ISOLATION, PURIFICATION AND PARTIAL CHARACTERIZATION OF SOME ISOENZYMES OF HUMAN SERUM ALKALINE PHOSPHATASE

A Thesis

Presented to

the Faculty of the School of Sciences and Mathematics

Morehead State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

M. Sue Jones

July 13, 1977

Accepted by the faculty of the School of Sciences and Mathematics, Morehead State University, in partial fulfillment of the requirements for the Master of Science degree.

Director of Thesis

Master's Committee: _____, Chairman

_____

_____

_____

July 18, 1977
(date)

ABSTRACT

ISOLATION, PURIFICATION
AND PARTIAL CHARACTERIZATION OF
SOME ISOENZYMES OF HUMAN SERUM ALKALINE PHOSPHATASE

M. Sue Jones, M. S.
Morehead State University, 1977

Director of Thesis: _____

An 8% polyacrylamide gel provided the matrix for isolation and purification of the isoenzymes of alkaline phosphatase(ALP) from human sera. Reconstituted extracts were analyzed for protein and carbohydrate content. Protein was found to vary from 2.7 to 9.0 mg/ml and carbohydrate from 270 to 600 µg/ml. Liver ALP isoenzymes demonstrated varied protein/carbohydrate ratios, from 6.7/1 to 28.6/1. Both liver and bone ALP isoenzymes were verified glycoproteins.

Accepted by:

_____, Chairman

_____

_____

_____

ACKNOWLEDGEMENTS

The author expresses appreciation to the companies which presented gratis materials for research purposes: Ames Company, Division Miles Laboratories, Inc., Elkhart, Indiana; Beckman Instruments, Inc., Fullerton, California; Boehringer Mannheim Corporation, Indianapolis, Indiana; Dade Division, American Hospital Supply Corporation, Miami, Florida; and Worthington Biochemical Corporation, Freehold, New Jersey. Gratitude is extended to Clinical Laboratory, Central Baptist Hospital, Lexington, Kentucky, for serum specimens for analyses. The author also acknowledges Morehead State University for the use of research facilities and reagents and her graduate committee for time and assistance in research. A special indebtedness is owed to Dr. David T. Magrane who served as chairman of that committee.

It is necessary to recognize several individuals for their encouragement, understanding, and advice through the years and in seeking this degree: Franklin Day, Dr. Daryl S. Bates, Patricia C. Motley, Dr. Irene E. Roeckel, Fran Turner, and Allen R. Riebau. The author especially acknowledges Howard Jones, her brother, who

has shared in her dreams, trials, and triumphs, and Caroline S. Jones, her mother, who has accepted these dreams, so often without understanding them. The author extends a word of gratitude to Philip R. Jones, her husband, who typed the thesis manuscript, and to Dr. M. E. Pryor for assistance in proofreading. All photographs were taken by Dean Rector.

So often a life is directed and guided by the lives and work of others. This thesis is a direct result of the teaching of two individuals. It is to those individuals that this work is dedicated: Tilden H. Jones, Sr., who, as an educator, tried to stimulate originality in all his students; and to Dr. Robert L. Scarry, who, because of his belief in a student, her abilities and goals, has given his time, encouragement, and experience.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

INTRODUCTION

Isoenzymes of alkaline phosphatase (ALP, E.C. 3.1.3.1, orthophosphoric monoester phosphohydrolase) are present in human serum at varying levels. Although these levels vary according to the sources of the enzymes, the quantity of each isoenzyme falls within specific levels designated "normal." Accurate interpretation of elevated total serum ALP levels is an unresolved problem in clinical situations. Disease may be indicated when the total ALP value is elevated, relative percentages of isoenzymes are altered, or isoenzymes not normally found in serum are detected, e.g., Regan isoenzyme. The difficulty of interpretation lies in determining the cause of elevation in patients not showing any distinct disease state and in those patients showing more than one diseased organ. ALP isoenzymes have been isolated from many different organs, therefore, it is expected that some of these isoenzymes may be differentiated following isolation from serum. Isoenzymes from bone, liver, and intestines normally occur in serum. An isoenzyme from the placenta may be found in serum during the last trimester of

pregnancy. These are only some expected variations in serum ALP isoenzyme patterns.

Differentiation of ALP isoenzymes in serum using biochemical inhibitors and/or activators has met with little success. Electrophoretic separation and characterization using various media has been somewhat successful, but an overlap in interpretation, particularly in young and elderly patients, still exists. For this reason, it is necessary to perfect a method for accurate, rapid, and positive identification of ALP isoenzymes. Whereas most methods of ALP interpretation have been indirect, i.e., characterization by activities of fractions, this research purposes to identify isoenzymes on the basis of their carbohydrate composition.

Isolation and purification of any enzyme is paramount to its characterization. The specific objectives of this research are to:

1. isolate isoenzymes of serum alkaline phosphatase from clinical specimens using polyacrylamide gel electrophoresis;

2. purify isolates by elution and re-electrophoresis on polyacrylamide gel; and

3. characterize these isolates on the basis of carbohydrate content and specific activity.

LITERATURE REVIEW

Alkaline phosphatase consists of a group of enzymes, or isoenzymes, reacting at an optimum pH of 9.8 (Tietz, 1970) to hydrolyze esters of phosphoric acid (Rhone et al., 1973; Lehninger, 1975). The enzyme has been shown to have pyrophosphatase activity, particularly when using sodium pyrophosphate as substrate (Moss et al., 1966, 1967). Natural substrates for in vivo reactions remain uncertain, but are thought to include ethanolamine phosphate or phosphatidyl ethanolamine (Tietz, 1970). Substrates for in vitro reactions include a variety of substances possessing similar structures (Lehninger, 1975). Amador and Urban (1972), Hausamen et al. (1967), and Skillen and Harrison (1973) indicate that p-nitrophenylphosphate is the substrate of choice in measurement of total ALP. Diethanolamine (DEA) or 2-ethylaminoethanol (EAE), as buffers, activate ALP as they have pK values close to the pH optimum of the enzyme (Hausamen et al., 1967). Skillen and Harrison (1973) point out that it is most important to use a buffer system that has the ability to accept

phosphate groups by transphosphorylation, since ALP shows phosphorylase activity. Amador and Urban (1972) suggest that dephosphorylation of the enzyme is the rate controlling step of *in vitro* ALP measurements. All human tissues contain ALP, and many organs have more than one isoenzyme that react differently with different *in vitro* substrates (PetitClerc, 1976). Thus, the problem involves an attempt to identify the source, or sources, of serum enzyme.

Numerous researchers have used heat inactivation to separate ALP into a heat stable fraction and a heat labile fraction (Neale *et al.*, 1965; Posen *et al.*, 1965a, 1965b; Kerkhoff, 1968; Fennelley *et al.*, 1969; Quigley *et al.*, 1970; It-Koon Tan *et al.*, 1972; Cadeau and Malkin, 1973; Nath and Saha, 1974; Whitby and Moss, 1975; and PetitClerc, 1976). They found that there was overlapping in relative percentages of the total serum isoenzyme value, thus making precise separation and interpretation impossible. Other workers have used urea, L-phenylalanine, neuraminidase, ethylenediaminetetraacetic acid (EDTA), and L-leucine as biochemical inhibitors and/or activators for isolating various isoenzymes on the basis of differential inhibitions. Bahr and Wilkinson

(1967), Horne et al. (1968), Fennelley et al. (1969), Righetti and Kaplan (1971), Winkelman et al. (1972), Gerhardt et al. (1974), Moss (1975), and O'Carroll et al. (1975) used urea in their work and documented the sensitivity of liver, bone, and kidney isoenzymes and resistance of the placental form to this chemical. Fishman et al. (1963), Fishman and Ghosh (1967), Yong (1967), Stolbach (1969), Winkelman et al. (1972), Moss (1975) and O'Carroll et al. (1975) demonstrated the resistance of liver and bone and the sensitivity of intestinal, placental and Regan isoenzymes to L-phenylalanine. Liver and placental ALP were shown to be sensitive to neuraminidase, while intestinal isoenzyme was resistant, by the work of Robinson and Pierce, 1964; Moss et al., 1966; Etzler and Law, 1967; and Righetti and Kaplan, 1971. Although much work has been done involving biochemical and physical inhibitors of ALP as a tool for isoenzyme differentiation, it is quite evident that little information can be gained by further investigation in this area.

Davis (1964) published a detailed account of the method and application of disc electrophoresis, using polyacrylamide gel, to separate serum proteins. Electrophoretic isolation and separation of ALP has

been successful using starch gel (Smith et al., 1968), cellulose acetate (Fritsche and Adams-Park, 1972), agar gel (Haije and DeJong, 1963), and polyacrylamide gel (Davis, 1964). Fritsche and Adams-Park (1972) suggested that ALP isoenzymes were better resolved on cellulose acetate than with polyacrylamide gel, but other workers have presented contrasting evidence. Connell and Dinwoodie (1970), using the method of Smith, Lightstone and Perry (1968) for polyacrylamide gel electrophoresis of ALP, have reported better resolution than with starch gel, agar gel, paper, or cellulose acetate. Canapa-Anson and Rowe (1970) and Kaplan and Roger (1969) successfully used polyacrylamide gel electrophoresis for separation. Unger and Petersen (1972) reported that polyacrylamide gel electrophoresis was relatively more difficult by their findings. With various media (starch gel, polyacrylamide gel, polyvinyl chloride-polyvinyl acetate copolymer, agarose, acrylamide disc and agar) both liver and bone ALP isoenzymes. Moss and King (1962), Moss et al. (1961), Keiding (1964), Cunningham and Rimer (1963), Chiandussi et al. (1962) used starch gel electrophoresis in their analyses of ALP. Boyer (1963) indicated that starch gel electrophoresis of a non-specific serum isoenzyme offered little

promise in diagnosis, and that other methods must be used simultaneously. Each of these workers, regardless of media employed, suggested electrophoretic separation as a definitive diagnosis. It is precisely that point that Boyer questions.

