CHEMICAL AND PHYSIOLOGICAL EFFECTS OF *CANDIDA ALBICANS* TOXIN ON TISSUES

BY

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ABSTRACT

CHEMICAL AND PHYSIOLOGICAL EFFECTS OF *CANDIDA ALBICANS* TOXIN ON TISSUES

Michael Peter Berk

Master of Science

Youngstown State University, 1993

Today, with the prevalence of acquired immunodeficiency syndrome (AIDS) and other immunosuppressed patients, *Candida albicans*, specifically, has come to the forefront of medicine as the most common fungal pathogen. Once a human host's immune system becomes compromised *Candida albicans* cells begin to proliferate rapidly and are capable of causing the disease candidiasis (or candidosis) in virtually any tissue of the body. *Candida albicans* may initiate a pathologic state under such circumstances as: physiological changes such as pregnancy, newborns that have not yet established a resident flora, invasive procedures, catheters, immunosuppressive therapy, malignancy, granulocytopenia, broad-spectrum antimicrobial agents, and especially the increased incidence of immune-deficiency diseases such as AIDS.

Previous research in this area has determined that a potent toxin is associated with *Candida albicans*. However, neither the extent of the tissue damage nor the means of toxin's action had been assessed. Our research conducted thus far has shown that high

infection and mortality rates can be achieved in laboratory rats utilizing intraperitoneal inoculations of *Candida albicans* cells as well as the presumed toxin alone. In addition, our study has shown that the intraperitoneal mode of infection results in numerous lesions and even tumorous conditions in the infected rats. The study at hand involves the correlation of increases in acid phosphatase levels with changes in mitotic activity of infected rats.

ACKNOWLEDGEMENTS

The author wishes to thank the following individuals: Scott Howell and Joe Restivo for technical assistance in preparation of some techniques, Drs. James R. Toepfer and Daryl W. Mincey for critical review of this manuscript, and especially Dr. John J. Yemma, without his guidance and leadership this work would not have been possible.

The author would like to dedicate this thesis to his parents, Peter and Olga Berk, whose love, support, and understanding made this study a reality.

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CHAPTER I

Introduction

Members of the genus *Candida* represent a diverse group of medically important yeasts. *Candida* species exemplify a group of ubiquitous human pathogens, causing localized, invasive, or disseminated disease states in normal and especially in immunocompromised hosts (Scherer and Stevens, 1986). Today, with the prevalence of AIDS and other immunosuppressive diseases, *Candida albicans*, specifically, has come to the forefront of medicine as a common fungal pathogen. Considered as part of the normal human flora, this pleomorphic yeast resides mainly in the alimentary tract and/or vagina of the healthy human host (Christensen, 1975). However, once a human host becomes compromised, it begins to proliferate rapidly as a human pathogen and is capable of causing the disease candidiasis (or candidosis) in virtually any tissue of the body (Bodey and Fainstein, 1985; Odds, 1978). Not only is it the major cause of vaginal and oral yeast infections (Gentry and Price, 1985; Knight and Fletcher, 1971; Masterson et al., 1976; Sobel, 1984; Tapper-Jones et al., 1981), but it has evolved into a major systemic pathogen of compromised hosts (Bodey and Fainstein, 1985; Chandler, 1985; Degregorio et al., 1982; Myerowitz et al., 1977; Parker et al., 1976). *C. albicans* may initiate a pathologic state under

circumstances such as: physiologic changes in pregnancy, newborns that have not established a resident flora (Rippon, 1974), invasive procedures, catheters, immunosuppressive therapy, malignancy, granulocytopenia, broad-spectrum antimicrobial agents, and especially the increased incidence of immune-deficiency diseases such as AIDS (Gottlieb et al., 1983; Scherer and Stevens, 1986; Ghannoum and Abu-Elteen, 1990). "The pathologic states are extremely varied and include (1) infections of mucocutaneous tissues (oral cavity, bronchial and pulmonary tissues, vaginitis) (Kam and Giacola, 1975), (2) infections of cutaneous tissue (between fingers or toes, in various body folds, under nails, or generalized over the entire body), (3) systemic involvement involving multiple organ systems (Myerowitz et al., 1977), and (4) allergic responses to the presence of yeast cells that may appear as eczema, asthma, gastritis, or candidids (Rippon, 1974)" (Moore and Jaciow, 1979). Such circumstances may lead to mortality, and as a consequence it is critical that the molecular biology of *Candida albicans* be well understood so that human candidiasis may be properly treated and possibly prevented.

Although early in the twentieth century only *C. albicans*, within the genus *Candida*, was considered pathogenic (Odds, 1988), in the 1950's and 1960's more critical investigations were performed demonstrating that pathogenicity extended to other species in this genus (Hasenclaver and Mitchel, 1961; Hurley, 1965; Hurley and Winner, 1962). Today, there are fifteen species of the genus *Candida* that are considered pathogenic (TABLE 1), (Ghannoum

TABLE 1

Candida albicans (including *Candida stellatoidea*)	*Candida dattila* (formerly *Torulopsis dattila*)
Candida catenulata (formerly *Candida brumpti*)	*Candida famata* (formerly *Torulopsis candida*)
Candida guilliermondii	*Candida glabrata* (formerly *Torulopsis glabrata*)
Candida kefyr (formerly *Candida pseudotropicalis*)	*Candida inconspicua* (formerly *Torulopsis inconspicua*)
Candida krusei	
Candida lusitaniae	
Candida parapsilosis	
Candida pulcherrima	
Candida tropicalis	
Candida zeylanoides	

Facultative pathogenic Candida species

and Abu-Elteen, 1990). Due to the fact that such a vast array of *Candida* species can be responsible for candidiasis, it is essential that these organisms be differentiated from one another through the identification of several morphological and biochemical characteristics. Of these characteristics the most important proves to be the ability of *C. albicans* to form both germ tubes and chlamydospores as wellas its ability to assimilate specific sugars (Evans and Richardson, 1989).

It should be noted that studies of human candidiasis involve animal model systems whereby the disease process can be experimentally simulated and studied in vivo. This study, as well as the vast majority of studies involving pathogenic yeasts to date, utilize *C. albicans* as the etiologic agent of choice (Kettner et al., 1983). It is also important to note that in this study, as is true of other studies involving the manipulation of pathogenic micro-organisms, it is imperative that a model system is used which offers the researcher a viable mode of infecting the specific system or organs of choice. It is also advantageous that the model system chosen has low-cost maintenance capabilities. Thus, with this in mind, the model system chosen for this study involves the intraperitoneal (IP) inoculation of rats with *C. albicans*. The major consideration that led to this choice was the fact that earlier studies have shown that high infection and mortality rates are achieved in rats utilizing inoculations of *C. albicans* (Kettner, 1983; Salvin, 1952), and the rat model system is highly cost-effective. Thus, since the present study includes the

detection of changes in deoxyribonucleic acid (DNA) levels within the kidney and liver cells, as well as changes in serum composition, the mode of IP inoculation to infect rats was ideally suited for this purpose.

Generally, three specific and distinct areas of research are prominent in the literature today which deal with the virulent properties of *C. albicans*. These areas involve adherence properties, hydrolytic enzyme content and release, and toxin production (Ghannoum and Abu-Elteen, 1990). The focus of this study will concern itself with the topic of possible toxin production by *C. albicans*, and its physiological and cellular results in view of earlier studies reported in the literature.

A major investigator of toxin production by *C. albicans* is Kazuo Iwata of Japan who maintains that the symptomatology and histopathology, particularly in systemic clinical and experimental infections by certain species of pathogenic fungi, suggest the possible production of toxins in invaded tissues. Still other research has dealt with endotoxin activity and evidence that it labilizes the membrane of lysosomes in vivo (Martini, 1959). "Also, others (Weissman and Thomas, 1962; Weissman and Thomas, 1964; Szilagyi et al., 1976) had shown that endotoxin promoted the liberation of lysosomal enzymes from lysosomes" (Kettner, 1983).

Upon duplicating the Iwata et al. experiments, Chattaway and Odds (1971) found that their strains of *C. albicans*, upon purification on DEAE-cellulose, gave identical protein elution

profiles. The canditoxin eluent from the Japanese strain proved to be acid phosphatase (Ph 3.6). It is also interesting to note that canditoxin, upon heating, loses its toxic effects and has alkaline phosphatase activity (Iwata and Uchida, 1967a,b, 1968). This data alone leads one to believe that possibly hydrolytic enzymes may have a more prominent role in the pathogenicity of *C. albicans* than possible toxin production itself, or be responsible for the observed toxic affect.

However, the release of toxic extracellular glycoproteins by *C. albicans* into growth media has long been suspected, but not adequately proven (Mankowski, 1968). In fact, Masler et al. (1966) reported the composition of a water-soluble, extracellular polysaccharide-protein complex (PPC) obtained from growth media of virulent strains of *C. albicans*. Other investigators have also isolated PPC entities from the cell wall of *C. albicans* which exert a shock-like state following IV administration (Cutler et al., 1972; Kettner et al., 1983).

