels were stained for 30 minutes at room temperature then distilled water was substituted for the staining buffer for twenty-four hours, after which a final change with distilled water was made.

The activity appeared as a transient, quickly diffusing yellow band, followed by the appearance of a milky white band at the site of activity, due to the deposition of calcium phosphate.

(2.6) NEURAMINIDASE TREATMENT

Neuraminidase was made up in Buffer A at the strength of one percent. Another solution was also made up, identical to the first except for the subtraction of the Triton.

The protocol called for the use of 1 mg. of Neuraminidase per 1 ml. of solubilized enzyme solution. This mix was incubated at room temperature for twelve hours. Then it was stored at 4 degrees C for approximately another ten hours before use. The neuraminidase solutions were always made up fresh before use (112,113).

These solutions were used for some alkaline phosphatase activity studies.

The Neuraminidase-treated extracts destined for electrophoresis were prepared somewhat differently from above. The change consisted of incubating the alkaline-phosphatase-neuraminidase mixture at 36 degrees C for two hours, followed by twenty hours at 4 degrees C. At this point it was used for electrophoresis.

All electrophoresis experiments, including those performed with neuraminidase were done in duplicate.

(2.7) INHIBITION AND ACTIVATION OF ALKALINE PHOSPHATASE
ACTIVITY

Six amino acids including L-phenylalanine and L-Homoarginine, as well as, $MgCl_2$, $ZnCl_2$, $CdCl_2$ and Urea were added to the standard alkaline phosphatase assay to gauge their inhibitory or activating effect on the various enzyme extracts tested. They were dissolved in Buffer A at 15 times the desired concentration in the assay. The alkaline phosphatase assay was adjusted to contain: 1.35 mls of substrate buffer, 0.05 mls. of enzyme extract and 0.10 mls. of the test compound. This reaction mixture was well mixed and the change in optical density recorded, as usual, at 400 nm. Results were expressed as % activity compared to a control run concurrently without additives. All results are the average of duplicate experiments.

(2.8) HEAT STABILITY

In this test, a small amount of the solubilized enzyme solution was incubated at 56 degrees C for various times up to 90 minutes. The samples were then assayed for alkaline phosphatase activity at 37 degrees C. Results were expressed as the % of activity remaining after "x" minutes of incubation at 56 degrees C, with an untreated control, run concurrently, serving as the 100 % standard (46,51). All observations were done in duplicate.

SECTION III - RESULTS

(1) - PRELIMINARY DATA

The first part of this work was concerned with establishing conditions for the assay and electrophoresis for our extracts. For these preliminary studies the testicular tissue of one adult rat was used. The tissue was homogenized in Buffer A, as well as Buffer A minus Triton. The homogenate was only given a light centrifugation using an International Clinical Centrifuge, Model CL at Speed 3 for five minutes. This served to remove large debris and the supernatant was used for further work.

(1.1) THE EFFECT OF TRITON X-100 IN SOLUBILIZING
ALKALINE PHOSPHATASE

In the first place we wanted to see if the inclusion of Triton X-100 at 1 % in the buffer system prior to homogenization would have any effect on enhancing alkaline phosphatase activity in the supernatant fraction. Standard alkaline phosphatase assays on both supernatants indicated that the Triton containing extract exhibited 2.4 times the activity of the extract containing no Triton. The Lowry protein Assay showed little difference in protein content between these two extracts. The non-Triton extract gave a value of 5.19 mgs. protein per ml. of extract, and the sample with Triton showed 5.48 mgs protein per ml. of extract; a variation of only 5.6 %.

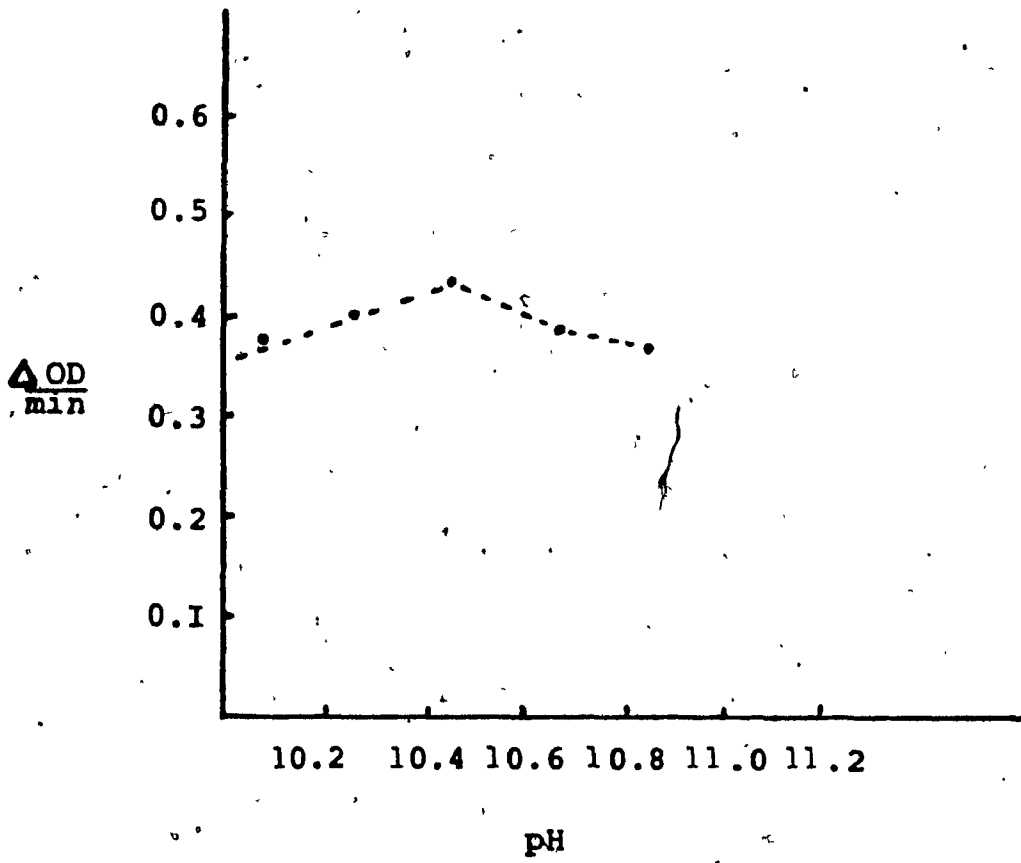
(1.2) pH - EFFECT ON ALKALINE PHOSPHATASE ASSAY

The optimum pH of the alkaline phosphatase assay was determined by varying the pH of the standard assay from 10.1 to 10.9 in increments of 0.2. Results are shown in Figure 6.

Testes alkaline phosphatase definitely shows pH sensitivity. the highest activity was found at pH:10.5. Therefore, for the balance of all further experiments this was the pH used.

FIGURE 6

Alkaline phosphatase assay - pH optimum.

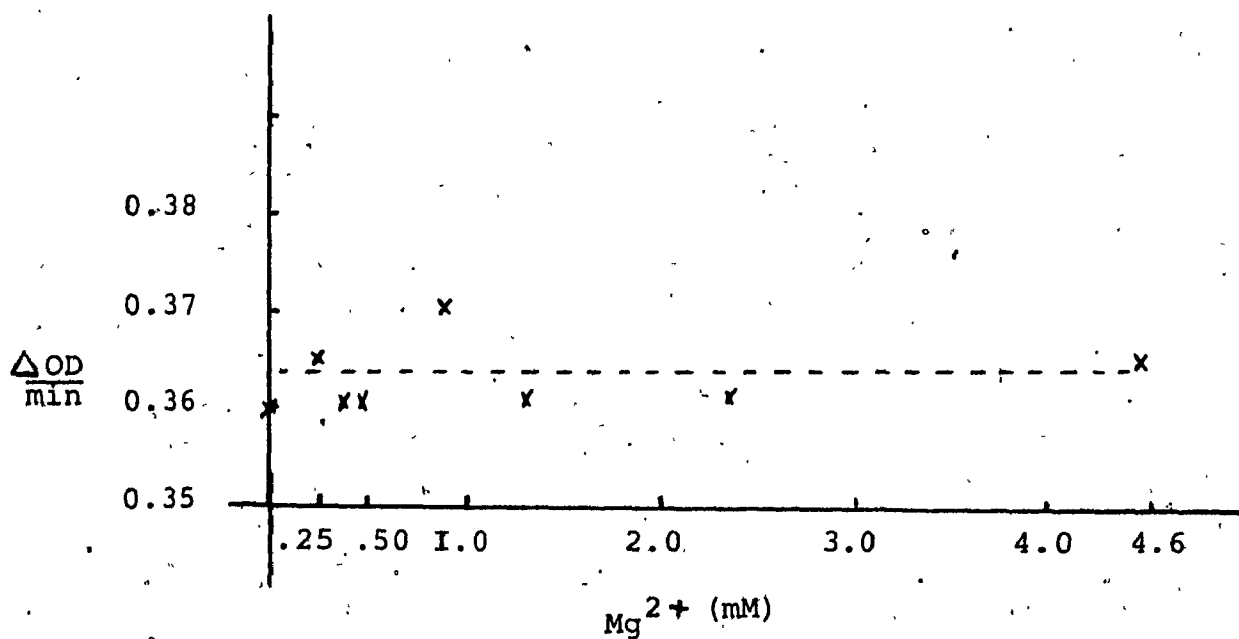


(f.3) Mg^{2+} - EFFECT ON ALKALINE ASSAY

Next, the effect of Mg^{2+} concentration on the alkaline phosphatase assay was checked. With all other variables kept constant, the Mg^{2+} concentration was varied between 0.00 mM and 4.60 mM. See Figure 7. We found no significant differences in activity with any of these Mg^{2+} concentrations.

FIGURE 7

2+
The effect of Mg concentration on alkaline
phosphatase activity



(1.4) STUDIES ON ACTIVITY-STAINING PARAMETERS IN
ELECTROPHORESIS

The literature surveyed showed that various dyes have been used as indicators of alkaline phosphatase activity on gels (107). We wanted to ascertain which dye would yield the best results with our extract. For this experiment we were now using the solubilized testicular extract from an adult rat using the Buffer A extraction and centrifugation as described in Methods.

The dyes assayed were Fast Blue B, Fast Blue BB, Fast Blue RR, and Fast Violet B. Identical quantities of extract were used for all experiments. Gels incubated with Fast Blue B showed no activity at all; Fast Blue RR showed faint staining; Fast Blue BB showed discernible but still faint bands of activity, but Fast Violet B gave strong staining of bands. In all cases this activity was demonstrated as bands of a reddish-copper colour.