Most work leading to separation and purification of ALP isoenzymes, has involved extraction of isoenzymes from various organs, rather than from serum. Cunningham and Rimer (1963) concentrated serum ALP by dialysis against Carbowax before fractionation by starch gel electrophoresis, in an effort to demonstrate heterogeneity of serum ALP. In extraction of ALP from different organs, Ahmed and King (1960) used Morton's (1955) butanol extraction to purify placental ALP, but were unsuccessful using ion exchange resin (Dowex-2). Twomey and Sweet (1976) employed polyacrylamide gel electrophoresis in a three step purification of serum alpha-fetoprotein and used immunoabsorption and counterimmunoelectrophoresis to monitor the purity of their extracts. Their findings indicated no denaturation of protein resulted, and a highly purified product was obtained. Debach and Neuhaus (1966) purified ALP from bovine synovial fluid using cellulose ion exchanges; Brenna et al. (1975) used Sepharose 4B derivatives as selective

absorbents. Dawson and Hemington (1967), working with a similar plant enzyme, phospholipase D, were successful in purifying it using heat treatment, acetone precipitation, and electrophoresis. Similar procedures were applied to ALP work by Birkett et al. (1966), Boyer (1961), Sussman et al. (1968), using enzyme-antienzyme reactions in the discrimination of the source of ALP.

Boyer (1963) suggested that there were two immunologically distinguishable classes of human serum ALP. This provided the groundwork for a new area of enzyme research. Birkett (1966) developed an antibody to placental ALP in rabbits, which had no cross-reactive activity with ALP extracted from Escherichia coli or bone. Fishman et al. (1968) showed that Regan isoenzyme (tumor) migrated electrophoretically with placental ALP, and cross-reacted with anti-placental antiserum. Stolbach, Krant, and Fishman (1969) reported no differences in placental and Regan isoenzymes. Jacoby and Bagshawe (1971) concluded that these isoenzymes were identical fractions, based on results obtained from electrophoresis, immunological difference, heat stability, and L-phenylalanine sensitivity. However, they did find that tumor enzyme was more sensitive to L-leucine and

EDTA than placental ALP by electroimmunoassay, incorporating the efforts of other workers in this area.

Much of the research concerning ALP isoenzymes is contradictory. This, in part, is due to lack of standardized methods of testing, and inadequate purification of fractions. Engstrom (1961a, 1961b, 1961c), in studies on calf intestinal ALP, reported a molecular weight of 100,000 and varying amounts of neutral sugars and hexosamine, but no sialic acid. Robinson and Pierce (1964), in studies of action of neuraminidase, suggested that isoenzymes differ in carbohydrate content. Hiwada and Wachsmuth (1974a, 1974b) showed that the heterogeneity of kidney ALP was due to different degrees of glycosylation at polypeptide chains, but these were lost when this isoenzyme was treated with neuraminidase. Wachsmuth and Hiwada (1974) indicated that porcine kidney ALP isoenzyme is a tetrameric glycoprotein consisting of identical subunits, having 20% (w/w) carbohydrate and a molecular weight of 150,000 - 156,000. However, Cathala et al. (1975a, 1975b) indicated that bovine kidney ALP, although a glycoprotein with a molecular weight of 172,000, is a dimer of similar or identical subunits of 87,000 daltons each.

Much work has been done to characterize ALP isoenzymes extracted from various animals and various organs. Until these isoenzymes can be identified directly, as in the recent work of Hiwada and Wachsmuth (1974b), Wachsmuth and Hiwada (1974), and Cathala et al. (1975, 1975b), their activities cannot be fully understood. Study of activity, without direct evaluation of composition and structure, cannot increase the understanding of ALP isoenzymes and their significance in disease. Before knowledge can be advanced, development of adequate isolation and purification methods must be effected. It was the purpose of this research to define a procedure employing polyacrylamide gel electrophoresis to isolate, identify, and partially characterize ALP from human serum.

MATERIALS AND METHODS

Serum Specimens and Case Histories

Serum specimens were obtained from Central Baptist Hospital, Clinical Laboratory, Lexington, Kentucky. Specimens were chosen on the basis of their alkaline phosphatase (ALP) elevation as determined during routine chemical screening using the SMA-12/60 (Technicon Corporation, Tarrytown, New Jersey). Case histories were obtained including age, sex, diagnosis, and other SMA-12/60 results, i.e., calcium, phosphorus, glucose, urea nitrogen, cholesterol, total protein, albumin, total bilirubin, glutamic oxalacetic transaminase, and lactic dehydrogenase. All specimens were stored at -20° C pending analysis.

Alkaline Phosphatase Activity

Measurement of ALP activity was by the Beckman (Beckman Instruments, Inc., Fullerton, California) kit based upon the procedure by Bowers and McComb (1966) using p-nitrophenylphosphate (pNPP) as substrate buffered by 2-amino-2-methyl-1-propanol (AMP), pH 10.1, 30° C. After determining the change in optical density per minute (ΔOD/min) at 405 nm using

the Bausch and Lomb Spectronic 70 Spectophotometer (Bausch and Lomb Analytical Systems Division, Rochester, New York), was calculated as follows:

$$\text{I.U./liter} = \frac{\Delta OD/min \times V_t \times T_c \times 1{,}000 \times 1{,}000}{(18.75 \times 10^3) \times V_s}$$

where,

V_t = Total Volume (ml)

T_c = Temperature Coefficient (Table I)

18.75×10^3 = Molar Extinction Coefficient of p-nitrophenol at 405 nm

V_s = Sample Volume (ml)

The same procedure was used in determining ALP activities of serum and each isolated fraction.

Table I

Coefficient for Correction to 30° C (Beckman Procedure)

Reaction Temperature °C	T_c
21	1.65
22	1.56
23	1.48
24	1.39
25	1.31
26	1.24
27	1.18
28	1.13
29	1.07
30	1.00
31	0.95
32	0.90
33	0.85
34	0.80

Total Protein

Protein concentration was determined by the method of Lowry *et al.* (1951) using folin-phenol reagent. Standards were prepared using Bovine Serum Albumin (BSA, Sigma Chemical Company, St. Louis, Missouri), 1.0 mg/ml as given in Table II. Each standard was assayed in duplicate. Average optical densities were plotted against concentration on linear graph paper (Fig. 1). Protein concentration was calculated directly from the standard curve. Total protein of ALP extract was determined by the same procedure, but the dilution factor of the final volume/sample volume, i.e., 3.0 ml/0.2 ml = 15, was incorporated into the final calculation.

$$\text{Protein} = \frac{\text{Concentration (mg/ml)} \times \text{Sample Volume (ml)} \times \text{Total Specimen Volume (ml)} \times \text{Dilution Factor}}{\text{Sample Dilution Volume (ml)} \times \text{Original Serum Volume (ml)}}$$

$$= \frac{\text{Concentration (mg/ml)} \times 0.1 \text{ ml} \times 3.0 \text{ ml} \times 2}{0.5 \text{ ml} \times 0.2 \text{ ml}}$$

$$= \text{Concentration (mg/ml)} \times 6$$

Table II

BSA Standards for Lowry
Method of Protein Determination

BSA (ml)	Water (ml)	Final Concentration (mg/ml)
0.00	0.50	0.00
0.01	0.49	0.02
0.02	0.48	0.04
0.04	0.46	0.08
0.10	0.40	0.20
0.20	0.30	0.40

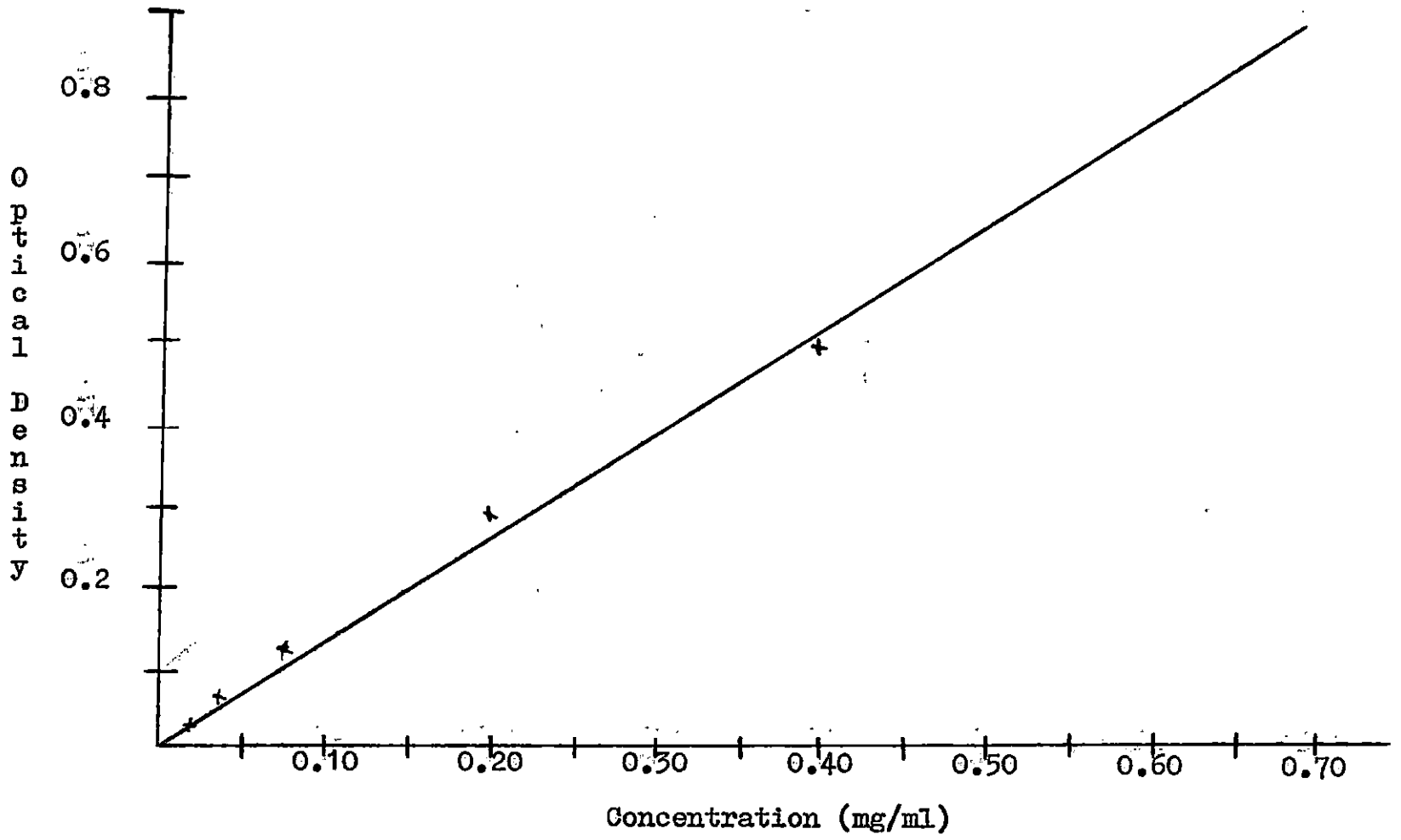

Figure 1

Standard curve for total protein by folin - phenol method

Controls

Normal and abnormal assayed control sera (Dade, Division of Smerican Hospital Supply Corporation, Miami, Florida) were used for monitoring assay procedures in all total protein and ALP determinations. Dade isoenzyme marker, containing intestinal and placental ALP, was included in each electrophoretic procedure.