In this study, an attempt to elucidate what type of chemical is released into liquid media culture by a known virulent *C. albicans* strain in humans is examined. Experimental groups of rats are then subjected to different intraperitoneal inoculations: (1) whole cells of *C. albicans*, (2) lyophilized media with toxic material, (3) physiologic saline (control). Individual rats are sacrificed at a specific interval post-inoculation via a direct cardiac puncture, and the resultant serum stored in liquid nitrogen for HPLC, electrophoretic, and spectrophotometric analysis

of lysosomal enzyme release into the bloodstream. The hepatic, and renal tissues of these rats are then fixed for sectioning so that the cells can be treated for quantitative cytophotometry employing the Feulgen nuclear reaction (Feulgen and Rossenbeck, 1924). This cytochemical technique allows for the quantitative measurement of DNA changes, and thus other changes within the cells (Anderson and Kjellstrand, 1971, 1972, and 1975; Decosse and Aiello, 1966) utilizing the two-wavelength method of microspectrophotometry (Patau, 1952). In summary, the purpose of this study is threefold: (1) to examine the release of toxic products into the growth media by *Candida albicans*, (2) to ascertain if this toxic product has an endotoxin-like effect on the lysosomes of the different tissues studied, thereby causing direct tissue damage, (3) to determine whether this toxic product has an effect on the nuclear activity of hepatic and renal cells which are known to be infected within this model system.

CHAPTER II

Materials and Methods

Organism

Candida albicans culture was obtained from St. Elizabeth's Hospital from a human source and maintained on Sabourad's dextrose agar. Culture purity was checked by means of the germ tube production test (Kern, 1985), as well as API 20C methodology (Analytab Products, 1982). See Figure 1 for API 20C procedure. Pure culture was then subcultured onto Sabourad's dextrose agar and grown at 25 degrees centigrade.

Growth Curve Determination

For the purpose of growth curve determination, several test tubes containing Sabourad's dextrose broth were inoculated with 55,000 *C. albicans* cells and grown in a Precision shaking water bath at 37 degrees centigrade at 50 oscillations per minute. Each day for a period of seven days following the inoculation, cell counts were done using a Bright-Line hemacytometer manufactured by American Optical. The data was graphed and log phase was determined (FIGURE 2).

Cells were grown to log phase by the method outlined above and subsequently spun down at 2,000 rpm for ten minutes. The supernatant was removed, pooled, and prepared for lyophilization in a Labconco model 77530 freeze dry system.

FIGURE 1

API 20C Clinical Yeast
Identification System Procedure

API 20C CLINICAL YEAST SYSTEM

Purpose

The API 20C system is a microtube method utilizing 19 assimilation tests for the identification of most clinically significant yeasts and yeast-like organisms.

Storage

The API 20C system should be stored at 2-8 degrees centigrade until use.

Materials

Beaker
API 20C Strips
Report Sheets
API 20C Basal Medium Ampules
Incubation Trays and Covers
Sterile Wooden Applicator Sticks
Sterile Pasteur Pipettes, 5 mL
Squeeze Bottle with Tap Water

Procedure

1. **Preparation of strips**
   a. Set up incubation tray and cover.
   b. Dispense 10 mL of water into the incubation tray with the squeeze bottle.
   c. Place one API strip in each incubation tray.

2. **Preparation of yeast suspension**
   a. Allow basal medium ampules to reach room temperature before use.
   b. Place ampules in beaker of water and bring to a boil. Allow ampules to remain boiling for five minutes after the medium appears melted.
   c. Remove ampule from boiling water and allow to cool for 10-20 minutes.
   d. Place the plastic breaker cap on the ampule, and snap the top off by applying thumb pressure in an outward motion.
   e. Prepare a yeast suspension in the API Basal Medium from a fresh culture of 48 to 72 hours growth on Sabourad's dextrose agar. The yeast suspension is standardized to a density just below 1+ on a Wickerham card (lines appear clearly distinguished but have rough edges).

FIGURE 1 (CONT'D)

3. Inoculation of the API 20C strips

a. With a sterile Pasteur pipette, inoculate each cupule by placing the pipette tip against the side of the cupule, while avoiding bubble formation. The cupule should be completely full, even slightly convex, in order to avoid possible misreading of the assimilation test.
b. Cover the incubation tray with the lid.

4. Incubation of the API 20C strips

a. Incubate at 30 degrees centigrade for 72 hours.

5. Reading the API 20C strips

a. Reactions are read and recorded at 24, 48, and 72 hours incubation.
b. The O cupule serves as a negative control, and any cupules showing more turbidity than it are considered positive. Positive reactions should compare in turbidity to the GLU cupule which serves as a positive growth control.

6. Identification of the organisms

a. Identification of the organisms can be made with the API 20C Analytical Profile Index.

7. Disposal

a. The entire incubation unit must be placed in a biohazard container to be autoclaved.

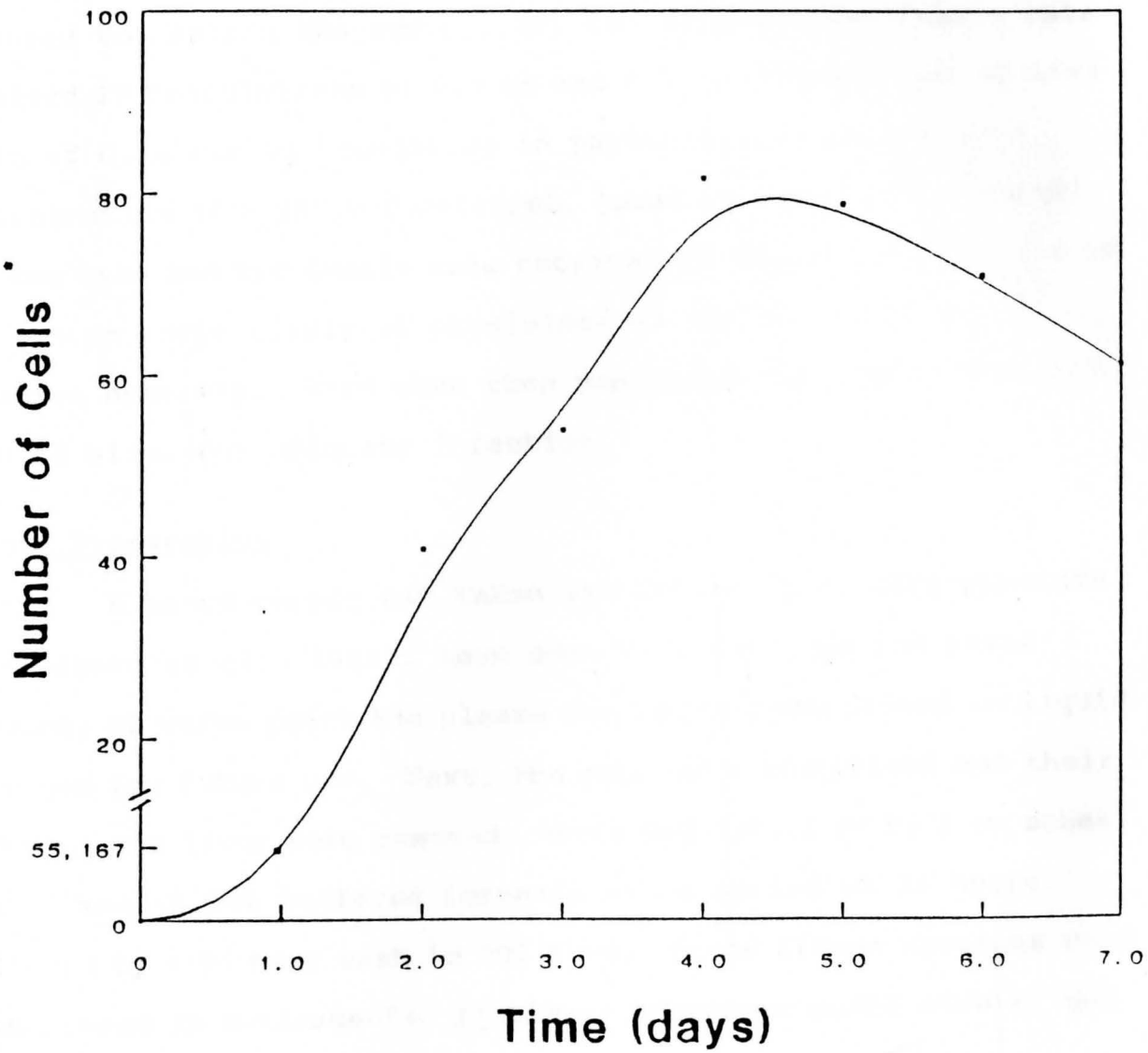
Reference: Analytab Products, API 20C Clinical Yeast System, 1985.

FIGURE 2

Growth curve for *Candida albicans*.

Candida albicans

Growth Curve



• all values (except day 1) $\times 10^6$

Injection/Infection

Male and female Long-Evans rats were obtained and injected in the following manner: (1) two male and two female rats received intraperitoneal (IP) inoculations of 5.0 cc and 2.5 cc respectively of lyophilized cellular supernatant from several test tubes reconstituted with physiological saline (this supernatant is presumed to contain the toxin), (2) two male and two female rats received IP inoculations of 5.0 cc and 2.5 cc respectively of live cells of *C. albicans* brought up in physiological saline to a concentration of 2,250,000 cells/mL, (used as a positive control), (3) two male and two female rats received IP inoculations of 5.0 cc and 2.5 cc respectively of physiological saline, (used as a negative control). Rats were then monitored for a period of six days to allow for adequate infection.