One other method of visualizing the alkaline phosphatase activity on gels was tried. This was the use of the p-nitrophenyl phosphate-CaCl₂ activity stain as described in
2
Methods.

Identical gels stained with Fast Violet B and the CaCl_2 stain showed no differences in results between the two, but since the Fast Violet B gave more permanent bands of activity, this method was used in all further work.

The literature suggested that these various dyes stained best at elevated temperatures such as 37 degrees C; needed long staining times, up to two hours; and had to be performed in the dark. We set out to see if these parameters fit our system as well. In a series of electrophoretic experiments we tried various combinations of staining times from 0.5 hrs. to 2 hours; two temperatures: 20 degrees C and 37 degrees C; and ascertained whether ordinary indoor fluorescent lighting had any effect on staining.

The summary of this set of data resulted in the finding that darkness was not a prerequisite for optimum staining, that staining worked equally well at 20 degrees C as at 37 degrees C, and that one half hour staining was quite sufficient for full staining activity. Further work was therefore performed at 20 degrees C with one half hour staining under regular lighting conditions.

(1.5) ENZYME KINETICS

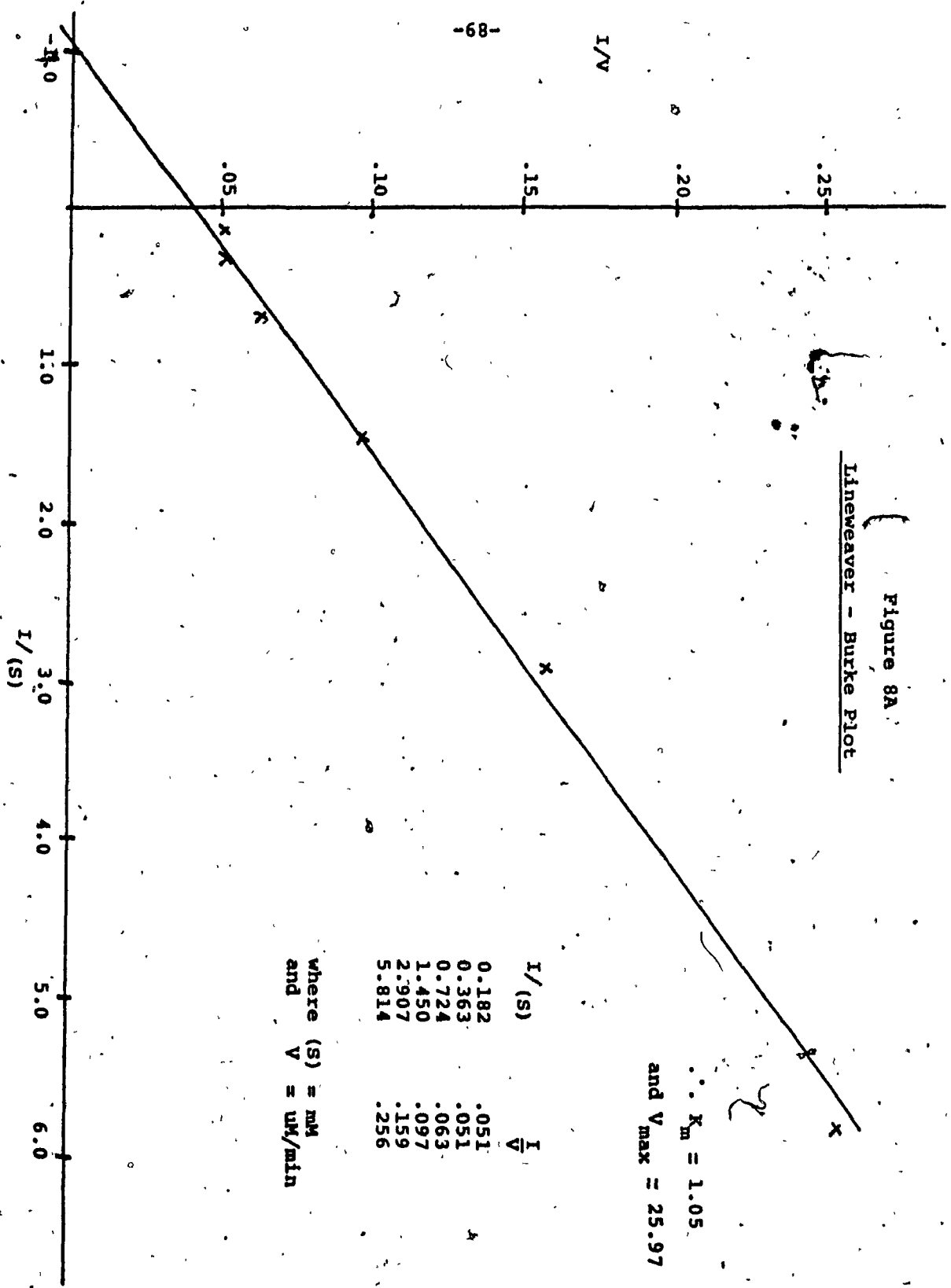
K_m and V_{max} were determined for this extract. The resulting values were plotted in three ways. See figures 8A, B, and C. The Lineweaver-Burke plot gave values 1.05 mM for K_m and 25.97 $\mu\text{M}/\text{min.}$ for V_{max} . The Eadie-Hofstee plot corroborated these results quite well, although the slope of the line could be varied; however I drew the best fit for this plot. Using this line, K_m had a value of 1.00 and V_{max} was 26.00 (114).

The nature of the Eisenthal-Cornish-Bowden plot is such that unless one obtains a perfect set of readings to plot, the values K_m and V_{max} can only be expressed as a range of values. In this case, the values I obtained were the following:

$$K_m = 0.85 - 1.12 \text{ and } V_{max} = 25.6 - 27.6.$$

As far as the V_{max} values are concerned, I must mention that I was working with impure alkaline phosphatase.

Figure 8A
 Lineweaver - Burke Plot



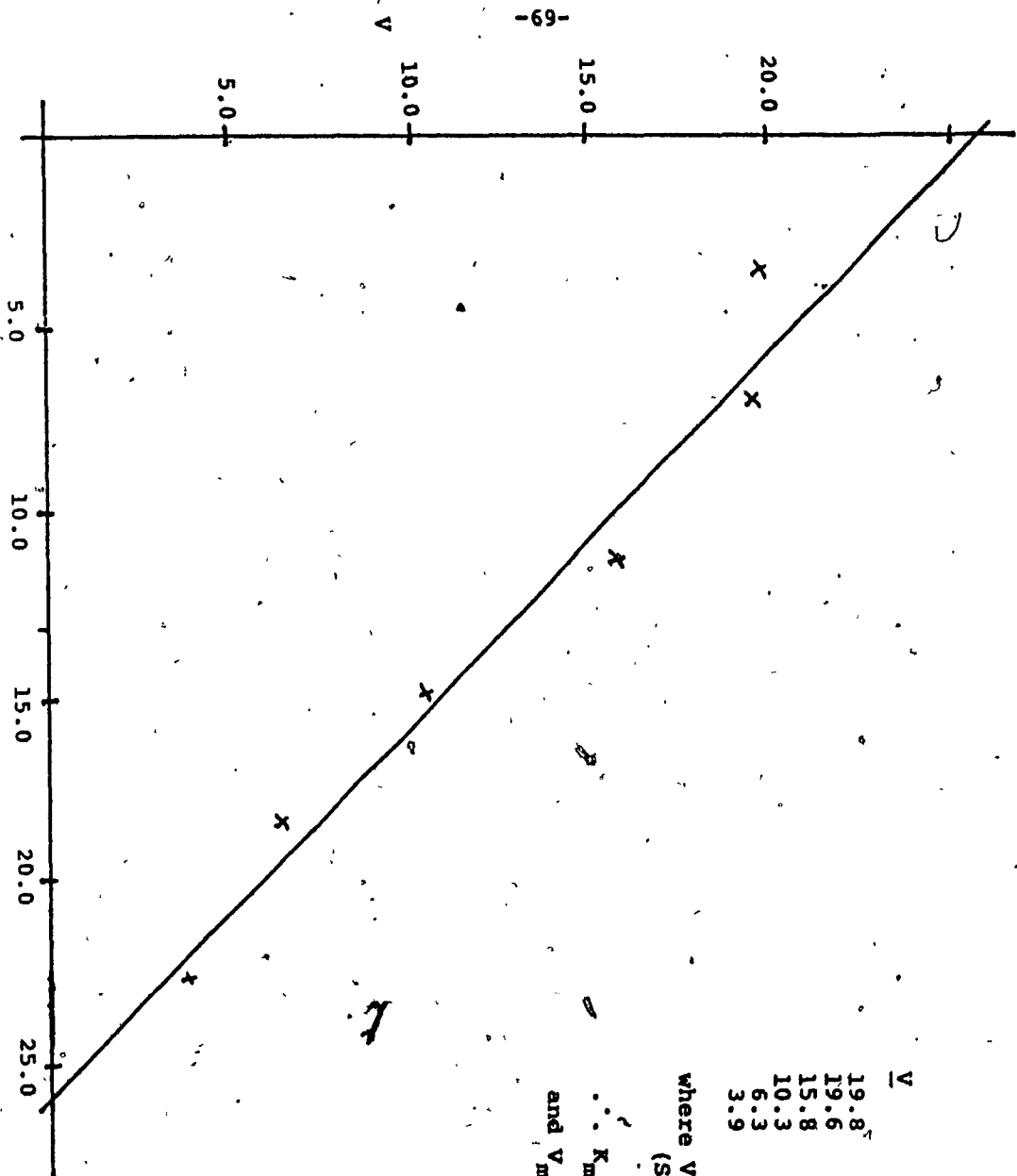
$\therefore K_m = 1.05$
 and $V_{max} = 25.97$