Polyacrylamide Gel Electrophoresis

Separation of serum ALP isoenzymes was accomplished using polyacrylamide gel electrophoresis according to a modification of the method of Smith et al. (1968) based upon the work of Davis (1964). An 8.0% gel was employed as compared to the 7.0% given in those procedures. Neither loading nor sample gels were used. Gels were prepared in lots of 12 by mixing gel buffer, gel monomer solution, and ammonium persulfate solution in the proportions of 1:1:2 v/v (Table III), followed by free radical polymerization for 30 min. (Fig. 2). A layer of distilled water, in uniform glass tubes, was employed to permit polymerization and proper gel surface. Tubes were 75 mm long x 5 mm (i.d.) x 8 mm (o.d.). A total volume of 16.0 ml, i.e., 4:4:8 ml, was adequate for one lot of gels by this procedure. Each electrophoretic run employed a single lot of gels.

Table III

Preparation of Polyacrylamide Gels

Reagent	Composition	Ratio
Separating Gel Buffer	45.5 g tris (hydroxymethyl-aminomethane in 900 ml distilled water. Adjust pH to 9.5 with saturated boric acid. Complete dilution to 1000 ml. Add 1.2 TEMED (N,N,N',N'-tetramethylene diamine).	1
Gel Monomer Solution	31.54 g acrylamide and 0.46 g bisacrylamide (N,N'-methylenebisacrylamide) in 100 ml distilled water. STORE IN AMBER BOTTLE	1
Ammonium Persulfate Solution	0.2 g ammonium persulfate diluted to 100 ml with distilled water. MADE FRESH WEEKLY.	2

All solutions stored at 2-8° C

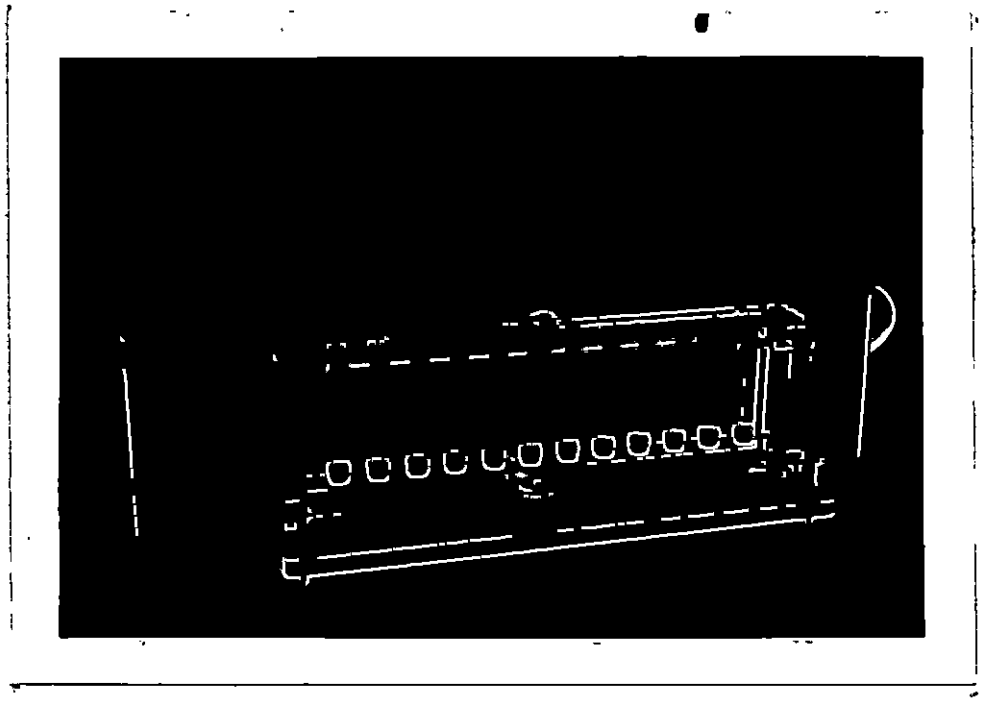

Figure 2

Gel Polymerization Rack

The electrophoretic system employed an anionic, 0.38 M tris-borate, continuous buffer prepared by dissolving 45.5 g tris (hydroxymethlaminomethane) in 900 ml distilled water. Saturated boric acid was added to adjust the pH to 9.5 and total volume was brought to 1000 ml with distilled water. Before use, electrode reservoir buffer was diluted 1:3 v/v with distilled water, yielding an ionic strength of 0.13.

Densities of serum specimens were increased with crystalline sucrose. Gels were layered with 20µl specimens beneath upper buffer. The upper chamber contained approximately 400 ml buffer, while the lower chamber contained 600 ml buffer. The electrophoretic apparatus was the Polyanalyst (Buchler Instruments, Division of Searle Analytic, Inc., Fort Lee, New Jersey) capable of holding 12 uniform size gel tubes (Fig. 3). Electrophoresis was initiated by application of current at 1 mA/tube for 5 min. The current was increased to 3 mA/tube for the remainder of the 30 min. running time. Power was supplied by a Buchler D.C. power supply (Fig. 4). Temperatures of both upper and lower chambers were determined immediately upon completion of electrophoresis. Sample gels were then removed and stained according to the required information.

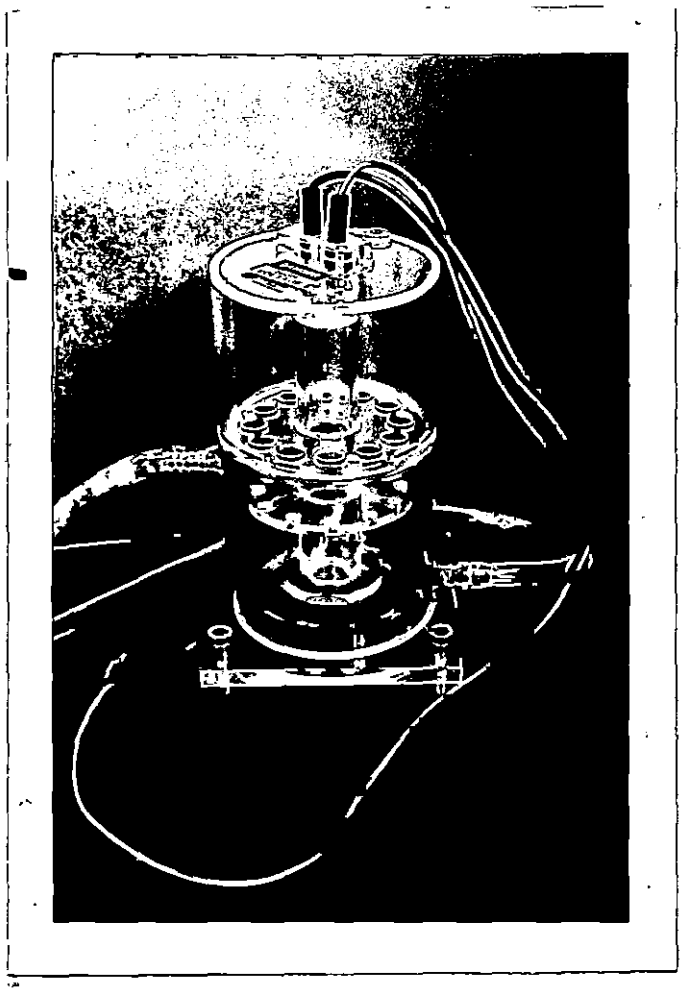

Figure 3

Polyanalyst Electrophoresis Chamber

Figure 4

Buchler D.C. Power Supply

Staining

Electrophoresced samples and controls were stained for ALP activity using p-toluidinium 5-bromo-4-chloro-3-indoxyl phosphate in 2-amino-2-methyl-1,3-propanediol (AMPD) buffer, pH 10.2 (Dade procedure). Reconstitution of each stubstrate was by manufacturers' instruction, yielding a solution of 2.0×10^{-3} M indoxyl phosphate in 2 M AMPD and 2.0×10^{-3} M $MgCl_2$. Gels were stained in 10 x 75 mm tubes containing 2 ml of stain solution. Staining was completed after 1-2 hrs. at 37° C. The reaction was terminated by rinsing the gel with 7% acetic acid. Stained gels were also stored in 7% acetic acid. Stained gels were scanned densitometrically using Gelman DCD-16, Digital Computing Densitometer (Gelman Instruments Company, Ann Arbor, Michigan) (Fig. 5).

Extraction of ALP Fractions

Each electrophoretic run contained one control sample of Dade Marker (intestinal and placental) and eleven test samples. The control gel and one patient gel were stained for ALP activity. The stained patient gel was placed beside the ten unstained gels. The area of unstained gel which corresponded to the stained area was cut out of

Figure 5

Gelman DCD-16 Densitometer

each patient gel with a razor blade. Gel sections were pooled and homogenized in distilled water with mortar and pestle. The resulting mulch was transferred to a small beaker and agitated with a mechanical stirrer for 15 min. The extracted material was pervaporated to dryness in dialysis tubing. The resulting residue was dissolved in 3.0 ml distilled water. Total carbohydrate and total protein contents were determined on each extract.