Sample Preparation

A blood sample was taken via intracardiac (IC) puncture (Hitzelberg et al., 1985), spun down in a Serofuge for seven minutes, at which point the plasma was removed and stored in liquid nitrogen for future use. Next, the rats were sacrificed and their kidneys, and liver were removed, sectioned into 1 cm by 1 cm cubes, then fixed in 10% buffered formalin for a period of 24 hours, followed by a 24 hour wash in 70% EtOH. These tissue sections were then placed in a Tissue-Tec II tissue processor model 4640-B, and run through the following solutions:

90% EtOH x2 (1 hr. each)

•

abs. EtOH x2 (1 hr. each)

•

xylene x2 (1 hr. each)

•

hot paraffin

The tissue cubes were removed from the hot paraffin and paraffin embedded utilizing a Tissue-Tec II tissue embedding center, model 4603. These paraffin embedded sections were then sectioned on an American Optical "820" Spencer microtome to a thickness of 3 microns, floated onto pre-albuminized slides, and allowed to thoroughly dry.

DNA Staining

The above slides were stained for stoichiometric DNA measurements through the use of the Feulgen nuclear reaction (Feulgen and Rossenbeck, 1924).

The staining reagents were prepared as follows:

(1) **Schiff's Reagent-** add 10.0 g Basic Fuchsin (Fisher Scientific Co.) and 22.0 g potassium metabisulfite to 1,000 mL of 1N HCl, stir mechanically for two hours in a dark environment and let stand overnight in a dark place. Four grams of activated charcoal were added to the solution and then filtered until clear.

(Store at 5 degrees centigrade)

(2)10% Potassium Metabisulfite- add 30.0 g potassium metabisulfite to flask and bring volume to 300 mL with distilled water.

(3)Hydrochloric Acid Rinse- 5N HCl

The previously prepared slides were run through the staining procedure as follows:

STEP	REQUIRED TIME
1. xylene	10 minutes
2. absolute EtOH	10 minutes
3. 95% EtOH	10 minutes
4. 70% EtOH	10 minutes
5. distilled water	3 minutes
6. 5N HCl (critical)*	45 minutes
7. stain**	2 hours
8. 10% K-meta rinse	5 minutes
9. 10% K-meta rinse	5 minutes
10. distilled water	1 minute
11. 70% EtOH	3 minutes
12. 95% EtOH	3 minutes
13. absolute EtOH	3 minutes
14. xylene	5 minutes
15. permount/coverslip	

* (Yemma and Therrien, 1972)

** Note: the stain is to be fortified with 10% K-meta to create an 80% solution of Schiff's reagent, just prior to staining.

Note: Trichloracetic acid (TCA) treated controls and experimental slides were stained simultaneously in order to eliminate any variations in staining.

Microspectrophotometry

The microspectrophotometer used for the quantitative cytophotometric measurements of nuclear DNA was a Zeiss Universal Type 01 model with the objective of choice being a 100x Planachromatic oil immersion lens. The two wavelength method of Patau (1952) was chosen for the DNA measurements. The procedure is as follows:

A homogeneous area is chosen from which an absorption curve is plotted (Figure 3) and a maximum absorbing wavelength is determined. A second wavelength is then selected so that:

$$2E1 = E2$$

Where:

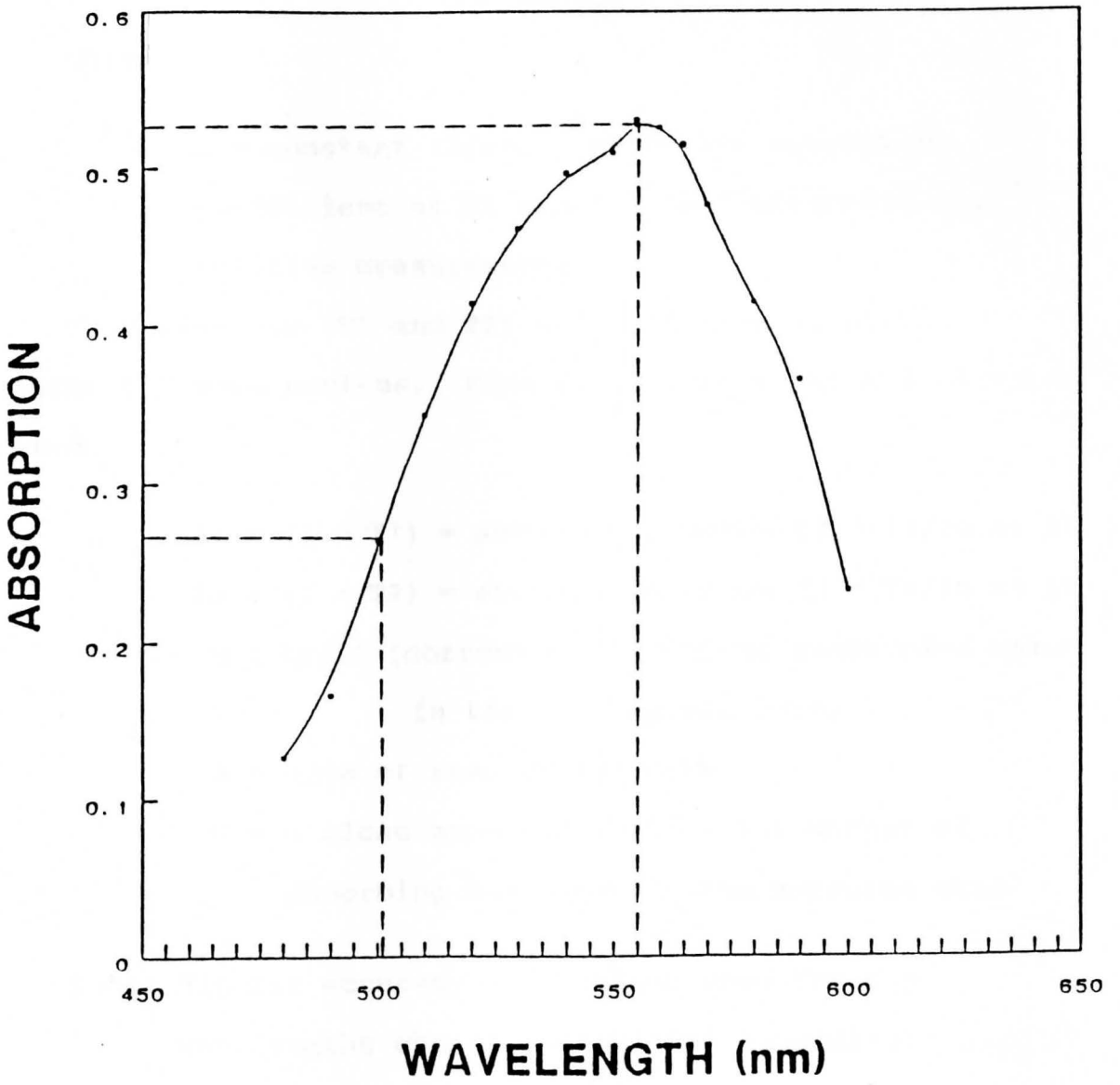
E1 = Log Io/Is at $\lambda 1$ (maximum wavelength)

E2 = Log Io/Is at $\lambda 2$ (half maximum wavelength)

Io is background light and Is is light passing through the specimen.

FIGURE 3

Absorption spectrum of Feulgen stained nuclei of *Candida albicans*.


ABSORPTION
0.6
0.5
0.4
0.3
0.2
0.1
0
450
500
550
600
650
WAVELENGTH (nm)

After the two wavelengths have been determined, inhomogeneous regions may be measured. The amount of dye (DNA) in the measured area (A), regardless of its distribution, is given as:

$$M = KAL1Q$$

Where:

K = constant (1/e), e being the extinction coefficient at $\lambda 1$ and may be disregarded for relative measurements.

Transmissions (T1 and T2) were obtained at both wavelengths for each nucleus. From these values, L1 and L2 were calculated.

L1 = (1 - T1) = absorption, where T1 = Is/Io at $\lambda 1$

L2 = (1 - T2) = absorption, where T2 = Is/Io at $\lambda 2$

Q = L2/L1 (correction factor for unoccupied space in the reading aperture)

A = area of reading aperture

M = a close approximation to the number of absorbing molecules in the measured area

Note: Highest accuracy is obtained when the two wavelengths chosen do not give exceedingly high or low transmissions, therefore, the values of L2/L1 should fall above 1.5. In addition, the microspectrophotometer pulses each cell 280 times per second and consequently the transmission or

optical density reading is actually the average of these pulses. Also, the selection of nuclei to be read was completely random.

Acid Phosphatase Assay

The acid phosphatase assay performed in this study followed the protocol of Barnett and Heath (1977), utilizing a Perkin Elmer Lambda 2 UV/VIS spectrophotometer which interfaces with an Epson Equity III+ computer.

The substrate for the reaction was bis (p-nitrophenol) phosphate (Sigma Chemical CO., N-1256). A positive control was run substituting the plasma sample with 0.3 milliliters of a solution of o-nitrophenol (Sigma Chemical Co., N-9256) at a concentration of 92.7 micrograms/milliliter.

The previously frozen serum samples were removed from the liquid nitrogen and allowed to thaw. The initial incubation mixture was comprised of 1.2 mL of 5mM sodium acetate (pH 5.0), 0.5 mL of 5mM p-nitrophenyl phosphate, and 0.3 mL of respective plasma samples. This mixture was allowed to incubate at room temperature for 20 minutes. Next, the reaction was terminated utilizing 100 microliters of a solution consisting of 1M Tris, 1M potassium phosphate, and 0.25M EDTA (pH 10.5) in equal volumes. This solution contained a concentrated amount of phosphate in order to inhibit alkaline phosphatase activity from interfering with acid phosphatase activity. Absorbance was measured at 420 nanometers against a distilled water blank, and data was recorded.