$I/(s)$	$\frac{1}{V}$
0.182	0.051
0.363	0.051
0.724	0.063
1.450	0.097
2.907	0.159
5.814	0.256

where (S) = mM
 and V = $\mu M/min$

Figure 8b

Edie - Hofster Plot



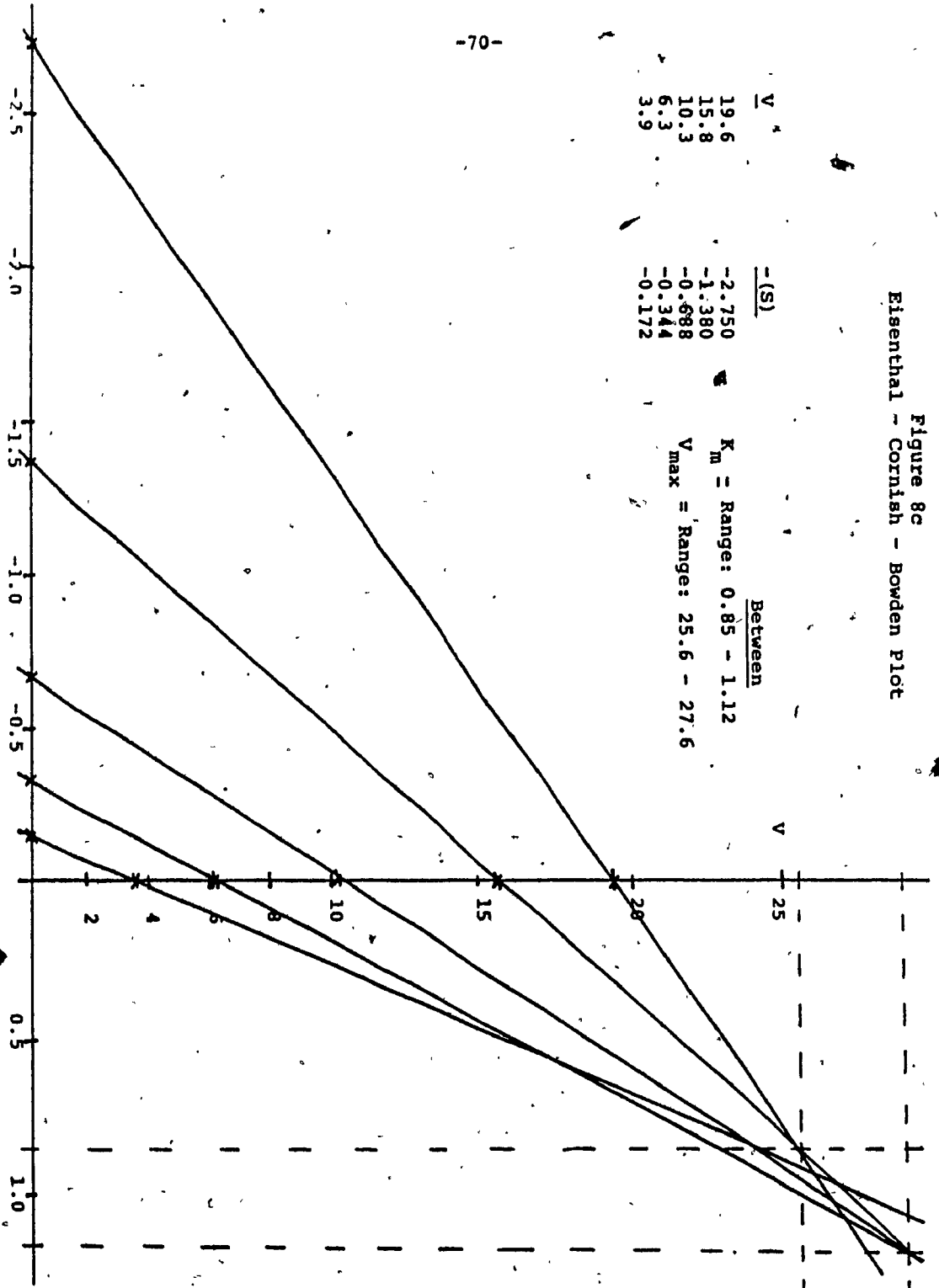
where $V = \mu\text{M}/\text{min}$
 $(S) = \text{mm}$
 $\therefore K_m = 1.00$
 and $V_{\text{max}} = 26.00$

Figure 8c
Eisenthal - Cornish - Bowden Plot

V	-(S)
19.6	-2.750
15.8	-1.380
10.3	-0.688
6.3	-0.344
3.9	-0.172

Between
 $K_m = \text{Range: } 0.85 - 1.12$
 $V_{max} = \text{Range: } 25.6 - 27.6$

-70-



5

2 - ACTIVITY STUDIES

(2.1) STUDIES ON TOTAL EXTRACTS BEFORE CENTRIFUGATION

Total alkaline phosphatase activities; total protein content per gram of wet tissue weight, as well as the specific activities were calculated for samples extracted by the Buffer A and Buffer B methods.

These data are presented in Table 5. Rats of two different ages; juvenile (13-day-old) and adult (105-day-old) were used.

Results showed that the testes of rats contained substantial quantities of alkaline phosphatase.

Looking at the Buffer A extracts only, the total activity of adult rats exceeded that of 13-day-old-rats by some 30 %, however, the quantity of total protein in the adult testes was also elevated by some 8 %. Overall, total activity, protein and specific activity of the 13-day-old rat were lower than the adult, although the differences were not great.

The buffer B extraction method showed broadly similar findings. The levels of total activity found for both ages were lower than comparable findings by the Buffer A method, as were the levels of total protein, however the same ratios between the adult and the 13-day-old rat persisted. One must note though, that the differences, in any case, are not major, amounting to some 20 - 30 % and within the statistical deviations calculated.

The specific activities for both ages are lower than those found by the Buffer A method, but the difference between the values for the adult and infant rats are less. In this case, the difference was only 16 %.

Comparing the two extraction methods in terms of the values obtained from the crude extract, the Buffer A extract shows higher total activities, higher total proteins and slightly higher specific activities. These differences are however comparatively small and within the limits of error.

Samples of liver tissue from adult rats was included as a comparative tissue and since it has been reported to contain one of the major isozymes. This sample showed much much less alkaline phosphatase activity; showed double the total protein values of testes, and thus liver specific activities were quite low.

TABLE 5

Activity Studies I

Tissue	Total Activity (μ moles/min./ gr. tissue)	Total Protein (mgs/gr. tissue)	Spec. Activity (μ moles/min/ mg. protein)
--------	--	-----------------------------------	--

A) Buffer A Extract

13-day-old rat testes (4)	5.59 + 0.92	103.03 + 20.22	0.054 + 0.014
Adult rat testes (6)	7.27 + 1.38	110.31 + 21.14	0.066 + 0.019
Adult rat liver (2)	0.70 + 0.03	218.80 + 30.09	0.003 + 0.000

B) Buffer B Extract

13-day-old rat testes (2)	4.40 + 0.22	89.31 + 8.24	0.049 + 0.007
Adult rat testes (4)	5.30 + 1.48	92.43 + 21.44	0.057 + 0.019
Adult rat liver (1)	0.28	171.76	0.0016

Numbers in parentheses are the number of determinations for that set of values.

(2.2) RELATIVE ALKALINE PHOSPHATASE ACTIVITY OF EXTRACT FRACTIONS

As mentioned above, the crude extracts resulting from the Buffer A and Buffer B extractions were centrifuged, resulting in two pellets, labelled P₁ and P₂ and a supernatant fraction labelled S₂ as per Figure 4.

The amount of alkaline phosphatase contained in these fractions was quantified and compared as demonstrated in Table 6. At this point, it should be mentioned that, with the Buffer B extraction method only, at the end of the first centrifugation step, there was, in addition to the pellet P₁ and the supernatant S₁, a thin layer of a beige-light-brown colored, semi-soft substance between the aqueous and butanol layers in the centrifuge tube. This substance, labelled "Semi-solid" was collected and analyzed for both alkaline phosphatase activity and protein content.

Analysis of the data in Table 6 shows little activity in the pellets from the Buffer A extracted rats of both ages, with most of the alkaline phosphatase activity (70-80%) present in solubilized form in the supernatant.

A simple analysis of the Buffer B extracted testes is more problematical because of the presence of the semi-solid.

If we assume that this brown substance is an aggregate of butanol-lipoprotein-lipid-soluble alkaline phosphatase, then we can say that, as with the Buffer A extract, most of the alkaline phosphatase activity (80-95%) is present in the solubilized form. This assumption should be valid since the large majority of particulate matter should have sedimented in the first sedimentation of 10,000 g for thirty minutes.

TABLE 6

Activity Studies 2

<u>Tissue</u>		<u>% activity</u>	<u>% protein</u>	<u>Specific Activity</u> (<u>umoles/min/</u> <u>mg.protein</u>)
<u>A) Buffer A Extract</u>				
13-day-old rat testes	P1	23.2	30.0	0.041
	P2	7.2	13.0	0.030
	S2	69.6	57.0	0.066
Adult rat testes	P1	14.8	22.0	0.044
	P2	5.4	13.0	0.028
	S2	79.8	65.0	0.081
<u>B) Buffer B Extract</u>				
13-day-old rat testes	P1	3.4	13.0	0.013
	P2	1.4	2.0	0.038
	S2	50.9	15.0	0.172
"semi-solid"		44.3	71.0	0.031
Adult rat testes	P1	12.7	23.0	0.032
	P2	7.8	3.0	0.160
	S2	59.8	12.0	0.300
"semi-solid"		19.7	63.0	0.018

% activity and % protein is expressed as % of total activity and total protein as shown in Table 5.

(2.3) RELATIVE PROTEIN CONTENT OF EXTRACT FRACTIONS

This data is presented in tabular form in Table 6. A substantial portion of the protein extracted by the Buffer A method (both ages) was found in the soluble fraction (~56-65%). However, in the case of the Buffer B extracts, the "semi-solid" substance was found to contain the greater part of the protein (63-71%), whereas the soluble S₂ fraction contained little.

(2.4) INHIBITION AND ACTIVATION OF ALKALINE PHOSPHATASE
ACTIVITY

The results in this and further sections is based on work performed with the soluble S fractions only. Ten small organic and inorganic molecules mentioned previously in the literature were included in alkaline phosphatase assays to gauge their inhibitory or activating effect on activity.

The results, expressed as the % activity of a control sample remaining after treatment are presented in Table 7.

L-Phenylalanine has little inhibitory effect on activity, whereas L-Homoarginine inhibits greatly. L-Leucine, L-Isoleu, L-Leuglygly and L-Tryptophan all inhibited to a modest degree.

CdCl_2 had very little inhibitory effect. MgCl_2 almost none, and ZnCl_2 was a definite activator.

Urea at 3M had no effect either way.

There were no significant differences in inhibition and activation patterns between 13-day-old and adult rats; and similarly little difference between the two extraction methods.

A sample of rat liver tissue, worked up in the same manner as rat testes showed very similar results when treated with the amino acids mentioned above.