Total Carbohydrate

Carbohydrate content was determined by a modification of the Dubois et al. (1956) procedure. The specimen to be analyzed (0.8 ml) was reacted with 0.02 ml phenol (80%) and 2.0 ml H_2SO_4 (concentrated) for 10 min. at room temperature. The solution was agitated and incubated 10-20 min. at 25-30° C. Optical density was determined at 480 nm against a distilled water blank using Bausch and Lomb Spectronic 70. Concentrations were determined from a standard glucose curve (Fig. 6). Concentration of carbohydrate in each extract was calculated to original sample size:

$$\text{Carbohydrate} = \text{Concentration } (\mu g/ml) \times \frac{\text{final volume}}{\text{sample volume}}$$

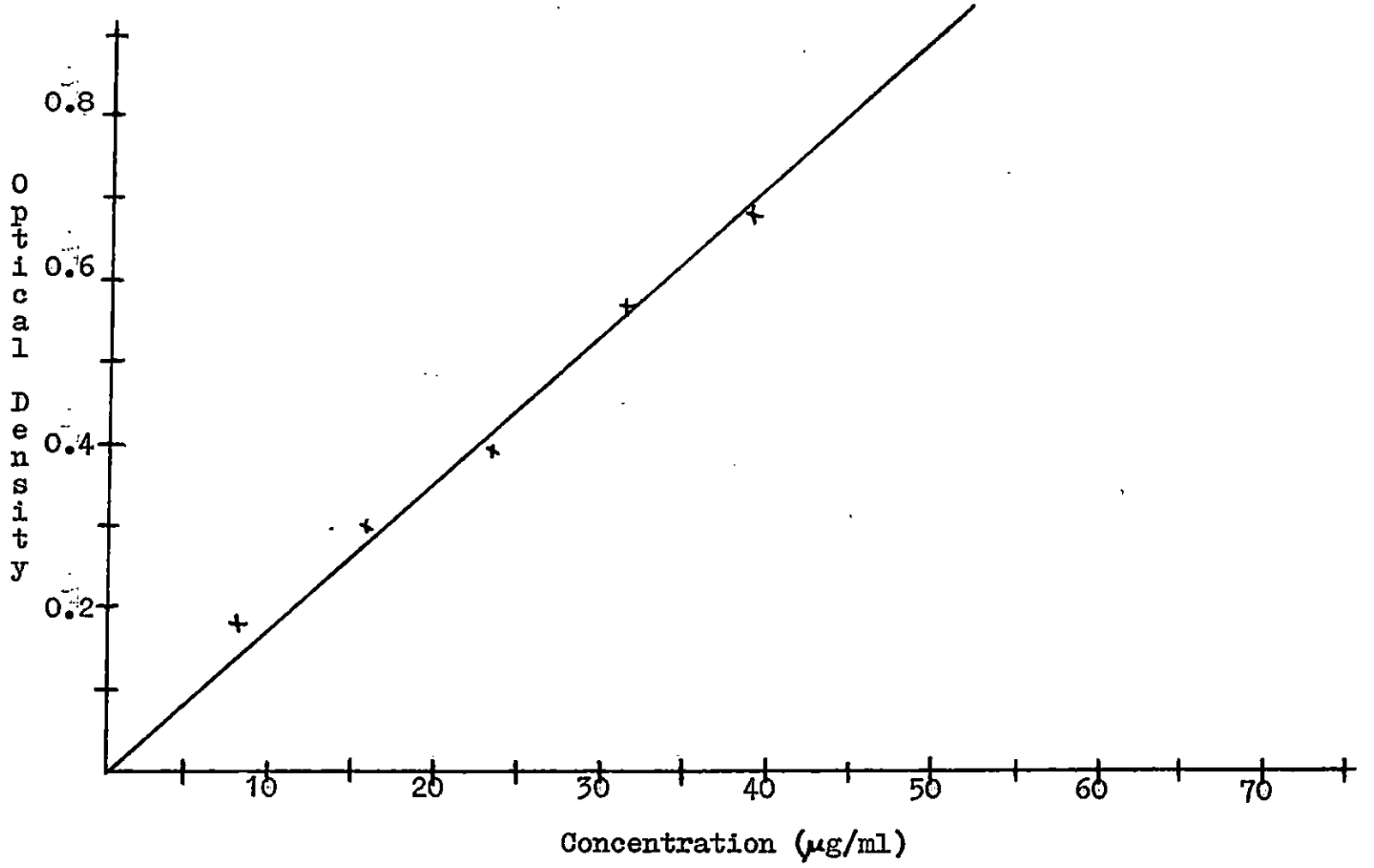

Figure 6

Standard curve for total carbohydrate by Dubois method

$$\text{Carbohydrate} = \text{Concentration } (\mu g/ml) \times \frac{3.0 \text{ ml}}{0.2 \text{ ml}}$$

$$= \text{Concentration } (\mu g/ml) \times 15$$

RESULTS

Results of total protein and carbohydrate analyses of extracts are summarized in Table IV. Protein contents range from 2.7 to 9.0 mg/ml. Carbohydrate values range from 270 to 600 μg/ml. Both values are adjusted to the original sample volume as described in the previous section. Table IV includes calculated protein/carbohydrate (P/C) ratios with a range of 6.7/1 to 28.6/1.

Table V presents calculated carbohydrate and protein quantities per unit of enzyme activity. Values correspond to the P/C ratios in the previous table. Carbohydrates range from 1.9 to 6.3 μg/mU of alkaline phosphatase (ALP). Protein content ranges from 26.4 to 96.8 μg/mU. Included in Table V is total serum protein as determined by folin-phenol procedure. Values range from 64 to 86 mg/ml.

Densitometric scans of patient specimens with corresponding controls are given in Figures 7 through 13. Scans confirm the sources of ALP fractions based upon migration relative to the control.

Table IV

Analysis of ALP Electrophoretic Fractions

Sample		Protein mg/ml	Carbohydrate μ g/ml	P/C Ratio
62877a*	Bone**	2.7	270	10.0/1
62877b	Liver	3.9	270	14.4/1
62977a	Liver	3.9	285	13.7/1
62977b	Liver	2.7	285	9.5/1
62977c	Liver	3.3	495	6.7/1
63077b	Slow Liver	5.4	600	9.0/1
	Fast Liver	9.0	315	28.6/1
63077c	Liver	6.0	390	15.4/1

* Numbers are codes employed to maintain patient confidentiality

** Source of ALP

Table V

Calculated Protein and Carbohydrate Composition of ALP Fractions

Sample		Total Serum ALP U/l*	C/ALP μg/mU	P/ALP μg/mU	Total Serum Protein mg/ml
62877a	Bone	100	2.7	27.0	79
62877b	Liver	145	1.9	26.8	64
62977a	Liver	50	5.7	78.0	86
62977b	Liver	57	5.0	47.4	79
62977c	Liver	125	4.0	26.4	75
63077b	Slow Liver	120	5.0	45.0	72
	Fast Liver	120	2.6	75.0	72
63077c	Liver	62	6.3	96.8	72

* As determined by Beckman Procedure

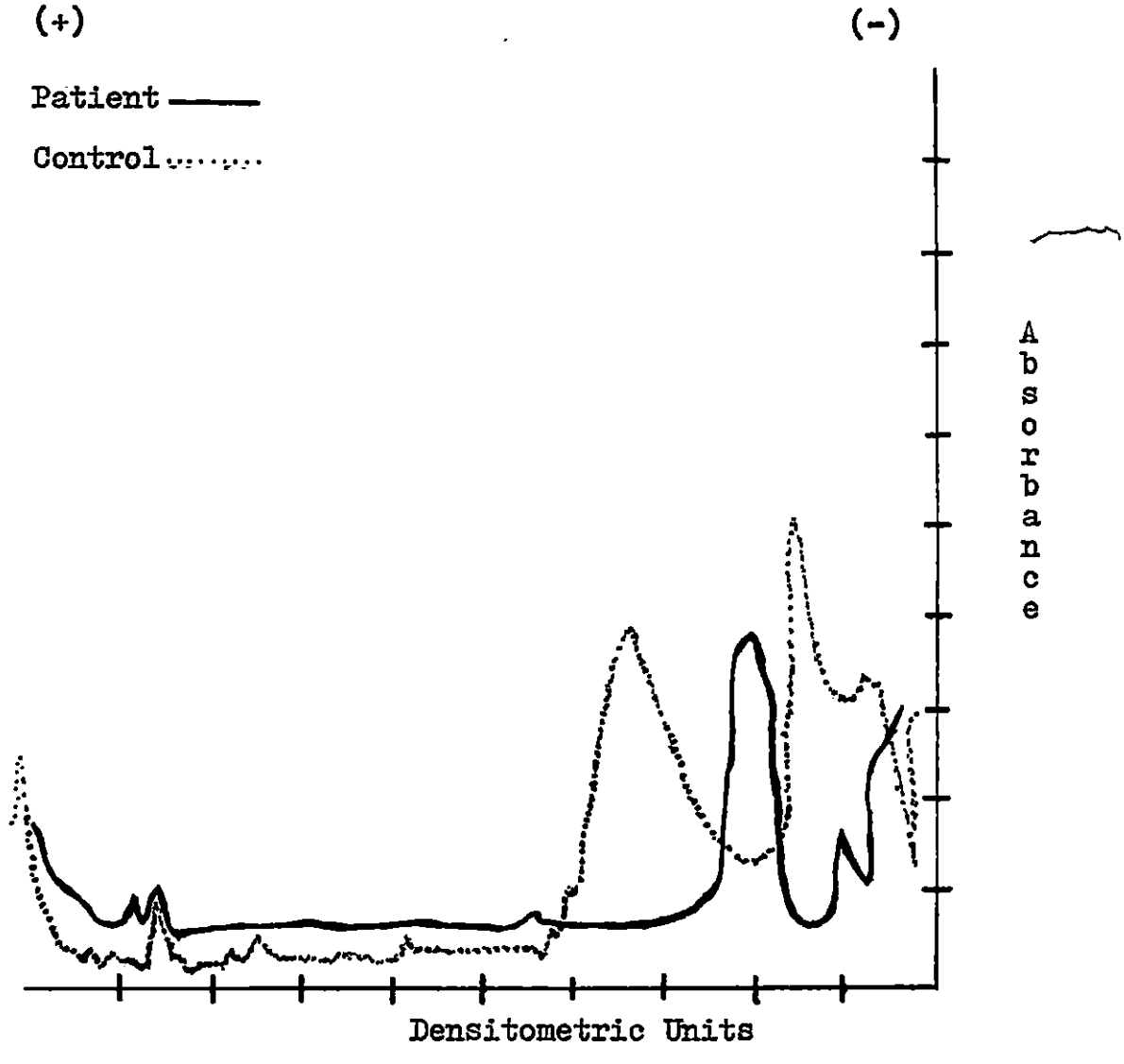

Figure 7

Densitometric Tracing - Dade Marker Control (Intestinal and Placental) and patient 62877 a, 75 year old male with diagnosis of carcinoma of the lung