Electrophoresis

Electrophoresis was performed utilizing the Helena Laboratories Titan Gel High Resolution Protein System in an attempt to isolate the different protein constituents within the different serum samples. The medium used was a Titan Gel High Resolution Protein Gel. The buffer used was the Titan Gel High Resolution Protein Buffer (pH 8.4-8.8).

The serum samples were removed from liquid nitrogen, thawed, and then a dilution of 1:2 was achieved with the buffer. These samples were then applied in duplicate onto the blotted gel in a 3 uL volume utilizing the template provided. The samples were allowed to diffuse for 5 minutes at which point the excess was blotted away. Next, the gel was moved onto a cooling plate (agarose side up) and placed into a previously prepared Helena Electrophoresis Chamber containing 150 mL of buffer in each outer well, and crushed ice in the inner wells to insure a cold environment. The Titan Gel wicks were placed onto the gel, the cover was placed on the chamber, and the gel was allowed to equilibrate for 60 seconds. After the equilibration period the chamber was plugged into a Helena Titan power supply, and electrophoresed at 250 volts for 20 minutes.

Gel Staining

The staining method chosen for this study was the silver stain method because of its increased sensitivity. A Titan Gel Silver Stain Kit was purchased from Helena Laboratories, Beaumont, Texas, and the gel was stained in the following manner:

(1) The plate was removed from the chamber and placed in a dish containing 100 mL wash solution, and gently swirled for 5 minutes.

(2) The plate was removed from the wash and thoroughly dried at 65 degrees centigrade in a Precision mechanical convection oven.

(3) When the plate was completely dry it was moved into a clean dish containing 50 mL Fixative Reagent and gently swirled for 10 minutes.

(4) The plate was then washed in two consecutive 5 minute washes of 100 mL Accelerator Reagent.

(5) The plate was removed from the last wash and the plastic backing was gently wiped off using a lint-free tissue.

(6) The plate was then placed in a clean dish containing 100 mL Reducing Agent and gently swirled for 20 minutes.

(7) While the plate was in the Reducing Agent, the Working Silver Stain was prepared (due to its extremely short shelf life).

(8) The plate was removed from the Reducing Agent and immediately placed into the Working Silver Stain for 5 minutes.

(9) The staining process was stopped by placing the plate in a dish containing 100 mL of Stop-Bath Solution for 10 minutes.

(10) The plate was removed from the Stop-Bath Solution and allowed to air dry.

Scanning

The dry plate was analyzed utilizing an LKB Bromma Ultrascan XL Enhanced Laser Densitometer, the peaks plotted, and analyzed.

High Performance Liquid Chromatography (HPLC)

The determination of relative acid phosphatase levels within the different serum samples was accomplished utilizing a Perkin Elmer series 400 chromatograph equipped with a Model LC75 variable-wavelength detector set at 280 nm. An acid phosphatase standard was obtained from Sigma Chemical Co. and was brought up in a 200:1 dilution. This standard was used as a method of comparison of the serum constituent peaks for determination of increased acid phosphatase levels, indicating cellular damage. The data was recorded and analyzed using a Perkin Elmer 7500 Professional computer.

The mobile phase used was (A) acetonitrile, (B) HPLC water, and (C) 0.02M phosphate buffer at a pH of 6.0. The acetonitrile and the HPLC water used to make up the phosphate buffer were obtained from Fisher Scientific Co., and the sodium phosphates were obtained from Sigma Chemical Company. Prior to each use, the solutions were first suction filtered through a 0.45 um pore size filter obtained from Pierce Chemical Co., and then degassed with pure helium.

The serum samples were again removed from liquid nitrogen and allowed to thaw, at which point a sample of each was diluted 50:1 with the mobile phase, and filtered through a 0.45 um filter (Gelman Sciences, Ann Arbor, Michigan) into a sample vial. Twenty microliters of each sample were then injected onto a C-18 Aquapore (220 mm x 4.6 mm x 7.0 um) RP 300 HPLC column obtained from Pierce Chemical Co. using a Perkin Elmer ISS-100 injector with a 200-ul loop. The mobile phase for the samples was delivered in 25 minutes at a flow rate of 1.0 mL/min., and in a linear gradient of 100% A - 50% A and 0% C - 50% C. In addition, the environment of the column was maintained at a constant temperature of 37 degrees centigrade by an oven encasing the column.

CHAPTER III

Results

The techniques of spectrophotometric and high performance liquid chromatography (HPLC) analysis were used to determine the levels of serum acid phosphatase for the control and experimental rats used in this study. The data obtained was directly compared with quantitative cytophotometric DNA measurements coupled with electrophoretic protein measurements in order to determine if a correlation could be obtained with DNA changes. As already mentioned, it has been previously reported (Kettner, 1983) that *Candida* toxin causes the release of lysosomal enzymes by injury to these organelles, and thereby causing pronounced changes in tissues.

Controls were of two types; first, untreated rats were designated as normal or as negative controls and secondly, rats treated with whole cells of *Candida albicans* were designated as positive controls. Experimental rats, treated with the toxin, were subsequently sacrificed and serum and tissue samples taken. These samples were then compared to the two different control groups in order to analyze the chemical and physiological effects of *Candida albicans* toxin only.

Double Beam Spectrophotometric Analysis of Serum Acid Phosphatase

The use of absorption spectrophotometry necessitates the

establishment of a standard curve for the particular assay being performed. The standard curve can be found in Figure 4, with absorbance on the abscissa and concentration on the ordinate. The values obtained from the assay are represented on Table 2. From these results it can be clearly seen that the levels of serum acid phosphatase are significantly higher in the positive controls and toxin-treated rats when compared to the normal (untreated) control animals.

High Performance Liquid Chromatographic Analysis of Serum Acid Phosphatase Levels

High performance liquid chromatographic analysis was carried out with the three serum samples within each group being pooled. This was done in order to verify any changes in the levels of acid phosphatase within the whole cell-treated controls and the toxin-treated animals when compared to the untreated (normal) animals. The results for both the acid phosphatase standard used and the experimental animals are shown in Figures 5-8.

Feulgen Absorption Curve

The use of the two-wavelength method of microspectrophotometry requires the establishment of an absorption curve (Figure 3). The maximum absorption for the Feulgen-DNA complex was at a wavelength of 555 nm, with the half-maximum wavelength value at 501 nm.

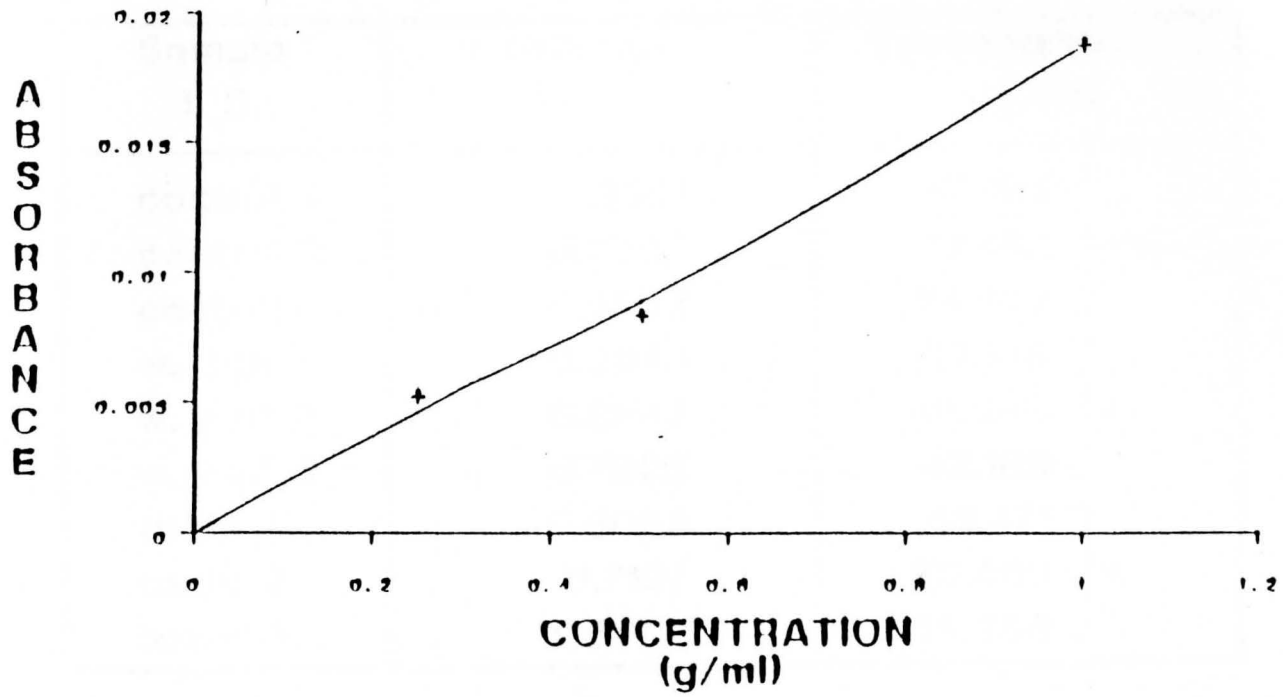
Microspectrophotometric Analysis of DNA Content

The deoxyribonucleic acid (DNA) concentrations were

FIGURE 4

Standard curve for acid phosphatase assay.