TABLE 7

Inhibition studies (in % activity of control)

Additive	Conc. (mM)	Adult Rat Testes (3)		13-Day-Old Rat Testes (2)		Adult Rat Liver (1)	
		A.	B.	A.	B.	A.	B.
L-Phe.	2.5	90.0	93.0	100.0	100.5	90.0	94.0
L-Leu.	5.0	76.7	73.3	81.0	81.5	55.0	76.0
L-Leuglygly	5.0	59.7	67.7	74.5	77.0	65.0	82.0
L-Har.	5.0	33.3	27.7	30.0	29.0	35.0	35.0
L-Try.	5.0	72.0	73.7	76.0	77.0	55.0	53.0
L-Isoleu	5.0	76.0	85.0	93.0	91.5	65.0	71.0
CdCl ₂	0.1	72.0	93.0	90.5	87.5	----	----
MgCl ₂	10.0	91.5	100.5	82.0	90.0	----	----
ZnCl ₂	1.0	105.0	116.5	109.5	114.5	----	----
Urea	3000.0	95.0	106.0	100.0	104.0	----	----

A. = Buffer A extraction. 1

B. = Buffer B extraction

Numbers in parenthesis refer to the number of different rat samples assayed and the results averaged.

(2.5) HEAT INACTIVATION OF ALKALINE PHOSPHATASE ACTIVITY

As mentioned above, heat treatment of alkaline phosphatase in various human and rat tissues was used by various researchers to partially characterise the enzyme. We subjected the solubilized enzyme derived from 13-day-old and adult rats to heat treatment. We also compared the effects of this treatment on the enzyme extracted via Buffer A and Buffer B.

These various samples were subjected to 56 degrees C in a water-bath for various lengths of time. Refer to Table B. After ten minutes at least 55 % of control activity was lost, and after 30 minutes, 90 % of the activity was gone, indicating that this form of the enzyme is highly heat-labile.

There were no differences in the rate of inactivation between adult and infant sample, however the alkaline phosphatase extracted by the Buffer B method was even more sensitive to heat than that obtained by the Buffer A method. These butanol extracts lost 98 % of activity after only 10 minutes. Within this group there were no differences between adult and infant extracts.

The adult liver sample added as a comparison extract behaved very similarly compared to the adult testes sample regardless of method of extraction.

TABLE 8

Heat Treatment of Alkaline Phosphatase
(in % activity remaining after treatment)

Time (min.)	Adult Rat Testes(3)		13-Day-Old Rat testes(2)		Adult rat Liver(1)	
	A	B	A	B	A	B
0	100.0	100.0	100.0	100.0	100.00	100.0
10	32.3	2.3	32.0	0.8	45.0	0.0
20	20.0	1.1	20.0	0.5	18.0	0.0
30	11.3	--	9.5	--	--	--
60	8.0	--	6.0	--	--	--
90	7.0	--	4.5	--	--	--

A = Buffer A Extraction

B = Buffer B Extraction

Numbers in parenthesis refer to the number of different rat samples assayed and the results averaged.

(2.6) EFFECT OF INHIBITORS ON HEAT-TREATED ALKALINE
PHOSPHATASE

Because of the findings of Chang (115), (see Discussion) and because we found at least some activity remaining after 90 minutes of heat treatment of the Buffer A extract, we decided to check if there was a second minor isozyme present in our Buffer A extract. The Buffer B extract presented no such question since 98 % of its activity was lost after only 10 minutes. The enzyme from one adult rat was used for this study. An aliquot of this was heat-treated at 56 degrees C for ten minutes and the loss of activity recorded. This treated sample was now considered as the control sample and L-Phe and L-Har were separately added to it, as described in Methods, and the degree of inhibition recorded. This data is shown in Table 9.

Results showed that the heat-treated alkaline phosphatase extracted by the Buffer A method, showed the same inhibition pattern with L-Phe and L-Har as did the untreated enzyme.

TABLE 9

Inhibition of heat-treated alkaline phosphatase by
L-Phe and L-Har (in % activity remaining after treatment)

<u>Treatment</u>	<u>Amino Acid only</u>	<u>Heat only</u>	<u>Heat & Amino Acid</u>
NIL	100.0	100.0	----
L-Phe(2.5mM)only	90.0	-----	-----
L-Har(5.0mM)only	33.3	-----	-----
56 degrees C for 10 min.	-----	37.8	100.0
56 degrees C for 10 min. + L-Phe(2.5mM)	-----	-----	92.0
56 degrees C for 10 min. + L-Har(5.0mM)	-----	-----	31.0

(2.7) EFFECT OF NEURAMINIDASE TREATMENT ON ALKALINE
PHOSPHATASE ACTIVITY

The soluble alkaline phosphatase activity of adult and infant rat testes extracted by both extraction methods was treated with neuraminidase as described in Methods (112).

The treated extracts were then assayed for alkaline phosphatase activity versus an untreated sample as control. Results are presented in Table 10.

Neuraminidase treatment had little effect on the activity of either the adult or infant samples extracted by the Buffer A method (less than 10 % loss of activity). Somewhat different results were observed with the samples extracted by the Butanol method. Neuraminidase deactivated the Adult sample by only 13 1/2%, however the infant sample was deactivated by 38 %.

TABLE 10

Alkaline phosphatase activity of Neuraminidase-treated extracts
(in % activity remaining after treatment)

	Adult Rat Testes (2)		Infant Rat Testes (1)	
	A	B	A	B
% Activity	93.5	86.5	90.0	62.0

A = Buffer A extraction

B = Buffer B extraction

Numbers in parenthesis refer to the number of different rat samples assayed and the results averaged.

3 - POLYACRYLAMIDE DISK GEL ELECTROPHORESIS

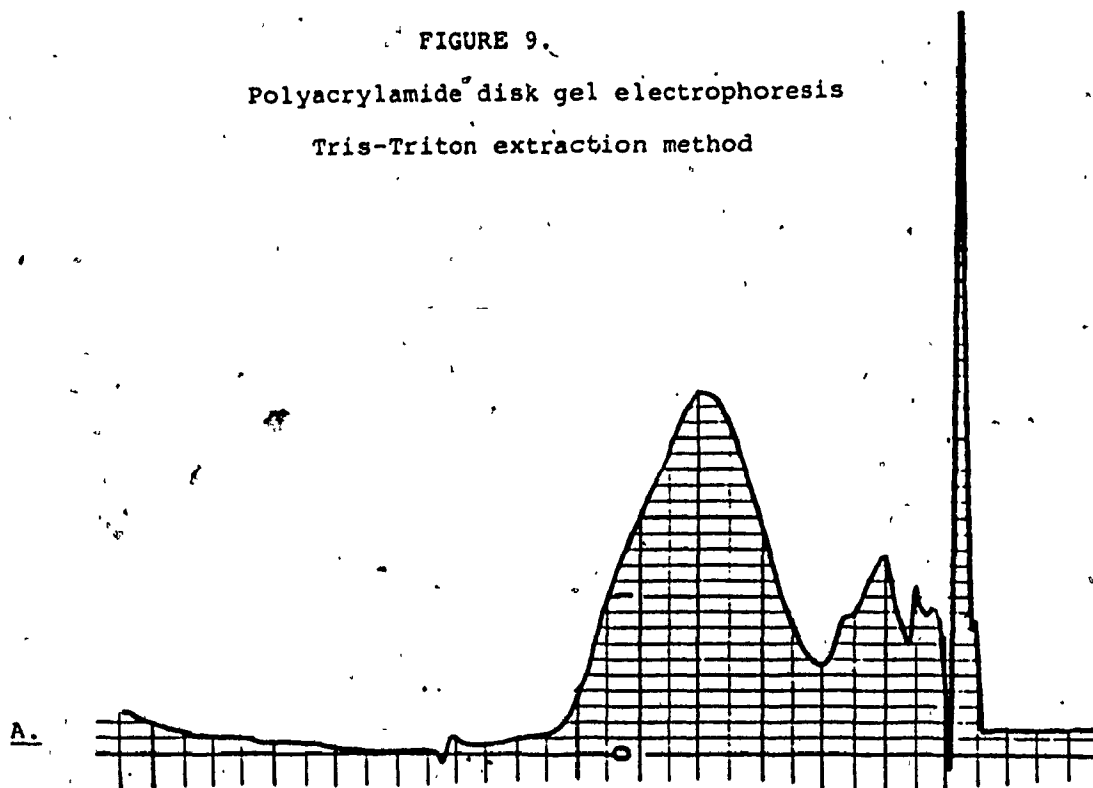
The solubilized enzyme extract obtained by both extraction methods was used for this study.

Polyacrylamide disk gels were prepared, and samples run and stained as described in Methods.

Considering the Buffer A extracts first, extracts from adult and 13-day-old rats gave extremely similar results. See figure 9. Two bands of alkaline phosphatase activity were observed; a quite sharp band close to the origin and a second, more diffuse band, but containing more alkaline phosphatase, somewhat farther into the gel.

Similarly, two bands of activity were noted with both the Adult and 13-day-old rat extracts prepared via the Buffer B method. See Figure 10. These two bands were, moreover, in the same positions as the bands seen with the previous method. However, their relative intensities had reversed. The band close to the origin was still the sharper one but it also had most of the activity, while the second band, which had moved further into the gel was more diffuse and fainter than the corresponding band seen with the Buffer A extracts.

FIGURE 9.
Polyacrylamide disk gel electrophoresis
Tris-Triton extraction method



A = Adult rat testes extracts, not neuraminidase treated
B = 13-day-old rat testes extracts, not neur. treated.