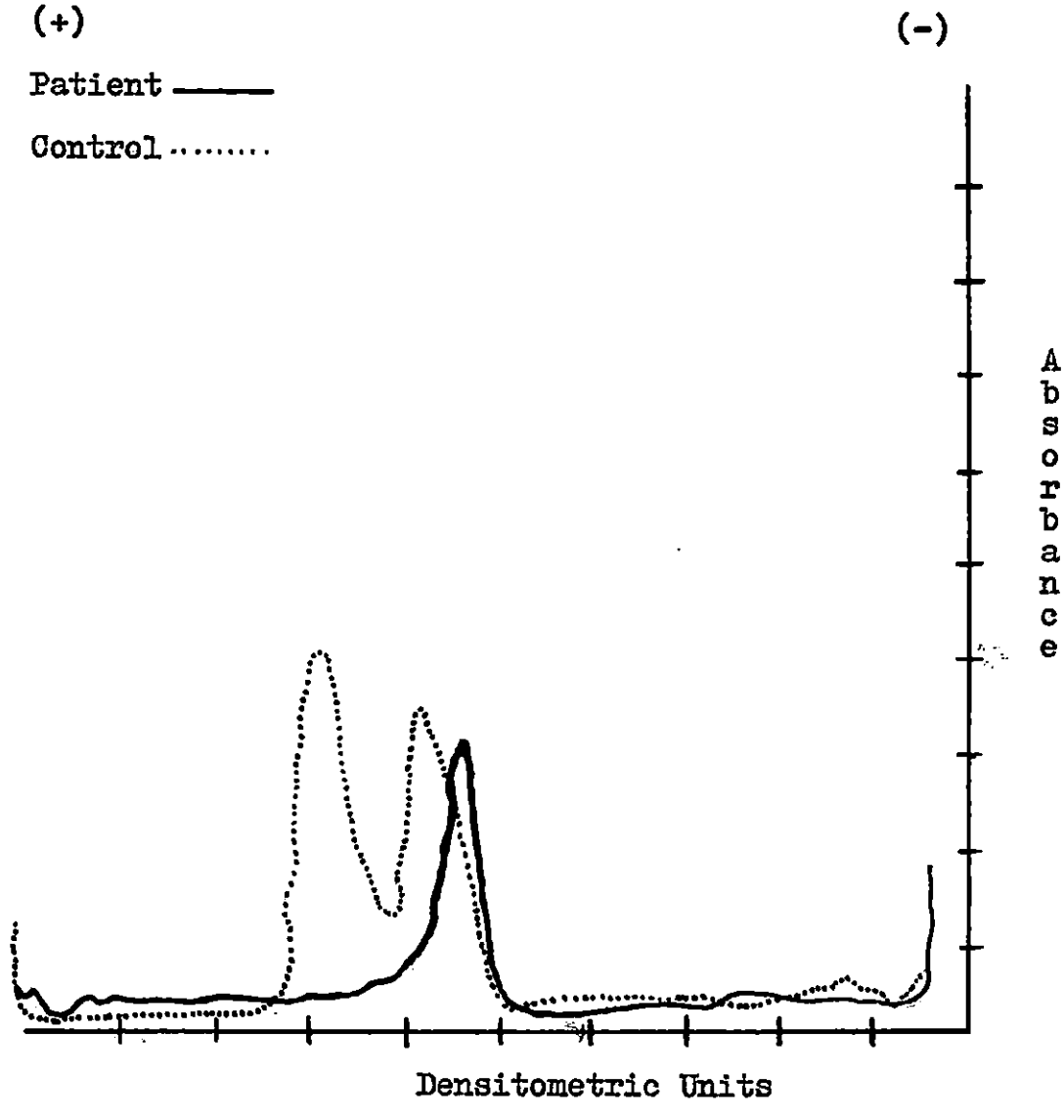

Figure 8

Densitometric Tracing - Dade Marker Control (Intestinal and Placental) and patient 62877 b, 79 year old female with diagnosis of carcinoma of the pancreas

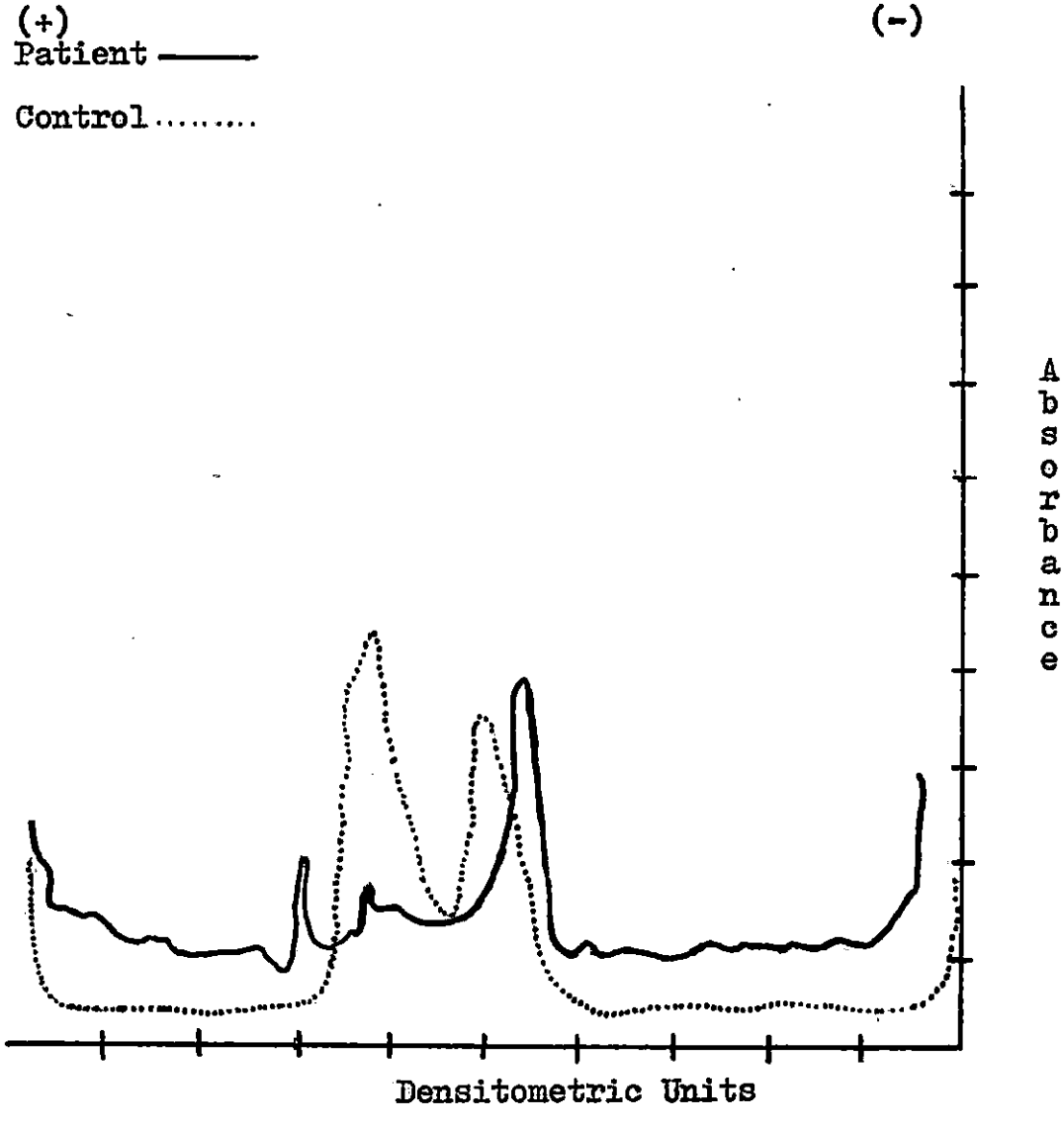

Figure 9

Densitometric Tracing - Dade Marker Control (Intestinal and Placental) and patient 62977 a, 76 year old male with diagnosis of carcinoma of the vocal cords

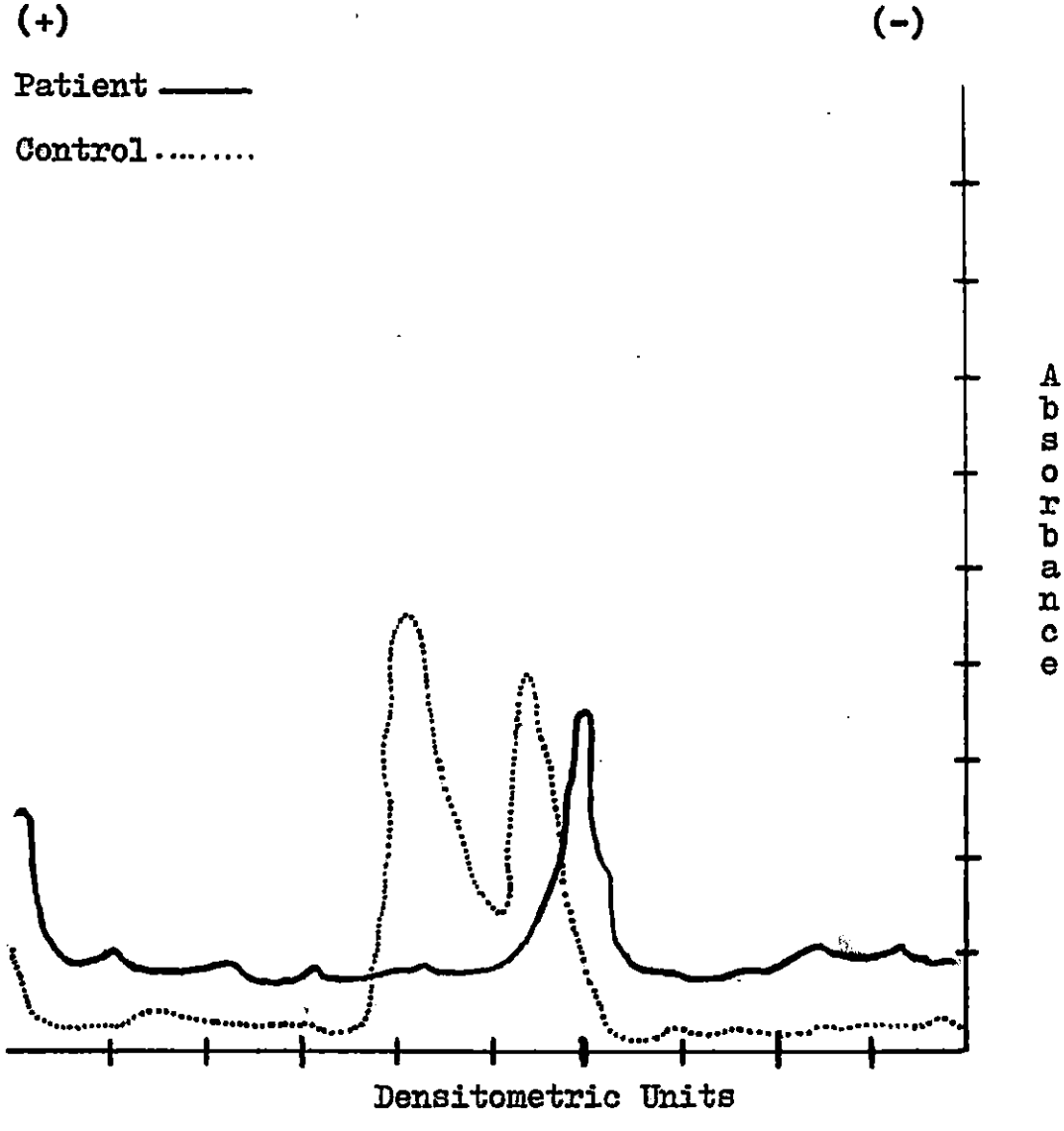

Figure 10

Densitometric Tracing - Dade Marker Control (Intestinal and Placental) and patient 62977 b, 82 year old female with diagnosis of adenocarcinoma of left Fallopian tube