ACID PHOSPHATASE ASSAY



standards at .25, .5, and 1.0

TABLE 2

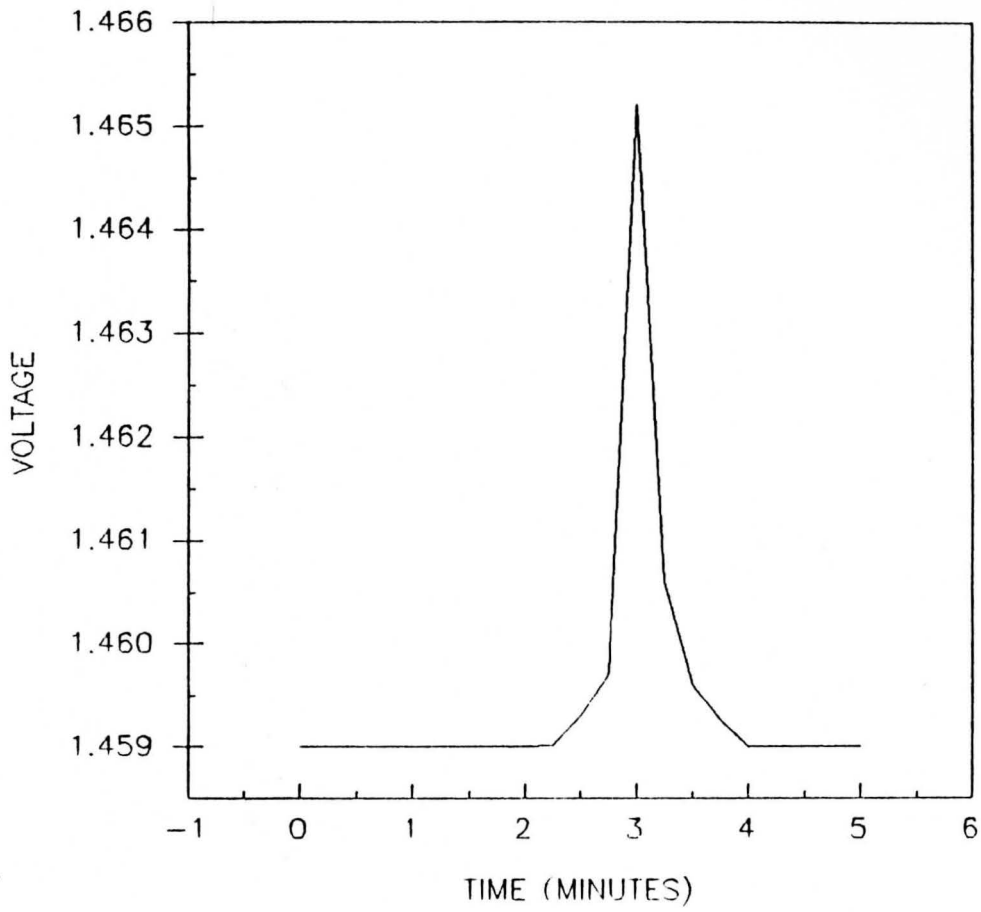
ACID PHOSPHATASE ASSAY RESULTS

Sample I. D.	Ordinate Value	Concentration (g/ml)	
control 1	0.3561	19.194	norm.
control 2	0.4223	22.762	
control 3	0.4533	24.433	
w. cell 1	0.9673	52.138	*
w. cell 2	0.8543	46.048	
w. cell 3	0.7826	42.183	
toxin 1	0.8065	43.471	+
toxin 2	0.7137	38.469	
toxin 3	0.6745	36.356	

* Significant at the .002 level
+ Significant at the .003 level
NOTE: compared to normal control.
*, + demonstrate no significant difference

FIGURE 5

High performance liquid chromatography assay of acid phosphatase standard.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1.461
1.460
1.459
VOLTAGE
−1
0
1
2
3
4
5
6
TIME (MINUTES)

FIGURE 6

High performance liquid chromatography assay of acid phosphatase levels in the serum of the untreated (normal) rats.

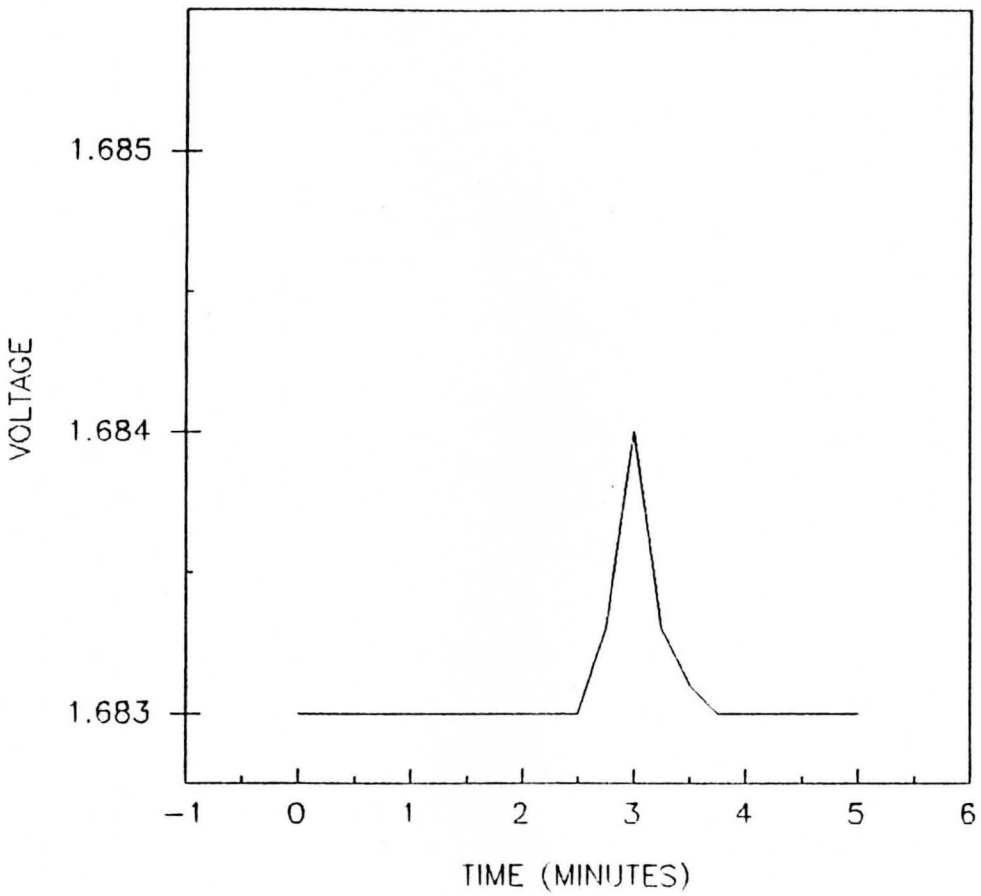

1.685
1.684
1.683
VOLTAGE
−1
0
1
2
3
4
5
6
TIME (MINUTES)

FIGURE 7

High performance liquid chromatography assay of acid phosphatase levels in the serum of the whole cell-treated rats.