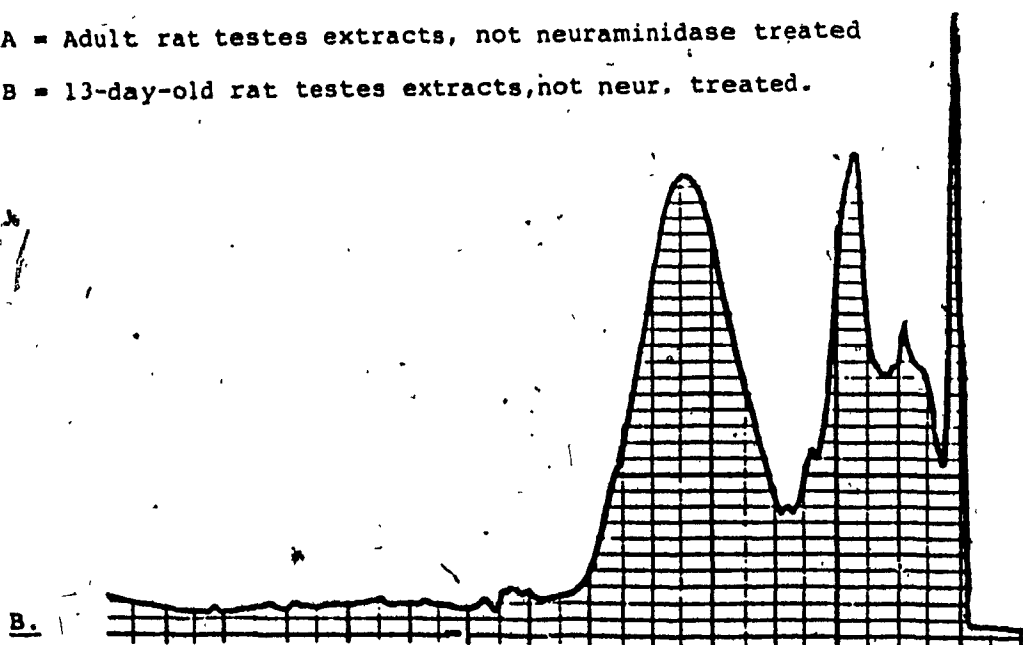
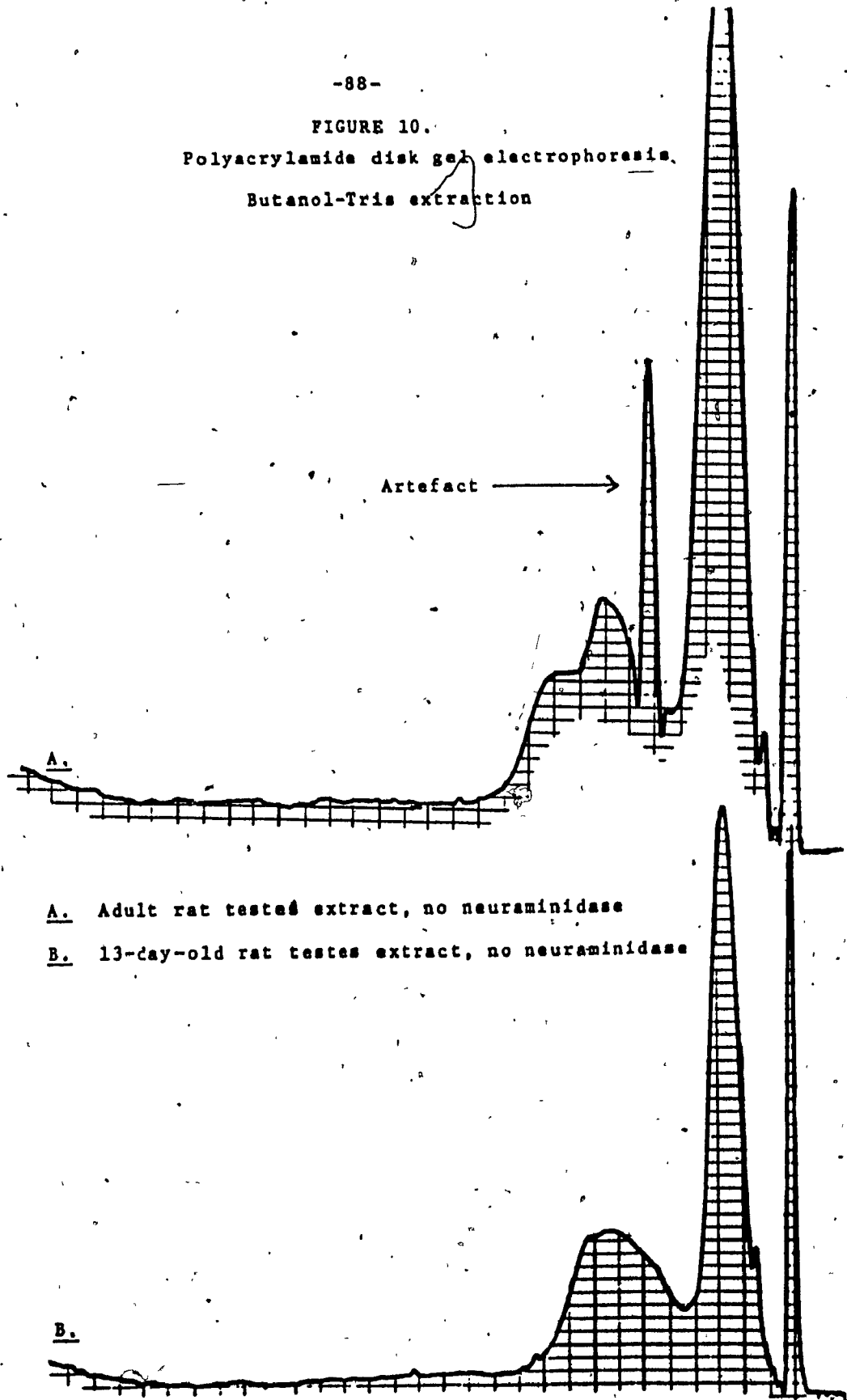


FIGURE 10.
Polyacrylamide disk gel electrophoresis,
Butanol-Tris extraction



A. Adult rat testes extract, no neuraminidase

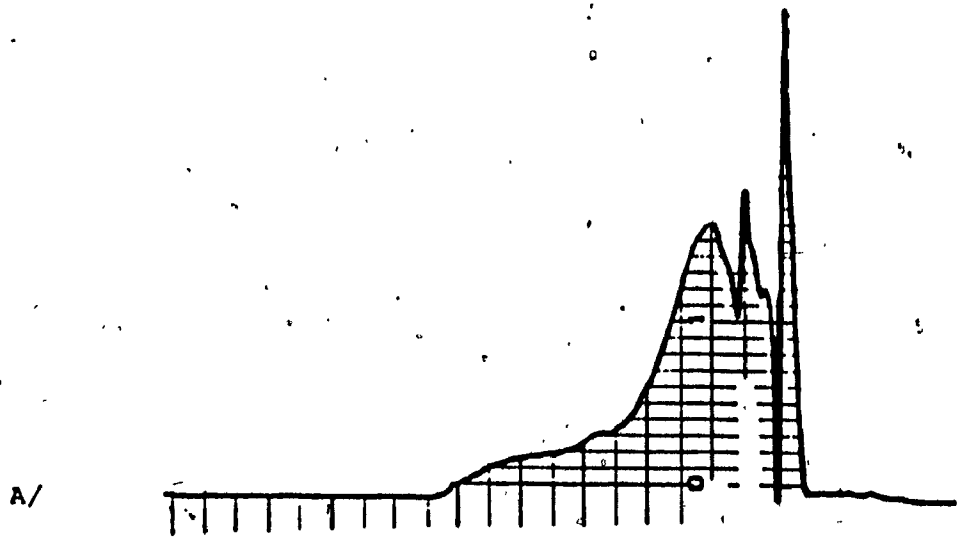
B. 13-day-old rat testes extract, no neuraminidase

(3.1) ELECTROPHORESIS OF NEURAMINIDASE
TREATED, ALKALINE PHOSPHATASE

Using the Buffer A extracts, one notes, See Figure 11, that the Adult and 13-day-old rat extracts still show quite similar bands. There are still two bands of activity but the mobility of both have been retarded. The majority of the activity is still in the band further from the origin.

The neuraminidase-treated Buffer B extracts show some differences from the above. See Figure 12. The infant rat extract shows two bands of activity, both displaced further towards the origin compared to the untreated samples. However, the band further from the origin has now become quite faint, and the band next to the origin is very strong and has hardly moved into the gel. The treated adult shows only one very strong band, again very close to the origin. There is perhaps a hint of a second shoulder band in the position where the corresponding second band is in the 13-day-old rat.

FIGURE 11
Polyacrylamide disk gel electrophoresis
Tris-Triton extract



A = Adult rat testes extract, neuraminidase-treated.
B = 13-day-old testes extract, neuraminidase-treated.