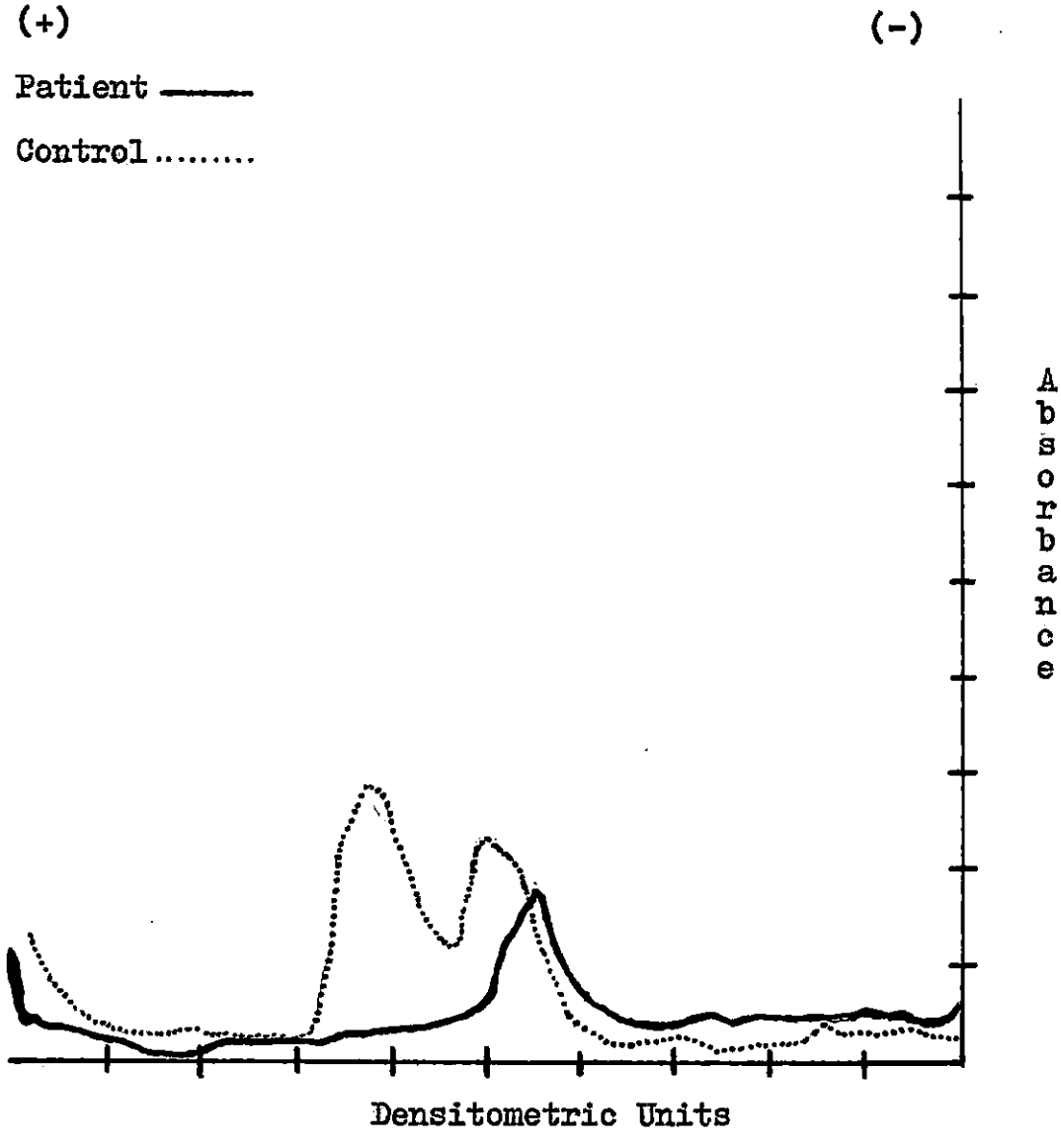

Figure 11

Densitometric Tracing - Dade Marker Control (Intestinal and Placental) and patient 62977 c, 16 year old male with diagnosis of acute rheumatic fever

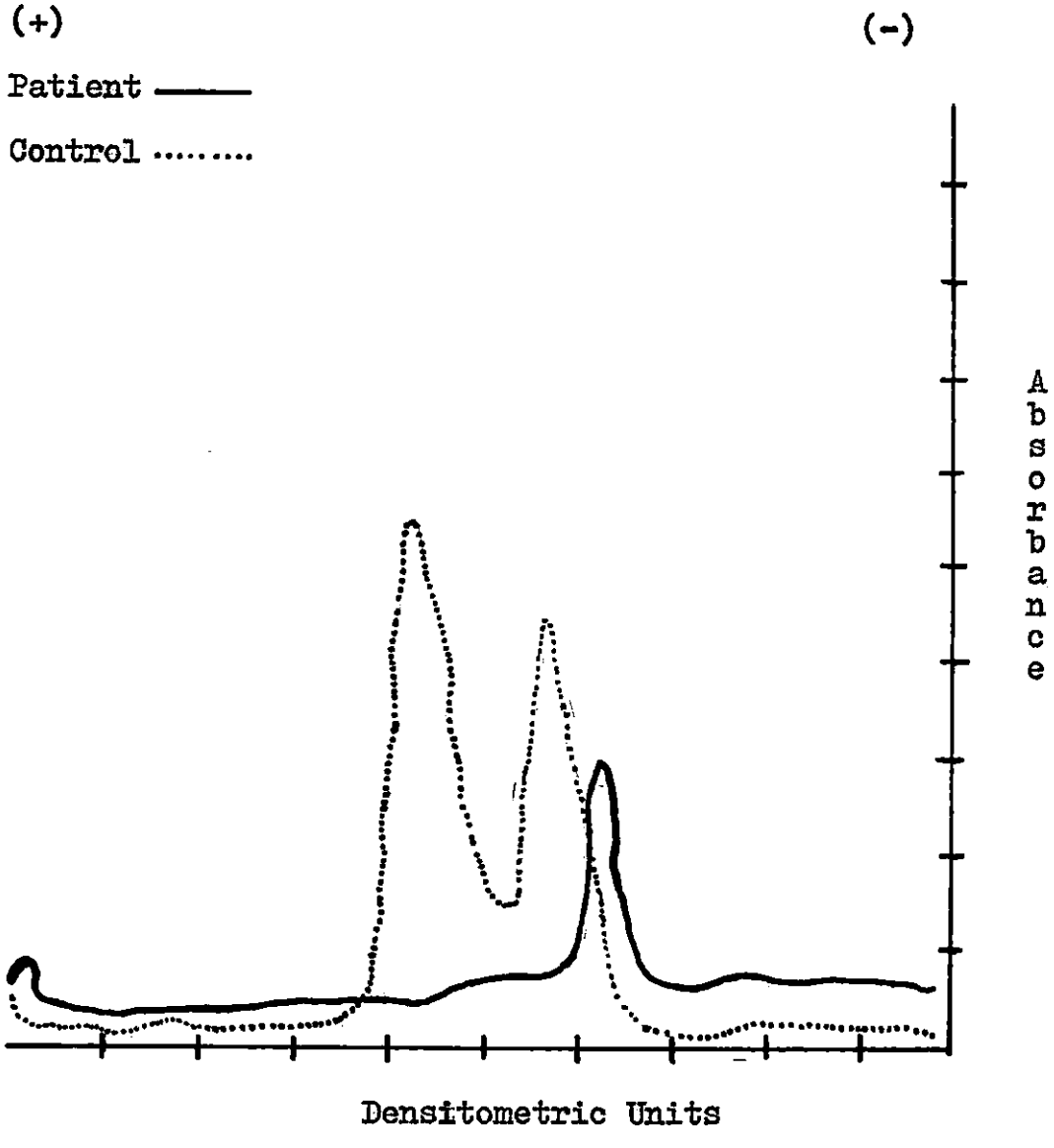

Figure 12

Densitometric Tracing - Dade Marker Control (Intestinal and Placental) and patient 63077 b, 61 year old female with diagnosis of diabetes mellitus, showing slow and fast liver bands