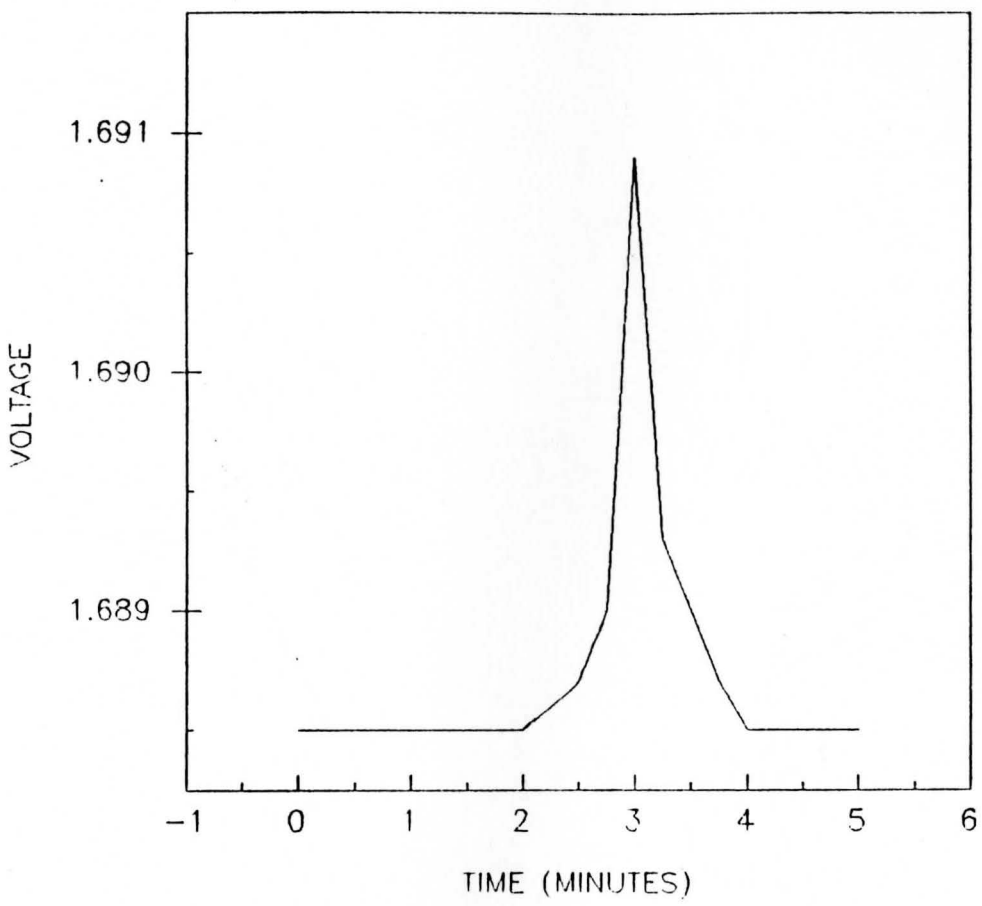
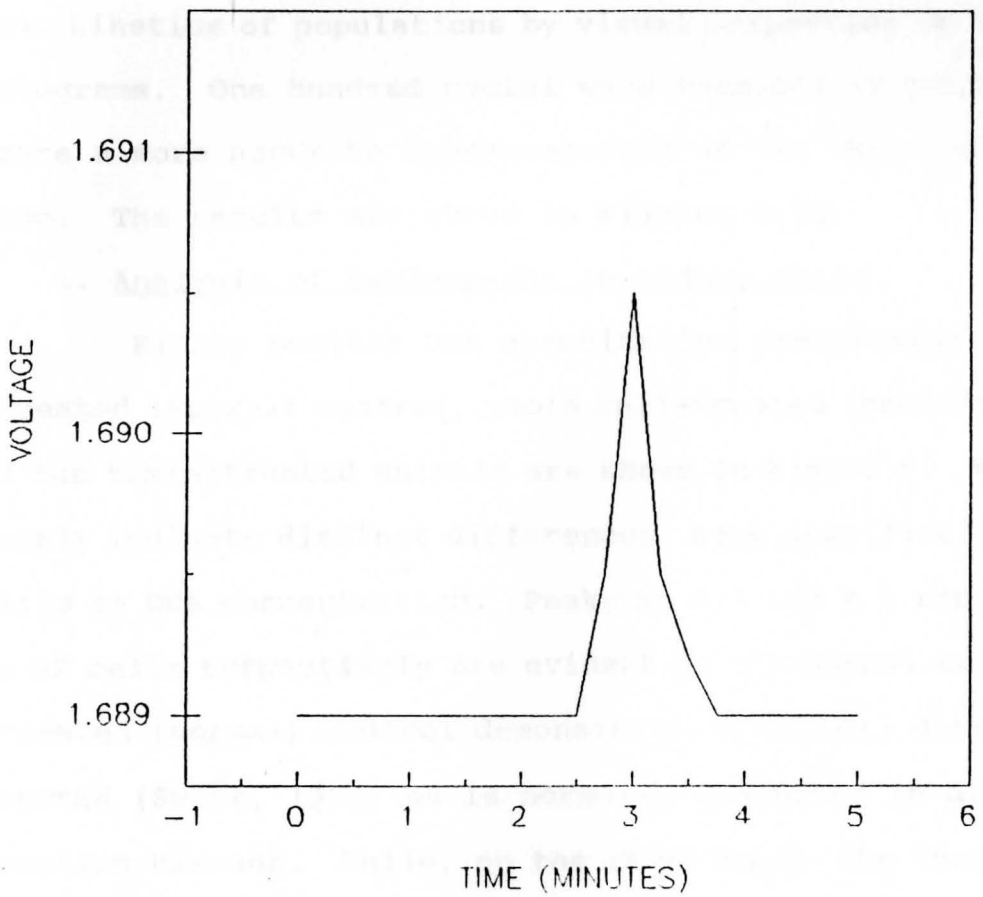

1.691
1.690
1.689
VOLTAGE
−1
0
1
2
3
4
5
6
TIME (MINUTES)

FIGURE 8

High performance liquid chromatography assay of acid phosphatase levels in the serum of the toxin-treated rats.


1.691
1.690
1.689
VOLTAGE
−1
0
1
2
3
4
5
6
TIME (MINUTES)

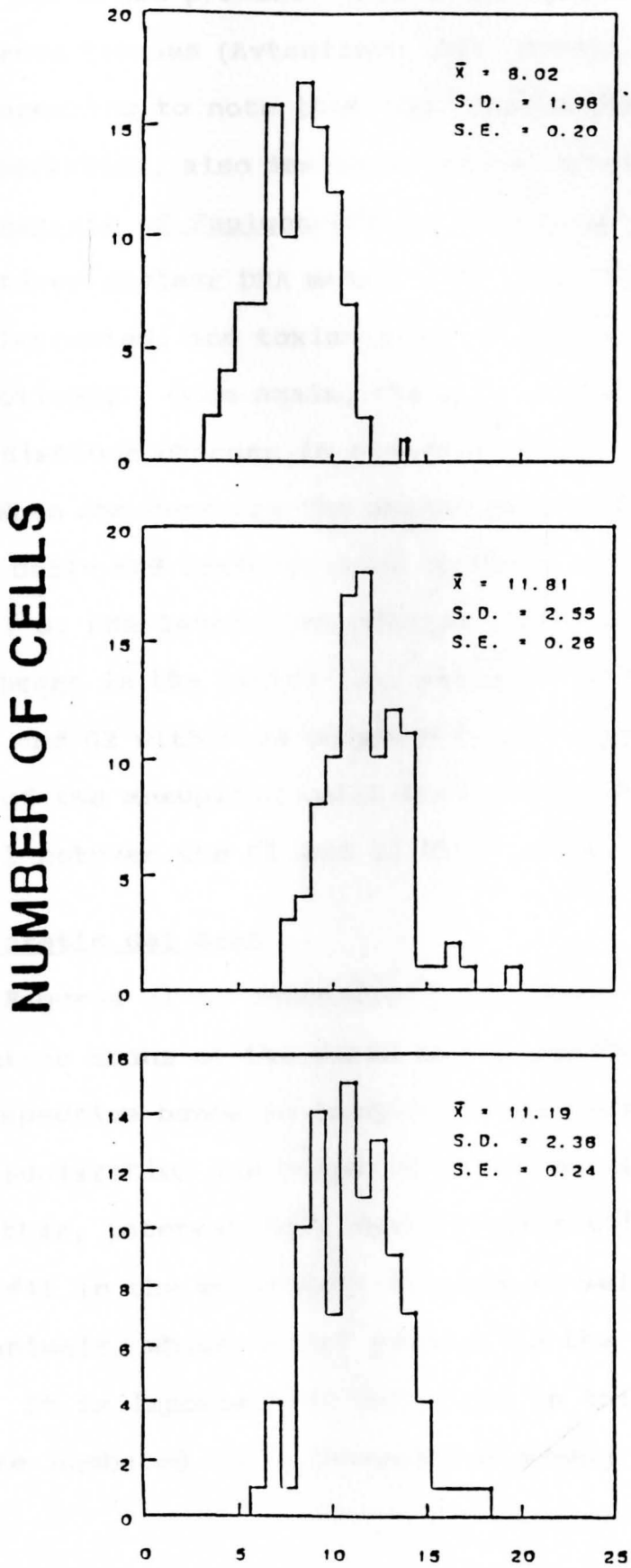
measured in the kidney and liver tissues of each of the three groups of animals. The data obtained is presented in the form of frequency histograms with the number of cells on the abscissa and the relative dye concentration on the ordinate. DNA profiles as exhibited, provide for the detection of changes regarding cell cycle kinetics of populations by visual inspection of the frequency histograms. One hundred nuclei were examined in each case, to insure a more accurate representation of the DNA content of each group. The results are shown in Figures 9-10.

1. Analysis of Feulgen-DNA in Kidney Cells

Kidney nuclear DNA quantitative measurements of the untreated (normal) control, whole cell-treated (positive) control, and the toxin-treated animals are shown in Figure 9. Measurements clearly indicate distinct differences, more specifically, dramatic shifts in DNA concentration. Peaks at 6.5 and 8.5 representing G1 and G2 cells respectively are evident in the normal controls. The untreated (normal) control demonstrates a bimodal distribution as expected (Swift, 1950) as is normally exhibited in diploid mammalian tissues. While, on the other hand, the whole cell- and toxin-treated animals clearly demonstrate a near total lack of cells at the normal G1 value, with some S cells present; however, most cells are at G2 DNA level. Interestingly many cells are above this value and some are clearly polyploid. Since DNA values of 6.5 represent G1 cells in the normal controls, one can observe that cells at this value are not represented in the experimentals. One can however note that in these populations many aneuploid and a few

FIGURE 9

Histograms representing Feulgen-DNA values for kidney nuclei of untreated (1), whole cell-treated (2), and toxin-treated (3) animals.


NUMBER OF CELLS
RELATIVE DYE CONCENTRATION
1.
X̄ = 8.02
S.D. = 1.98
S.E. = 0.20
2.
X̄ = 11.81
S.D. = 2.55
S.E. = 0.26
3.
X̄ = 11.19
S.D. = 2.38
S.E. = 0.24

polyploid cells are present. Since this profile is often found in pre-cancerous tissues (Avtanilov, 1976; Nowell, 1976; Stich, 1960), it is interesting to note that the tissues from our study, upon visual observation, also demonstrated the presence of small tumors.