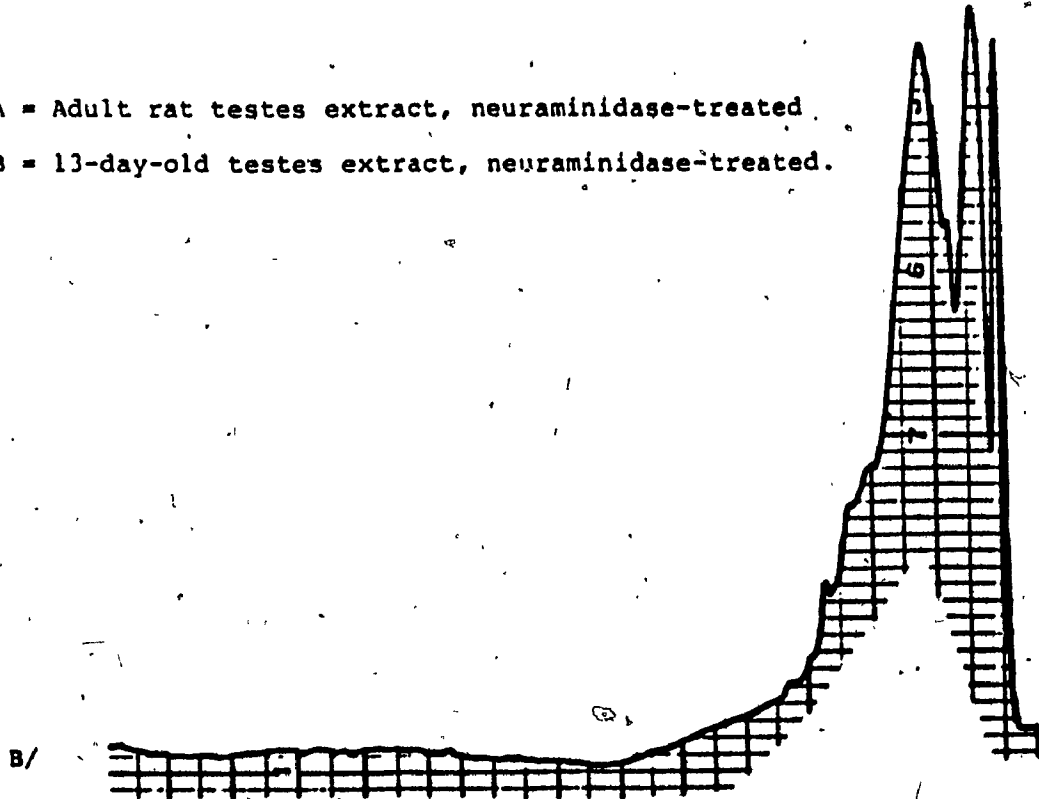
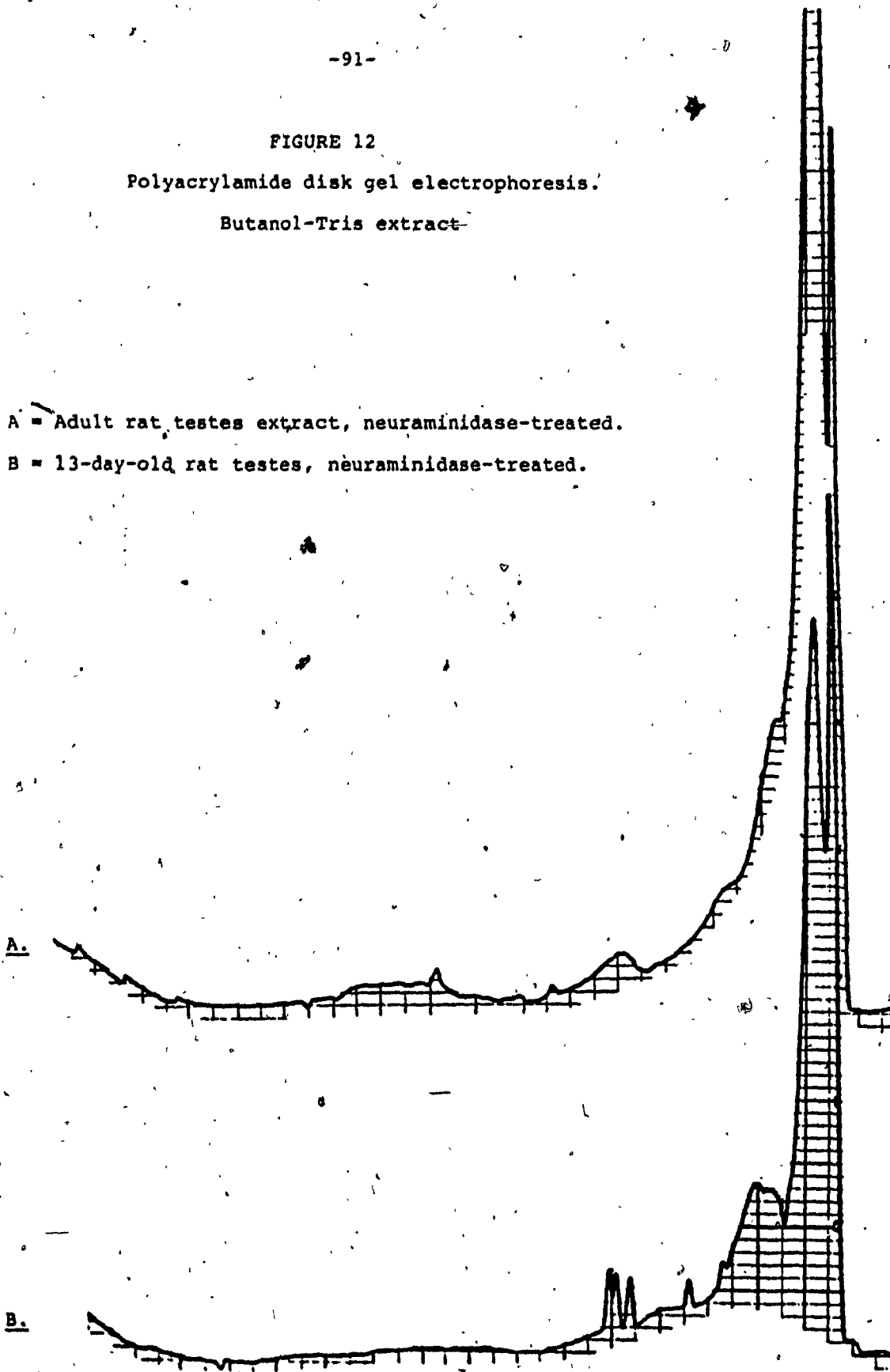


FIGURE 12
Polyacrylamide disk gel electrophoresis.
Butanol-Tris extract

A = Adult rat testes extract, neuraminidase-treated.
B = 13-day-old rat testes, neuraminidase-treated.



SECTION IV - DISCUSSION

This work had three purposes:

A) To characterise the alkaline phosphatase extracted from whole rat testes. This was achieved by measuring certain well-defined variables such as total and specific activity levels; total protein content, inhibition patterns and others;

B) To compare and contrast two methods of extracting this enzyme activity: the aqueous Tris-Triton buffer system used by a number of researchers (102), and the Butanol system introduced by Morton (103) and used by others (97,104);

C) And thirdly, we wanted to see if there were any developmental changes in the isozyme patterns of alkaline phosphatase as the rat goes from a very immature state, where the testes contain basically only spermatogonia, to fully mature rats, where all the stages of spermatogenesis coexist. This type of comparison has only been done for rat liver and placentas (116), not for testes.

The preliminary work, to determine optimum extraction and enzyme assay conditions, was performed to ensure that we would extract all or most of the enzyme from the tissues, and in order not to leave any uncharacterised. This work was performed on the extract from an adult rat using the Buffer A extraction method.

The presence of Triton X-100 in the extraction buffer significantly increased the amount of alkaline phosphatase in the soluble fraction. This result was expected due to the mildly detergent action of the Triton. We wanted the greatest possible concentration in the soluble fraction for the upcoming electrophoresis experiments.

Our findings indicated that varying Mg^{2+} concentration from 0.0mM to 4.6mM in the alkaline phosphatase assay had no effect on activity levels. This is consistent with the results of Pickering (11), examined five different rat tissues and reported that only the intestinal extract showed greatly increased alkaline phosphatase activity with added Mg^{2+} . Simon and Sutherland (13), working with rat livers also reported little increased activity of EDTA-treated extracts with increasing Mg^{2+} concentration. These result are not surprising given the native presence of this ion in a crude extract in both a free and also enzyme-bound state.

However, since all other workers added Mg^{2+} to their assays, we also included a discreet amount.

We found that the optimum pH for testes alkaline phosphatase was 10.5. This was in excellent agreement with the optimum pH reported by Yokota (117) and Nakasaki (16), both of whom worked with rat liver extracts. Fishman^B, using various human organ extracts, reported pH optima ranging from 9.0 to 10.7, while Van Belle (45) has recorded pH optima between 9.9 and 10.2 for various dog tissues.

Our calculated K_m for testes alkaline phosphatase was 1.01 mM. This compares with values of 2.4 mM and 2.8mM reported by Yakota (117) and Nakasaki (16) respectively, using rat liver extracts and p-nitro-phenylphosphate as substrate; and a value of 0.95 mM reported for rat intestines by Fishman (118).

The differences in specific activity between infant and adult rats was quite small. the infant rat showed some 16.18 % less specific activity than the adult regardless of the extraction method.

This somewhat greater specific activity in the adult might mean that somewhat more alkaline phosphatase is being produced in the adult, but in my opinion it does not amount to much in significance. Essentially, very similar amounts of alkaline phosphatase are produced by, both adult and infant rats, nor is alkaline phosphatase production turned on or off, as far as I can tell, at any time during the timespan studied.

Of more interest perhaps is the finding that there is less total alkaline phosphatase extracted by the Buffer B or Butanol method, regardless of the age of the animal, than by the Buffer A or Tris-Triton method. There is also less protein found, but the difference is not as great as the drop in alkaline phosphatase levels. Since duplicate extractions (i.e. both Buffer A and Buffer B) were usually performed on the same rat testes tissue, I would conclude from this that the Buffer B extraction method is harsher to the protein present since some of the alkaline phosphatase (as well as other proteins) is destroyed in the procedure. This interpretation agrees with Fraki (102) who, working with guinea pig thymus, reported that Tris-triton extracted 10X the activity that Butanol did.

However - it should be noted that the Buffer B method is more successful in solubilizing a greater proportion of proteins, including alkaline phosphatase than the Buffer A method (80-95 % vs. 70-80 %). A large proportion of proteins is trapped in the "semi-solid" phase leaving the Supernatant (S2) enriched in alkaline phosphatase and relatively low in other proteins. This fact could be put to good use in further efforts to purify the enzyme.

Treatment with neuraminidase did not appreciably affect activity levels, except for the Buffer B-infant extract, which lost 38 % of its original activity. However, this was only a single (duplicate) determination. Basically identical results with various human organ extracts was reported by Mulivor (35).

As mentioned in the Introduction, alkaline phosphatase has been postulated to exist in at least three different isozymic forms: the so-called liver-bone-kidney; the placental, and the intestinal forms. These three forms may be differentiated and characterized by amino acid inhibition (36,37,43,46), heat inactivation (46,50,51), polyacrylamide gel electrophoresis patterns (52,53,54), and the effect of neuraminidase treatment on these patterns (35,54,55).

The tests conducted with various inhibitors and activators yielded valuable and conclusive data. Of great importance was the finding that regardless of the method of extraction used, or the age of the rat, the pattern of inhibition found was the same.

L-Phenylalanine does not inhibit testes alkaline phosphatase activity, whereas L-Homoarginine inhibits greatly. L-Leucine, L-Leuglygly and L-Tryptophan were used by Mulivor (35) and Lin (119) to further characterize and differentiate the alkaline phosphatase from tissues they were working with. Their results are compared to my finding in Table 11.

The pattern of amino acid inhibition shown by testes alkaline phosphatase corresponds to the profile for the liver-bone-kidney isozyme. There is no contradictory evidence.

Our liver sample behaved as expected from the literature search; that is, it was also greatly inhibited by L-Homoarginine but not by L-Phenylalanine.

Fraki (102) used various chlorides and urea to differentiate the isozymes from various guinea pig tissues.

CdCl₂ inhibited all tissues except intestines; MgCl₂ activated all tissues except liver, and urea inhibited placental enzyme greatly (90 %) and all the others moderately (30-40 %). ZnCl₂ did not have much effect. I could not show any parallels to Fraki's work, CdCl₂ and MgCl₂ inhibited my extract but slightly; urea had no effect whereas ZnCl₂ was somewhat stimulatory. With minor variations all my extracts were inhibited to the same degree with these various salts.

The next step in characterising the enzyme was the treatment of our soluble extracts at 56 degrees C for various lengths of time. All samples, whether infant or adult or whether extracted by Buffer A or Buffer B showed great sensitivity to heat, however there were some differences in the degree of inactivation dependent on the method of extraction.

If the Buffer A or aqueous method was used, the alkaline phosphatase of both adult and infant extract was 90 % deactivated after half an hour of treatment. Samples extracted via the Buffer B or Butanol method were deactivated by 98 % after only ten minutes.

This greater heat-lability of the Butanol-extracted alkaline phosphatase might be due to the effect of butanol on the structure of alkaline phosphatase. Butanol may strip off certain surface lipids or lipophilic compounds which serve to stabilize the structure of alkaline phosphatase.

Once again, the sample of rat liver extract included in this study behaved very similarly to testes extracts.