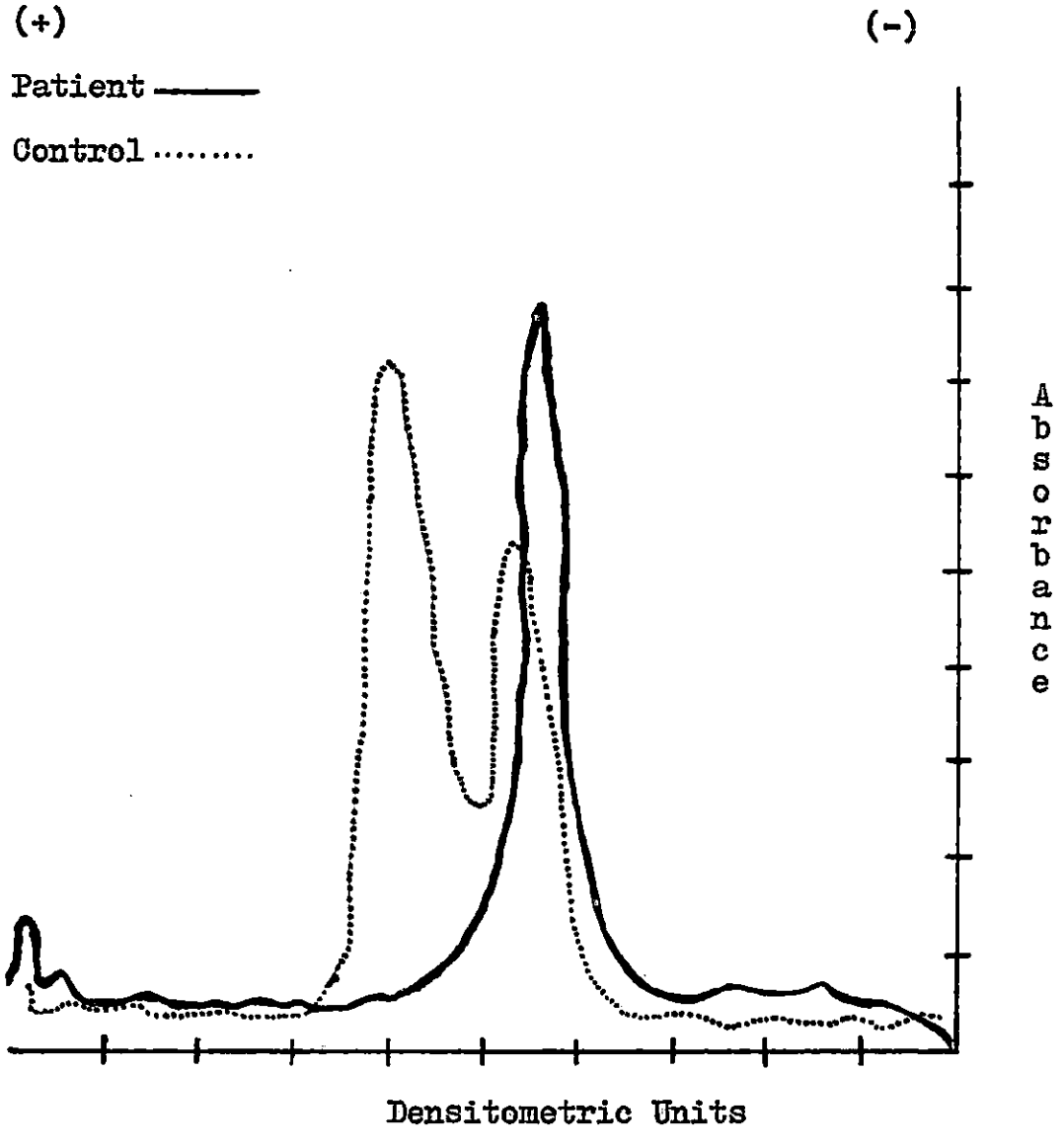

Figure 13

Densitometric Tracing - Dade Marker Control (Intestinal and Placental) and patient 63077 c, 30 year old male with diagnosis of hepatitis

DISCUSSION

Smith *et al.* (1968) used 5% polyacrylamide gels for routine use "as it combined maximum separation and sharpness of bands." Green *et al.* (1972) chose to use 7% gels. In the present study an 8% gel provided optimal separation of alkaline phosphatase (ALP) for characterization studies. The inclusion of Dade Marker in each electrophoretic run decreased possibilities of errors in interpretation due to minor variation in gel content of each lot, alteration in buffer ionic strength, uncontrollable fluctuations in current, or incomplete staining of bands.

Buffer pH and ionic strength, gel concentration, current and running time were varied to establish optimal conditions for ALP isoenzyme separation. Buffer ionic strength was varied from 0.095 M to 0.125 M with pH 9.5 to 10.0. Using ionic strength at only 0.095 M allowed increased resistance, thus increased ohmic heat production. Buffer pH 10.0 was out of optimum range for ALP and prevented sharp focus of bands. Gels of 7% concentration did not allow

distinct separation of ALP isoenzymes, whereas the decreased pore size of 8% gels permitted more precise separation. Initial trial separations were run at 5 mA/gel, i.e., 60 mA, maintaining constant current throughout. ALP migrated at this current, but the rate of migration was too rapid to permit adequate isoenzyme separation. Successive trials at reduced current indicated 1 mA/gel was optimal for time and focus if followed by an increase to 3 mA after 5 minutes running time. The five minute run at 1 mA/gel simulated a stacking effect. An increase of 3 mA/gel for actual migration of the isoenzymes provided optimal focusing.

Initially, all patient specimens were run in triplicate and stained for protein (Nigrosin, 0.02% in 7% acetic acid), glycoprotein (Schiff's), and ALP (Dade). Since much of the controversy in earlier literature revolved around carbohydrate content of ALP, it was deemed necessary to demonstrate that ALP was indeed glycoprotein. Though not an original objective of the present study, evidence obtained confirmed earlier work. Since these findings did conincide with reports of other workers (Hiwada and Wachsmuth, 1974a, 1974b; Wachsmuth and Hiwada, 1974), the data was not included in this text.

Actual analysis of extracts for protein and carbohydrate demonstrated variation in quantities and calculated P/C ratios. High value for carbohydrate (Table IV) was 600 μg/ml for a slow liver band, sample 63077b. This could be misleading, and would not be accurate without precise identification of carbohydrates in the total structure. Since sucrose should not migrate, and slow liver ALP band migrates only slightly anodic, the extract would be contaminated with sucrose added to original specimens. The same specimen showed increased protein in both slow and fast liver ALP bands. Patient 63077bb did not display increased total serum protein, but represented the only specimen containing both slow and fast liver isoenzymes. A distinct bile band often migrates slightly anodic to slow liver ALP. This, of course, does not stain for ALP and is not demonstrated on densitometric scanning (Fig. 12). Patient 63077b was a 61 year old female with proven diabetes mellitus. To date diabetes mellitus has not been regarded as a cause of ALP elevation. However, this study would suggest that such an association may exist.

ALP from specimen 62877a was positioned comparable to bone ALP. Taken from a 75 year old

male with diagnosis of carcinoma of the lung, one could surmise this was Regan ALP. Since heat inactivation studies of all samples revealed no heat stable (Regan) ALP, the tumor isoenzyme could not have been present. Densitometric scanning of stained patient gel and control gel (Fig. 7) indicates that the isoenzyme is of bone origin, possibly due to one of several causes, e.g., increased bone porosity with age, metastasis of primary tumor, etc. All other isoenzymes isolated were determined to be of "normal" liver origin by densitometeric scans compared to corresponding controls.

Interpretation of P/C ratios indicate that the quantity of protein proportional to carbohydrate content is within the values for glycoproteins. Preliminary results from Schiff's reagent studies suggested ALP to be a glycoprotein. Calculations based on carbohydrate and protein (Table IV) confirmed ALP a glycoprotein.

ALP separation using polyacrylamide gel electrophoresis as demonstrated here indicates that this procedure is more sensitive and precise than similar procedures employing cellulose acetate membranes as electrophoretic medium. Polyacrylamide gels also provide for ease in eluting specific bands

for further identification and flexibility in gels and buffers for distinct separation of components regardless of source. Future studies involving ALP should incorporate a wide variety of disorders and serum ALP levels. Findings from such a study could provide the matrix of characterization analysis. A characterization analysis would involve identification of the specific carbohydrate moieties, molecular weight determination, and isoelectric focusing of each isoenzyme. In the event that specific carbohydrate entities can be shown to vary between isoenzymes, a method for precise identification of isoenzyme source could be developed.

The above report then provides a means for purification of ALP isoenzymes. Purification represents the first step in a sequential study which when completed offers the clinical laboratory (and indirectly the patient) a precise means for identifying the source of increased serum ALP.

CONCLUSIONS

Alkaline phosphatase (ALP) isoenzymes can be adequately separated and purified for analysis by polyacrylamide gel electrophoresis using 8% gels, tris-borate buffer pH 9.5, ionic strength 0.13, with a constant current of 1 mA/gel for 5 min., increased to 3 mA/gel for 25 min. Isolated fractions may be recovered by dissolution in distilled water, concentrated by pervaporation in dialysis tubing, and analyzed as desired. ALP isoenzymes are glycoproteins with variations in protein and carbohydrate content. Further work must be done to expand the study and obtain sufficient data for statistical analyses and individual carbohydrate and/or protein evaluation.

LITERATURE CITED

Ahmed, Z. and E.J. King. 1960. Purification of placental alkaline phosphatase. Biochem. Biophys. Acta. 40: 320-328.

Amador, Elias and Jackie Urban. 1972. Transphosphorylation by human alkaline phosphatases. Am. J. Clin. Pathol. 57: 167-172.

Bahr, M. and J. H. Wilkinson. 1967. Urea as a selective inhibitor of human tissue alkaline phosphatases. Clin. Chim. Acta. 17: 367-370.

Birkett, D. J., J. Done, G. C. Neale, and S. Posen. 1966. Serum alkaline phosphatase in pregnancy: andimmunological study. Brit. Med. J. 1: 1210-1212.

Bowers, G. N., Jr. and R. B. McComb. 1964. A direct spectrophotometric assay for serum alkaline phosphatase (monoester phosphohydrolase). Clin. Chem. 10: 636.

Boyer, S. H. 1961. Alkaline phosphatase in human sera and placentas. Science. 134: 1002.

Boyer, S. H. 1963. Human organ alkaline phosphatases : Discrimination by several means including starch-gel electrophoresis of antienzyme-enzyme supernatant fluids. Ann. N. Y. Acad. Sci. 103: 938.

Brenna, O., M. Perrella, M. Pace and P. Pietta. 1975. Affinity chromatography purification of alkaline phosphatase from calf intestine. Biochem. J. 151: 291-296.

Cadeau, B. J. and A. Malkin. 1973. A relative heat stability test for the identification of serum alkaline phosphatase isoenzymes. Clin. Chim. Acta. 45: 235-242.

Canapa-Anson, R. and D. J. F. Rowe. 1970. Electrophoretic separation of tissue-specific serum alkaline phosphatases. J. Clin. Pathol. 23: 499.

Cathala, Guy, Claude Brunel, Danielle Chappelet-Tordo and Michel Lazdunski. 1975 a. Bovine kidney alkaline phosphatase: Purification, subunit structure, and metalloenzyme properties. J. Biol. Chem. 250: 6040-6045.

Cathala, Guy, Claude Brunel, Danielle Chappelet-Tordo and Michel Lazdunski. 1975 b. Bovine alkaline phosphatase: Catalytic properties, subunit interactions in the catalytic process, and mechanism of Mg^{2+} stimulation. J. Biol. Chem. 250: 6046-6053.

Cawley, L. P. and L. Eberhardt. 1967. Association of alkaline phosphatase isoenzymes with serum lipoproteins. Amer. J. Clin. Path. 47: 364.

Chiandussi, L., S. F. Greene, and S. Sherlock. 1962. Serum alkaline phosphatase fractions in hepatobiliary and bone diseases. Clin. Sci. 22: 425-434.

Connell, Margaret D. and Alison J. Dinwoodie. 1970. Diagnostic use of serum alkaline phosphatase isoenzymes and 5'-nucleotidase. Clin. Chim. Acta. 30: 235-241.

Cunningham, V. R. and J. G. Rimer. 1963. Isoenzymes of alkaline phosphatase in human serum. Biochem. J. 89: 50p.

Dabach, D. and O. W. Neuhaus. 1966. Purification and properties of bovine synovial fluid alkaline phosphatase. J. Biol. Chem. 241: 415.