2. Analysis of Feulgen-DNA in Liver Cells

Liver nuclear DNA measurements of the untreated control, whole cell-treated, and toxin-treated animals are shown in Figure 10, respectively. Once again, these frequency histograms clearly indicate distinct changes in ploidy levels between the different groups. When one compares the untreated control values to those of the whole cell- and toxin-treated animals, one can clearly see that the normal G1 DNA levels (as pointed out previously) are almost totally absent in the latter two, while the majority of their cells are in S, and G2 with some polyploid cells present. Clearly, the G1 cells of the aneuploid cells have predominantly high values which fall between the G1 and G2 DNA levels of the normals.

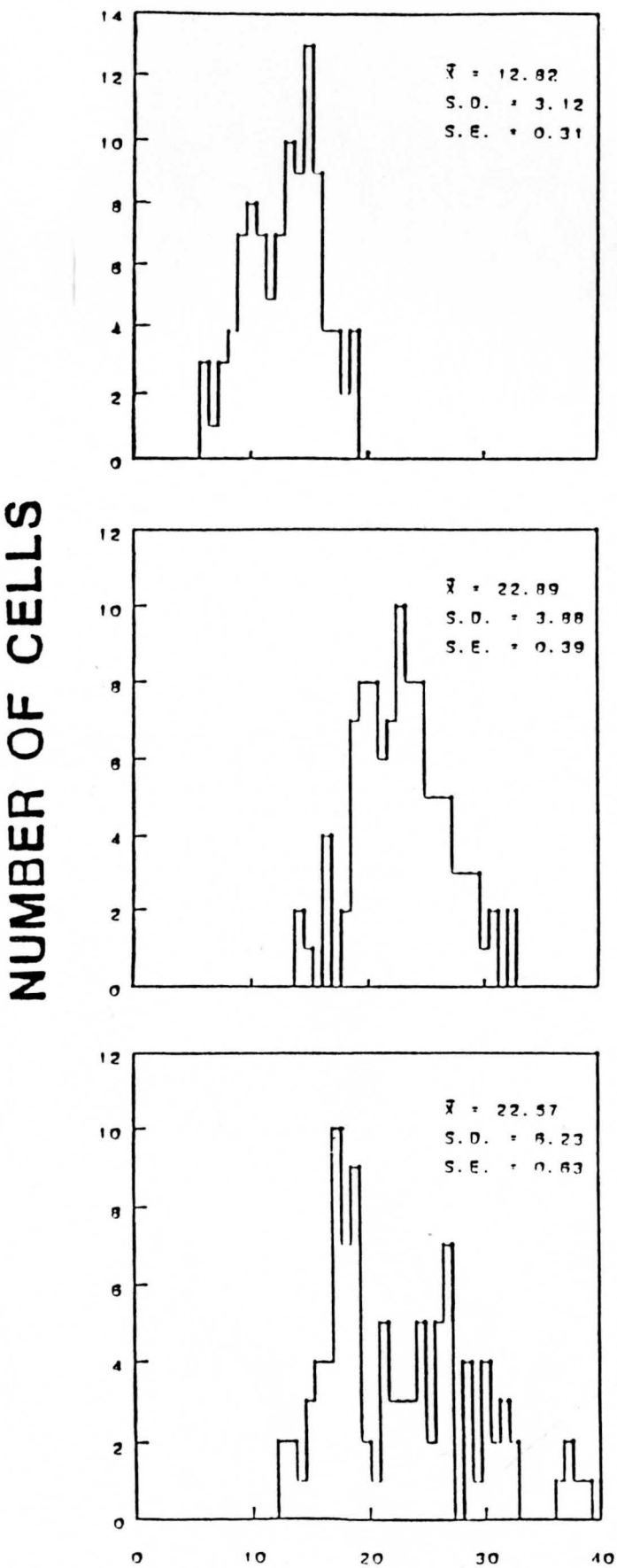
Electrophoretic Gel Scan

Figures 11-13 demonstrate graphs of the results of the densitometric scans of the serum electrophoretic gels. A diagram of the respective bands is included below each graph to allow for better visualization and comparison. These graphs, when compared to each other, interestingly show an unidentified additional peak or band (#4) in the whole cell-treated as well as in the toxin-treated animals, which is not present in the untreated (normal) animals. It is important to note that in this observation the peaks were numbered in a consecutive manner independent of each

other in each tracing. It can be observed that a new peak #4 arises in Figures 12 and 13 when compared to Figure 11. In addition, peak or band #2 appears to be the same as that of the glycoprotein (acid phosphatase) as has been previously reported in the literature by the Helena Company, where extensive work has been done on the migration of isoenzymes (Laurell, C. B., 1972). It should be noted that previous investigations had speculated (Kettner, 1983) that *Candida* toxin may damage cells by destroying lysosomes and releasing its very powerful hydrolytic enzymes in the cell, and hence into the serum. It is critical to note that the data from the HPLC work and of the spectrophotometric analysis directly supports this contention.

FIGURE 10

Histograms representing Feulgen-DNA values for liver nuclei of untreated (1), whole cell-treated (2), and toxin-treated (3) animals.


NUMBER OF CELLS
x̄ = 12.82
S.D. = 3.12
S.E. = 0.31
x̄ = 22.89
S.D. = 3.88
S.E. = 0.39
x̄ = 22.57
S.D. = 6.23
S.E. = 0.63
0
10
20
30
40
RELATIVE DNT CONCENTRATION

1.

2.

3.

FIGURE 11

Gel electrophoresis pattern and densitometer tracing of the serum proteins of the untreated (normal) animals.

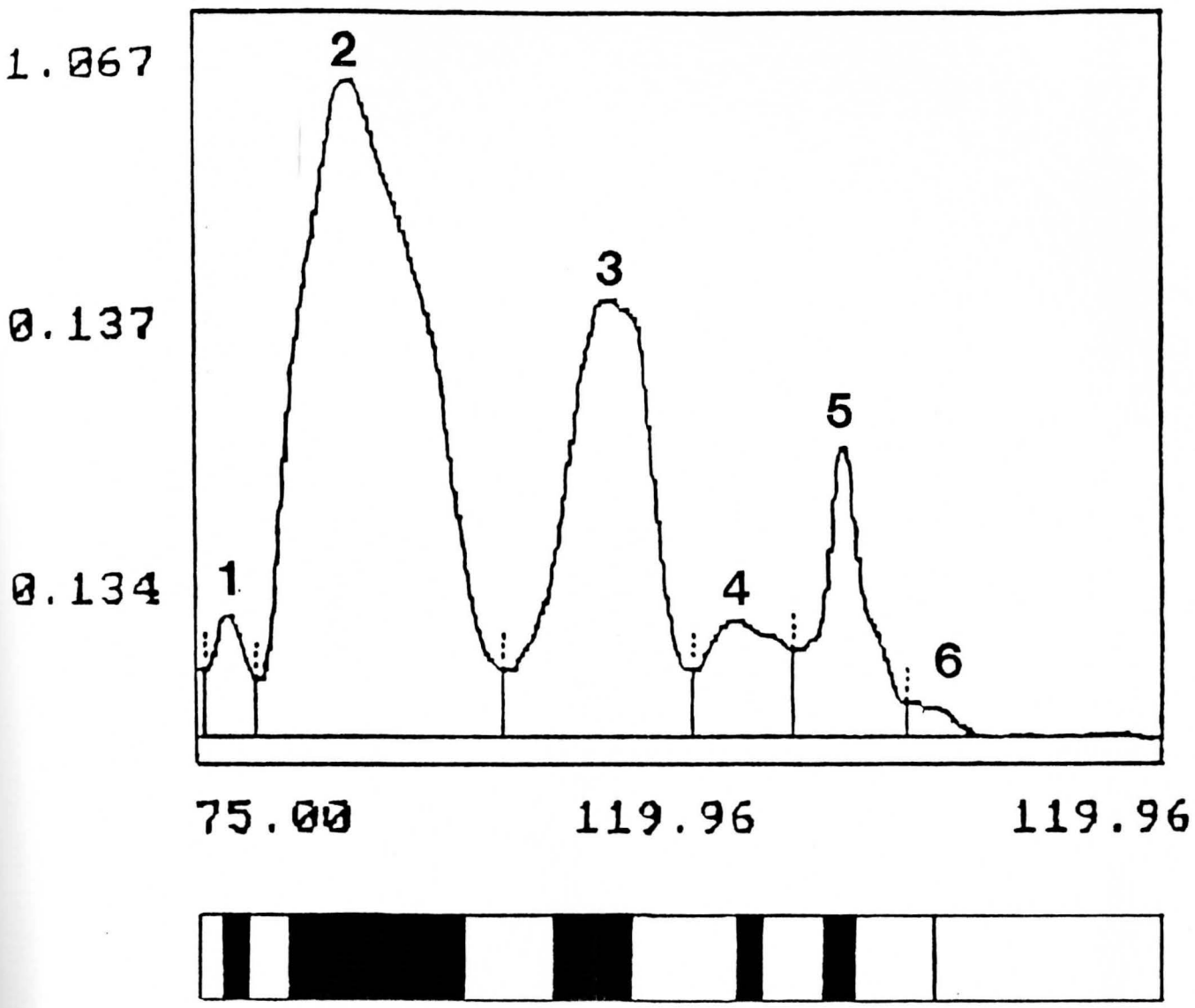

1.067
0.137
0.134
1
2
3
4
5
6
75.00
119.96
119.96

FIGURE 12

Gel electrophoresis pattern and densitometer tracing of the serum proteins of the whole cell-treated animals.