This demonstrated pattern of heat-lability is characteristic of the liver-bone-kidney isozyme (50).

Chang (115), working with human testes extracts found that this alkaline phosphatase was also strongly inhibited by L-Homoarginine and but slightly inhibited by L-Phenylalanine. It was also highly heat-labile, losing at least 95 % of total activity after thirty minutes of treatment. However, a small proportion (0.3 to 4.6 %) of the activity persisted and showed great resistance to heat denaturation. Suspecting a minor isozyme, Chang treated this heat-resistant portion with L-Phe and L-Har with the result that this alkaline phosphatase was found to be inhibited by L-Phe (63-93 % loss of activity), but basically unaffected by L-Har (0-10 % losses). This profile of heat stability corresponds to the placental and intestinal-type of isozyme.

Since our testes extracts also showed some residual activity even after 90 minutes of heat treatment we decided to see if Chang's result paralleled our own. Therefore, we treated the heat-resistant portion of our alkaline phosphatase extracts with L-Phe and L-Har. However this heat-stable portion showed the same inhibition pattern as the untreated enzyme, i.e., the liver-bone-kidney type. Thus we could find no evidence for a minor isozyme in our testes alkaline phosphatase.

Moreover, if some significant portion of the heat-treated alkaline phosphatase such as 10-20 % were present as a minor isozyme having a different (opposite) inhibition pattern to the major isozyme, then we should expect to see a drop in activity after treatment with L-Phe, and an increase in activity after treatment with L-Har as compared to the non-heat-treated sample. In fact such was not the case. If however a minor isozyme was present at very low levels, i.e. < 2 % of total alkaline phosphatase our test probably would not have detected it.

Polyacrylamide gel electrophoresis gave extremely similar results for all samples regardless of age or method of extraction: two bands of activity, one sharp well-focused band near the origin, and a second broad, more diffuse band further into the gel.

See figure 9 and 10.

Neuraminidase treatment reduced the mobility of both bands in all cases.

These findings indicate that the alkaline phosphatase extracted from rat testes contains N-acetylneuraminic acid. See Figures 11 and 12.

Although there are no other authors who have done similar work with rat testes, we can compare these findings with electrophoresis results from other rat tissues.

Yokota (117), working with rat liver alkaline phosphatase, found two extremely close bands on 7.5 % gels at the approximate position of our faster-moving band. Nakasaki (16), again using liver extracts, this time purified, found three bands but stated that the slowest-moving one, and incidentally the major band was an aggregate of high molecular weight.

There are numerous reports on human serum and human tissue extracts. Fishman (47) showed the liver-bone isozymes to be slower moving than the intestinal isozyme with both bands being in the first half of the gel. Similar results were reported by Smith (54) and Moss (113).

Some authors report an intense band of activity from any and all tissues, very close to the origin which they all ascribe to aggregates (52,57,58).

All authors agree that of all the various tissue extracts treated with neuraminidase only the intestinal isozyme does not lose mobility upon electrophoresis (35,52,53,54). Since our rat testes alkaline phosphatase definitely loses mobility on gels after neuraminidase treatment, this indicates that it is not an intestinal isozyme. All of these results reported above point to the conclusion that the alkaline phosphatase present in whole rat testes is similar if not identical to the liver-bone-kidney isozyme.

As reported so frequently by other authors, it is my belief as well that the first electrophoretic band, that is the one closer to the origin, is actually an aggregate of alkaline phosphatase with various lipoproteins. Some support for this conclusion comes from previous work with liver isozymes. Also, work subsequent to this thesis by this laboratory showed that when rat testes alkaline phosphatase was preincubated with Sodium Dodecylsulfate, only one band of activity resulted.

There were also no differences noted between the adult and 13-day-old-extracts. Moreover, a sample of rat liver that we extracted simultaneously with rat testes, showed only one band of activity at the same position as the faster-moving band of rat testes. Finally, whereas we found the faster-moving band always at the same position in the gel from rat to rat, the slow-moving band varied slightly in position.

Both methods of extraction used were equally effective in solubilizing the alkaline phosphatase although the Buffer B extraction method resulted in a supernatant enriched in alkaline phosphatase. We can demonstrate no differences in the nature of the alkaline phosphatase recovered by these two methods.

Finally, we were able to demonstrate no significant differences in any of the parameters measured when alkaline phosphatase from adult and 13-day-old rats was compared. This implies that alkaline phosphatase production is turned on very early in the development of rat testes, probably in the fetus, and then there is either a steady turnover, of the enzyme, maintaining overall levels constant, or, if there is no turnover, some additional alkaline phosphatase must be produced as the testes mature to keep specific activities constant.

I have not seen similar age comparison for other rat tissues, but Edwards (120) compared the alkaline phosphatase isozyme patterns from human fetal and adult livers and from early fetal and full-term placentas. In each case, the total level of enzyme activity was unchanged, and the isozyme patterns were the same. Mulivor (35) compared human adult and fetal liver, bone, kidney and intestinal extracts. No electrophoretic differences were observed with the liver, bone or kidney tissues. By contrast, fetal intestinal alkaline phosphatase gave a different pattern compared to adult.

Of interest, is the comparative finding that the specific activity of alkaline phosphatase in rat ovaries drops as the rat goes from immature to mature status, but this is due to a great increase in activity of the luteal and interstitial cells as the ovary grows. Indeed, determinations of phosphatase per follicle in young and mature rats suggested that the concentration of phosphatase during maturation remains relatively constant (121).

We may conclude that the enzyme activities and isozymes patterns of the testes isozyme do not change as the animal goes from fetal to adult status. It would be interesting to do such comparative age studies with other rat tissues.

According to my work, as well as others, isozyme patterns are established early.

At this point the question was asked as to exactly where in the testes this alkaline phosphatase activity located. Testes, as mentioned earlier, contain, in addition to the germinal cells, Sertoli cells, Leydig cells, cells of the basement membrane, endothelial cells of blood vessels and others. Since spermatocytes and spermatids comprise approximately 85 % of the cells present in the mature testes it was logical to assume that the alkaline phosphatase, or the greater part of it, came from germinal cells. This, however, proved not to be the case. In work performed after this thesis, Kornblatt (122) demonstrated that the alkaline phosphatase of the testes did not reside in spermatocytes, early spermatids, and presumably, late spermatids, but in the other cellular components of the testes. The likely, though not necessarily only candidate, since it takes up approximately 20 % of the total volume of the testis, is the Sertoli cell.

TABLE 11

Differentiation of alkaline phosphatase isozymes by means of
L-Leu, L-Leuglygly and L-Try
(expressed in % activity remaining.)

	Mulivor (34)		Lin (115)			My results
	Human Intestine	Human Liver-Kidney	Human Intestine	Human Placenta	Human Kidney	Rat Testes
No Treatment	100 %	100 %	100 %	100 %	100 %	100 %
L-Leu	30 %	75 %	-----	-----	-----	70 - 80 %
L-Leuglygly	60 %	80 %	-----	-----	-----	70 - 80 %
L-Try	-----	-----	20 - 40 %	20 - 40 %	80 %	70 - 80 %

SECTION V - CONCLUSIONS

There is considerable alkaline phosphatase activity in rat testes. This activity was characterised by means of amino acid inhibition patterns, heat inactivation, polyacrylamide gel electrophoresis and neuraminidase treatment. The evidence shows that there is but one form of alkaline phosphatase present in the testes of rats and that it shows the properties associated with the liver-bone-kidney isozyme.

There were no differences found in the type of the alkaline phosphatase extracted by both extraction methods discussed above, however lower total protein and alkaline phosphatase levels were measured from the Buffer B extracts. The alkaline phosphatase extracted by both methods showed the same amino acid inhibition pattern, reacted similarly to heat inactivation, showed the same electrophoretic pattern and behaved identically to neuraminidase treatment.

Finally, in all respects, except for specific activity, the alkaline phosphatase from adult and 13-day-old-rats were identical and even for specific activity, the differences were slight. One may conclude, therefore, that the isozyme to be found in infant rats is identical to that in the adult rat and that there are no significant changes in the alkaline phosphatase levels in the testes as the rat matures from infant to adult status.

REFERENCES

- 1) McComb, R.B. et al, Alkaline Phosphatase, Plenum Press, N.Y., 1979.
- 2) McWhinnier, D.J., & Saunders, J.W., Dev. Biol. 14, 169, 1966.
- 3) The Enzymes, Vol IV, 3rd. edition, Academic Press, p. 417, 1971.
- 4) McComb, R.B. et al, Alkaline Phosphatase, Plenum Press, N.Y., 1979.
- 5) Bradshaw, R.A., et al, Proc. Natl. Acad. Sci. USA, Vol. 78, No. 6, pp. 3473-3477, 1981.
- 6) Engstrom, L., Biochim. Biophys. Acta, 52, 49, 1961.
- 7) Falk, M.C., Bethune, J.L. & Vallee B.L., Biochemistry, Vol. 21; No. 7, p. 1471, 1982.
- 8) Fishman, W.H., Am.J.Med., 56, 617, 1974.
- 9) Simpson, R.T., & Vallee, B.L., Biochemistry 7, p. 4343, 1968.
- 10) Morton, R.K., Methods of Enzymology, Academic Press, Vol. 2, p. 538, 1955.
- 11) Fishman, W.H. & Ghosh, N.K., Adv. Clin. Chem., 10, 255, 1967.
- 12) Pickering, C.E., & Pickering, R.G., Arch. Toxicol., 39, 249, 1978.
- 13) Bosron, F.W., et al, Biochemistry 16, No. 4, 610, 1977.
- 14) Simon, F.R. & Sutherland, E., Enzyme, 22, 80, 1977.
- 15) Ghosh, N.K., & Fishman, W.H., Biochem. J., 108, 779, 1968.
- 16) Harkness, D.R., Arch. Biochem. Biophys, 126, 503, 1968.
- 17) Nakasaki, H., et al, J. Biochem., 86, 1225, 1979.
- 18) Reynolds, J.A., & Schlesinger, M.J., Biochem. 8, 588, 1969.
- 19) Reynolds, J.A., & Schlesinger, M.J., Biochem. 8, 4278, 1969.