Davis, D. J. 1964. Disc electrophoresis II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121: 404-427.

Dawson, R. M. C. and N. Hemington. 1967. Some properties of purified phospholipase D and especially the effect of amphipathic substances. Biochem. J. 102: 76.

Engstrom, Lorentz. 1961 a. Studies on calf-intestinal

alkaline phosphatase. I. Chromatographic purification, microheterofeneity, and some other properties of the purified enzyme. Biochim. Biophys. Acta. 52: 36-48.

Engstrom, L. 1961 b. Studies on calf-intestinal alkaline phosphatase. II. Incorporation of inorganic phosphate into a highly purified enzyme preparation. Biochim. Biophys. Acta. 52: 49.

Engstrom, L. 1961 c. Further studies on the incorporation of inorganic phosphate into calf-intestinal alkaline phosphatase. Biochim. Biophys. Acta. 54: 179.

Etzler, M. E. and G. R. Law. 1967. Effect of neuraminidase on isoenzymes of alkaline phosphatase and leucine aminopeptidase. Science. 157: 721.

Forman, D. T., D. W. Moss and Latrine B. Whitaker. 1976. Determination of human placental alkaline phosphatase by an electroimmunoassay ("Rocket") technique. Clin. Chim. Acta. 68: 287-290.

Fenelly, J. J., M. X. Fitzgerald, and K. McGeeney. 1969. Value of differential thermostability, urea inhibition and gel filtration of alkaline phosphatase in the identification of disease states. Gut. 10: 45.

Fishman, L. 1974. Acrylamide disc gel electrophoresis of alkaline phosphatase of human tissues, serum and ascitis fluid using Triton X-100 in the sample and gel matrix. Biochem. Med. 9: 309-315.

Fishman, W. H. and N. K. Ghosh. 1967. Influence of reagents reacting with metal, thiol and amino sites on catalytic activity and L-phenylalanine inhibition of rat intestinal alkaline phosphatase. Biochem. J. 105: 1163.

Fishman, W. H., S. Green and N. I. Inglis. 1963. L-phenylalanine: an organ specific stereospecific inhibitor of human intestinal phosphatase. Nature. 198: 685-686.

Fishman, W. H., N. I. Inglis, S. Green, C. L. Antiss, N. K. Ghosh, A. E. Reif, R. Rustigan, M. J. Krant and L. L. Stolbach. 1968. Immunology and

biochemistry of Regan isoenzyme of alkaline phosphatase in human cancer. Nature. 219: 697-699.

Fritsche, H. A. and H. R. Adams-Park. 1972. Cellulose acetate electrophoresis of alkaline phosphatase isoenzymes in human serum and tissue. Clin. Chem. 18: 417-421.

Gerhardt, W., M. Lykkegaard Nielsen, O. Vagn Nielsen, J. S. Olsen and B. E. Statland. 1974. Routine measurements of liver and bone alkaline phosphatase in human serum - Differential inhibition by L-phenylalanine and carbamide (urea) on the LKB 8600 reaction rate analyzer. Clin. Chim. Acta. 53: 281-290.

Haije, W. G. and M. DeJong. 1963. Isoenzyme patterns of serum alkaline phosphatase in agar gel electrophoresis and their clinical significance. Clin. Chim. Acta. 8: 620-623.

Hausamen, T. U., R. Helger, W. Rick and W. Gross. 1967. Optimal conditions for the determination of serum alkaline phosphatase by a new kinetic method. Clin. Chim. Acta. 15: 241-245.

Hiwada, Kunio and Ernest D. Wachsmuth. 1974 a. Catalytic properties of alkaline phosphatase from pig kidney. Biochem. J. 141: 283.

Hiwada, K. and E. D. Wachsmuth. 1974 b. Alkaline phosphatase from pig kidney. Microheterogeneity and the role of neuraminic acid. Biochem. J. 141: 293.

Horne, Michael, Coralie J. Cornish and Solomon Posen. 1968. Use of urea denaturation in the identification of human alkaline phosphatase. J. Lab. Clin. Med. 72: 905-915.

It-Koon Tan, Lee-Foon Chio and Lim Teow-Suah. 1972. Heat stability of human serum alkaline phosphatase in bone and liver diseases. Clin. Chim. Acta. 41: 329-334.

Jacoby, B. and K. D. Bagshawe. 1971. Placental-type alkaline phosphatase from human tumour tissue.

Clin. Chim. Acta. 35: 473-481.

Kaplan, M. M. and L. Rogers. 1969. Separation of human serum alkaline phosphatase isoenzymes by polyacrylamide gel electrophoresis. Lancet 2: 1929.

Keiding, N. R. 1964. The alkaline phosphatase fractions of human lymph. Clin. Sci. 26: 291.

Kerkhoff, J. F. 1968. A rapid serum screening test for increased osteoblastic activity. Clin. Chim. Acta. 22: 231-238.

Korner, N. H. 1962. Distribution of alkaline phosphatase in serum protein fractions. J. Clin. Pathol. 15: 195.

Lehninger, Albert L. 1975. BIOCHEMISTRY. 2 nd edition. THE MOLECULAR BASIS OF CELL STRUCTURE AND FUNCTION. Worth Publishers, Inc., New York, N. Y. p.218.

Lowry, O.H., A. L. Rosebrough, A.L. Farr and R. J. Randall. 1951. Protein measurement with folin-phenol reagent. J. Biol. Chem. 193: 265-275.

Moss, D. W. 1975. Alkaline phosphatase isoenzymes. Enzyme. 20: 20-34.

Moss, D. W., D. M. Campbell, E. Anagnostou-Kakaras and E. J. King. 1961. Characterization of tissue alkaline phosphatases and their partial purification by starch gel electrophoresis. Biochem. J. 81: 441-447.

Moss, D. W., R. H. Eaton, J. K. Smith and L. G. Whitby. 1966 a. Alteration in the electrophoretic mobility of alkaline phosphatase after treatment with neuraminidase. Biochem. J. 98: 32c- 33c.

Moss, D. W., R. H. Eaton, J. K. Smith and L. G. Whitby. 1966 b. Association of pyrophosphatase activity with human alkaline phosphatase preparations. Biochem. J. 99: 19p.

Moss, D. W., R. H. Eaton, J. K. Smith and L. G. Whitby. 1967. Association of inorganic-pyrophosphatase activity with human alkaline phosphatase preparations. Biochem. J. 102: 53.

Moss, D. W. and E.J. King. 1962. Properties of alkaline phosphatase fractions separated by starch-gel electrophoresis. Biochem. J. 84: 192-195.

Nath, R. L. and D. Saha. 1974. A thermostable serum alkaline phosphatase. Clin. Chim. Acta. 55: 5-9.

Neale, Frank C., John S. Clubb, Diane Hotchkiss and Solomon Posen. 1965. Heat stability of human placental alkaline phosphatase. J. Clin. Path. 18: 359-363.

O'Carroll, Donnan, Bernard E. Statland, Bernard W. Steele and Desmond Burke. 1975. Chemical inhibition method for alkaline phosphatase isoenzymes in human serum. Am. J. Clin. Pathol. 63: 564-572.

PetitClerc, Claude. 1976. Quantitative fractionation of alkaline phosphatase isoenzymes according to their thermostability. Clin. Chem. 22: 42-48.

Posen, S., F. C. Neale, and J. S. Clubb. 1965. Heat inactivation in the study of human alkaline phosphatases. Clin. Chem. 11: 789.

Posen, S., F.C. Neale, and J. S. Clubb. 1965 b. Heat inactivation in the study of human alkaline phosphatases. Ann. Int. Med. 62: 1234.

Quigley, G. J., R.T. Richards, and K. J. Shier. 1970. Heat stable alkaline phosphatase. A parameter of placental function. Am. J. Obstet. Gynec. 106: 340-351.

Rawstron, J. R. 1971. Rapid electrophoresis of alkaline phosphatase isoenzymes. Clin. Chim. Acta. 32: 303-304.

Rhone, Douglas P., Florence M. White and Helen Gidaspow. 1973. Isoenzymes of liver alkaline phosphatase in serum of patients with hepatobiliary disorders. Clin. Chem. 19: 1142-1147.

Righetti, A.B.-B. and M.M. Kaplan. 1971. The origin of the serum alkaline phosphatase in normal rats. Biochim. Biophys. Acta. 230: 504-509.

Robinson, J. C. and J. E. Pierce. 1964. Differential action of neuraminidase on human alkaline phosphatase. Nature. 204: 472.

Skillen, A. W. and J. Harrison. 1973. Serum alkaline phosphatase - Effect of pH and buffer on optimum substrate concentration. Clin. Chim. Acta. 45: 287- 291.

Smith, Ivor, P. J. Lightstone and J. D. Perry. 1968. Separation of human tissue alkaline phosphatases by electrophoresis on acrylamide disc gels. Clin. Chim. Acta. 19: 499-505.

Stolbach, Leo L. 1969. Clinical application of alkaline phosphatase isoenzyme analysis. Ann. N. Y. Acad. Sci. 166: 760-774.

Stolbach, L. L., M. J. Krant, and W. H. Fishman. 1969. Ectopic production of an alkaline phosphatase isoenzyme in patients with cancer. New Engl. J. Med. 281: 757.

Sussman, H. H., P. A. Small and E. Cotlove. 1968. Human alkaline phosphatase. Immunochemical identification of organ-specific isoenzymes. J. Biol. Chem. 243: 160-166.

Tietz, Norbert W., editor. 1970. FUNDAMENTALS OF CLINICAL CHEMISTRY. W. B. Saunders Company, Phikadelphia. pp. 394-405.

Twomey, Stanley L. and Randy V. Sweet. 1976. Purification of α-fetoprotein. Clin. Chem. 22: 1306-1309.

Unger, M. and P. H. Petersen. 1972. Electrophoretic separation and quantitation of some serum alkaline phosphatases in agar gel electrophoresis. Enzyme. 14: 238-256.

Wachsmuth, Ernest D. and Kunio Hiwada. 1974. Alkaline phosphatase from pig kidney. Method of purification and molecular properties. Biochem. J. 141: 273-281.

Whitby, L. G. and D. W. Moss. 1975. Analysis of heat inactivation curves of alkaline phosphatase isoenzymes in serum. Clin. Chim. Acta. 59:

361-367.

Winkelman, J., S. Nadler, and J. Demetriou. 1972. The clinical usefulness of alkaline phosphatase isoenzyme determinations. Am. J. Clin. Pathol. 57: 625-634.

Yong, J. M. 1967. Origins of serum alkaline phosphatases. J. Clin. Pathol. 20: 647-653.