- 20) Williams, A., & Naylor, R.A., J. Chem. Soc., B, 1973, 1971.
- 21) Schachter, H., Biochem. Soc. Symp., 40, 57, 1974.
- 22) Schlesinger, M.J., et al, Ann. N.Y. Acad. Sci., 166, 368, 1969.
- 23) Vreven, J., Lieberherr, M., & Vaes, G., Biochem. Biophys. Acta, 293, 170, 1973.
- 24) Emery, A.J., & Dounce, A.L., J. Biophys. Biochem. Cytol., 1, 315, 1955.
- 25) Fishman, W.H., & Lin, C.W., Membrane phosphohydrolases, Metabolic conjunction and metabolic hydrolysis, Academic Press, N.Y.; p. 387, 1973.
- 26) de Duve, C., J. Cell Biol., 50, 20D, 1971.
- 27) Brockman, R.W., & Heppel, L.A., Biochem., 7, 2554, 1968.
- 28) Done, J., et al, Biochem. J., 96, 27, 1965.
- 29) Rothstein, A., & Meier, R., J. Cell Compar. Physiol. 34, 97, 1949.
- 30) Sela, B.A., & Sacks, L., J. Cell Physiol., 83, 27, 1974.
- 31) Pekarthy, J.M., et al, J. Biol. Chem., 247, 1767, 1972.
- 32) Danielli, J.F., Nature (London), 168, 469, 1951.
- 33) Walsh, C., Enzymatic reaction mechanisms, W.H. Freeman and Co., San Francisco, -1979, p. 192.
- 34) Doellgast, G.J., & Benirschke., K., Nature, 280, 601, 1979.
- 35) Mullivor, R.A., Hannig, V.L. & Harris, H., Proc. Natl. Acad. Sci. U.S.A., Vol. 75, No. 8, p. 3909, 1978.
- 36) Fraser, D., Am. J. Med., 22, 730, 1957.
- 37) Badger, K.S., & Sussman, H.K., Proc. Natl. Acad. Sci., 73, 2201, 1976..
- 38) Wilkinson, J.H., Isoenzymes, J.B. Lippincott Co., Philadelphia, p. 239, 1970.
- 39) Latner, A.L., et al, Adv. Clin. Chem., 9, 70, 1967..
- 40) Moss, D.W., FEBS Symposium, 18, 227, 1970.

- 41) Fishman, W.H., Inglis, N.R., & Ghosh, N.K., Clin. Chim. Acta., 19, 71, 1968.
- 42) Fishman, W.H., Green, S., & Inglis, N.R., Nature, 198, 685, 1963.
- 43) Fishman, W.H., Plasma Proteins, 2, 59, 1960 edit. by F. Putnam, Academic Press, N.Y.
- 44) Fishman, W.H., & Sie, H.G., Enzymologia, 41, 141, 1971.
- 45) Byers, D.A., Fernley, H.N., & Walker, P.G., Eur. J. Biochem., 29, 197, 1972.
- 46) Cox, R.P., & Griffin, M.J., Arch. Biochem. Biophys., 122, 522, 1967.
- 47) Fishman, W.H., & Sie, H.G., Clin. Chim. Acta., 29, 339, 1970.
- 48) Lin, C-W., & Fishman, W.H., J. Biol. Chem., 247, 3082, 1972.
- 49) Johnson, R.B., Clin. Chem., 18, 110, 1972.
- 50) Moss, D.W., & King, E.J., Biochem. J., 84, 192, 1962.
- 51) Neale, F.C., et al, J. Clin. Pathol., 18, 359, 1965.
- 52) Kaplan, M.M., & Rogers, L., Lancet, 2, 1029, 1969.
- 53) Allen, J.M., & Hyncik, G., J. Histochem. Cytochem., 11, 169, 1963.
- 54) Smith, I., et al, Clin. Chim. Acta. 19, 499, 1968.
- 55) Shaw, C.R., Int. Rev Cytol., 25, 297, 1969.
- 56) Smith, A.F., & Fogg, B.A., Clin. Chem., 18, 1518, 1972.
- 57) Walker, A.W., & Pollard, A.C., Clin. Chim. Acta., 34, 19, 1971.
- 58) Beratis, N.G., Biochem. Genetics, 4, 689, 1970.
- 59) Moss, D.W., Nature, 193, 981, 1962.
- 60) Cornish, C.J., Studies on bone alkaline phosphatase, M. Sc. Thesis, Univ. of Sydney, 1973.
- 61) Eaton, R.H., & Moss, D.W., Enzymologia, 35, 31, 1968.

- 62) Robinson, J.C., & Pierce, J.E., Nature (London), 204, 472, 1964.
- 63) Saini, P.K., & Done, J., FEBS Letters, 7, 86, 1970.
- 64) Komoda, T., & Sakagishi, Y., Biochim. Biophys. Acta, 445, 645, 1976.
- 65) Steinberger, E. & Steinberger, A., Handbook of Physiology-Section 7, Vol. 5, pp. 1-19.
- 66) Roosen-Runge, E.C., The process of Spermatogenesis in animals, Cambridge University Press, 1977.
- 67) Bloom, W., & Fawcett, D., A Textbook of Histology, W.B. Saunders Co., Toronto, 1975.
- 68) Ross, M.H., Am. J. Anat., 121, 523, 1967.
- 69) Cavicchia, J.C., & Dym, M., Am. J. Anat., 150, 501, 1977.
- 70) Clermont, Y., & Perey, B., Am. J. Anat., 100, 241, 1957.
- 71) Clermont, Y., et al, Arch. Anat. Microsc. Morph. Exp., 48, 37, 1959.
- 72) Bellve, A.R., et al, J. Cell. Biol., 74, 68, 1977.
- 73) Steinberger, E., & Steinberger, A., Handbook of Physiology, Section 7, Vol. 4, Chap. 34, p. 325.
- 74) Means, A.R., The human testis, Advances in experimental medicine and biology, Vol. 10, p. 301, Plenum Press, 1970.
- 75) Leblond, C.P., & Clermont, Y., Amer. J. Anat., 90, 167, 1952.
- 76) Cooke, B.A., et al, Nature New Biology, 237, 255, 1972.
- 77) Wilson, J.D., New England J. Med., 287, 1284, 1972.
- 78) Dorrington, J.H., & Fritz, J.B., Biochem. Biophys. Res. Commun., 54, 1425, 1973.
- 79) Steinberger, E., & Duckett, G.E., J. Reprod. Fert., 2, 75, 1967.
- 80) Go, V.L.W., et al. Can. J. Biochem., 49, 768, 1971.
- 81) Kornblatt, M.J., Schacter, H., & Murray, R.K., Biochem. Biophys. Res. Commun., 48, 1489, 1972.
- 82) Kornblatt, M.J., et al, Can. J. Biochem., 52, 689, 1974.

- 83) Millette, C.F., & Bellve, A.R., J. Cell. Biol., 74, 86, 1977.
- 84) Letts, P.J., et al, Biochim. Biophys. Acta., 343, 192, 1974.
- 85) Fritz, I.B., Current Topics in Cellular Regulation, Vol. 7, p. 129.
- 86) Vernon, R.G., Go. V.L.W., Fritz, I.B., Can. J. Biochem., 49, 761, 1971.
- 87) Males, J.L., & Turkington, R.W., J. Biol. Chem., 245, 6329, 1970.
- 88) Males, J.L., & Turkington, R.W., Endocrinology, 88, 579, 1971.
- 89) Majumder, G.C., et al., Endocrinology, 96, 890, 1975.
- 90) Stafford, R.O., & Rubinstein, I.N., & Meyer, R.K., Proc. Soc. Expt. Biol. Med., 71, 353, 1949.
- 91) Allen, J.M., Anat. Rec., 129, 255, 1957.
- 92) Atkinson, W.B., Anat. Rec., 100, 731, 1948.
- 93) Dempsey, E.W., Greep, R.O., & Deane, H.W., Endoc., 44, 88, 1949.
- 94) Bern, H.A., Anat. Rec., 104, 361, 1949.
- 95) Turpeinen, P., et al, Endocrinology, 70, 731, 1962.
- 96) McAlpine, R.J., Anat. Rec., 121, 407, 1955.
- 97) Ahmed, Z., & King, E.J., Biochim. Biophys. Acta., 40, 320, 1960.
- 98) Lowry, O.H., et al, J. Biol. Chem., 193, 265, 1951.
- 99) Hausamen, T.U., Clin. Chim. Acta., 15, 241, 1967.
- 100) Fradola, W.J., Williams, R.D., & Austad, R.A., Am. J. Clin. Pathol., 43, 261, 1965.
- 101) Torriani, A., Biochim. Biophys., Acta., 38, 460, 1960.
- 102) Fräki, J., Ruuskanen, O., & Kouvalainen, J., Biochim. Biophys. Acta., 482, 370, 1977.
- 103) Morton, R.H., Biochem. J., 57, 595, 1954.
- 104) Sakujama, T., Robinson, J.C., and Chou J.Y., Arch. Biochem. Biophys., 191, 782, 1978.

- 105) Davis, B.J., Ann. N.Y. Acad. Sci., 121, 404, 1964.
- 106) Ohkubo, A., Langerman, N., & Kaplan, M.M., J. Biol. Chem., 249, 7174, 1974.
- 107) Boyer, S.H., Science, 134, 1002, 1961.
- 108) Smith, I., et al, Clin. Chim. Acta, 25, 17, 1969.
- 109) Smith, I., et al, Clin, Chim. Acta, 19, 499, 1968.
- 110) Hochberg, M.L., & Sargent, M.L., Anal. Biochem., 51, 516, 1973.
- 111) Schaffer, H.J., Scheurich, P., & Rathgeber, G., Z. Physiol. Chem., 359, 1441, 1978.
- 112) Gerbitz, K.D., et al. Z. Physiol. Chem., 358, 435, 1977.
- 113) Moss, D.W., Eaton, R.H., Smith, J.K., & Whitby, L.G., Biochem. J., 98, 32c, 1966.
- 114) Lehninger, A.L., Biochemistry, Worth Publishers Inc., N.Y., 1970, p. 157.
- 115) Chang, C.H., et al, Cancer Res., 40, 1506, 1980.
- 116) Edwards, Y.H., & Hopkinson, D.A., Isozymes, Vol. 1, p. 45, 1977, Alan R. Liss Incl, N.Y.
- 117) Yokota, Y., J. Biochem., 83, 1285, 1978
- 118) Fishman, W.H., & Ghosh, N.K., Biochem. J., 105, 1163, 1967.
- 119) Lin, C.W., Sie, H.G., & Fishman, W.H., Biochem. J, 124, 509, 1971.
- 120) Edwards, Y.H., & Hopkinson, D.A., Isozymes, Vol. 1, Alan R. Liss Inc., N.Y., 1977, p. 45
- 121) Ford, D.H., & Hirschman, A., Anat, Rec., 121, 531, 1955.
- 122) Korablatt, M.J., Klugerman, A., & Nagy, F.S., Biol. Reprod., 29, 157, 1983.