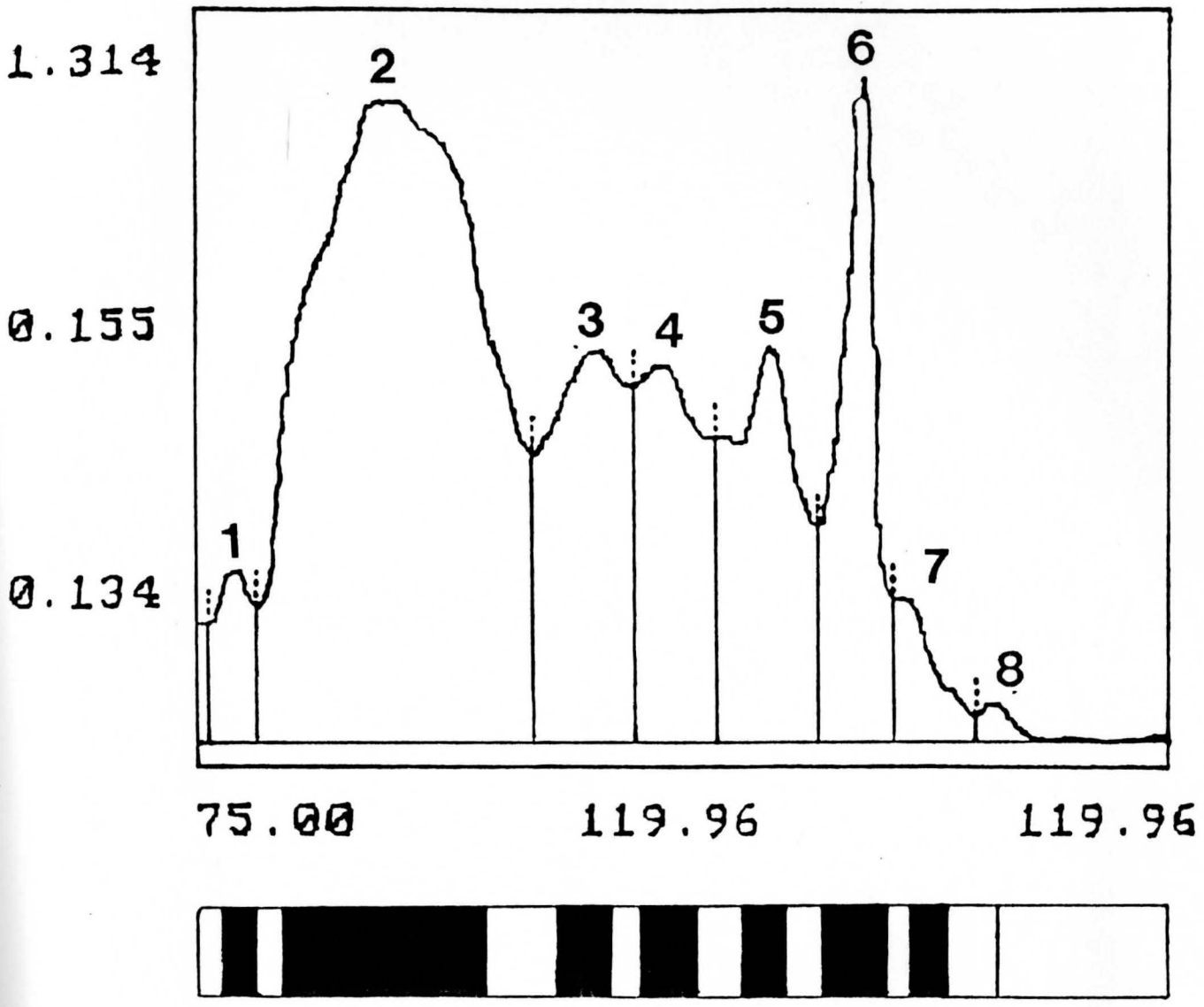
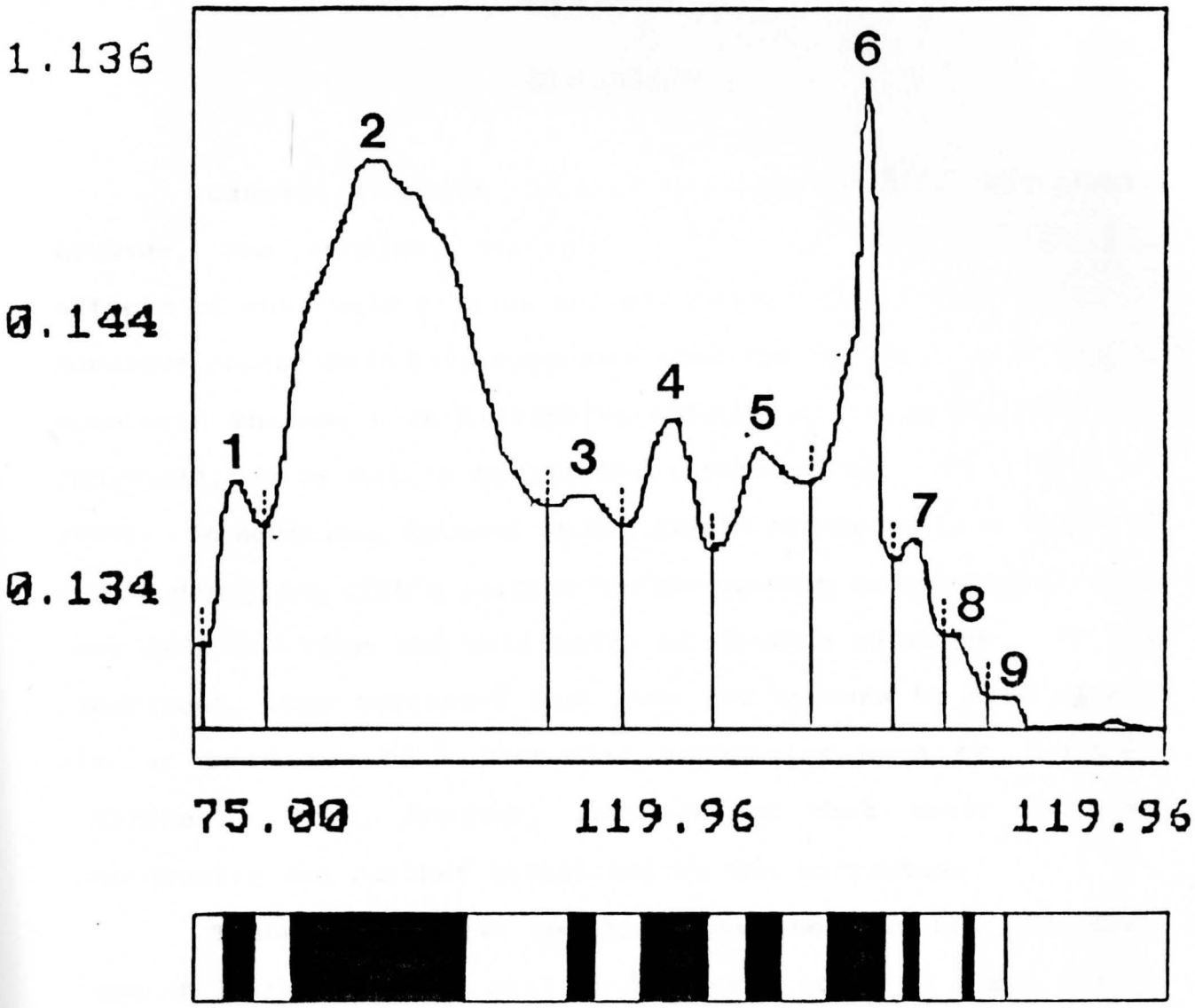

1.314
0.155
0.134
1
2
3
4
5
6
7
8
75.00
119.96
119.96

FIGURE 13

Gel electrophoresis pattern and densitometer tracing of the serum proteins of the toxin-treated animals.


1.136
0.144
0.134
1
2
3
4
5
6
7
8
9
75.00
119.96
119.96

CHAPTER IV

Discussion

Candida albicans toxin(s) has been studied extensively; however, the chemical characterization and the physiological effects of the toxin(s) have not been adequately demonstrated. Numerous researchers have suggested that the toxin(s) is in fact an endotoxin and may have disruptive effects on lysosomal membranes (Caldwell, 1975; Galvin et al.,1978; Farb et al., 1976; Martini, 1959). In addition, Kettner et al. (1983) attempted to demonstrate such a condition with a polysaccharide-protein complex (PPC) which they isolated from the cell walls of *Candida albicans*. In this experiment, they suggested that this PPC appears to have effects similar to that which bacterial endotoxins have on lysosomal membranes. They, however, pointed out that their data was inconclusive and further investigation was warranted.

These facts serve to illustrate the need for additional research in this area as well as its correlation to other areas involving the deleterious effects of *Candida albicans* toxin(s). With this in mind, the present study attempts to look at serum acid phosphatase levels and to directly correlate them with changes in DNA concentration within hepatic and renal tissues related to cellular damage.

Although initial attempts to infect the chosen model

system (Long-Evans rats) were unsuccessful (due to premature death, most likely due to the administration of high toxin levels), further experimentation soon developed successful techniques. Subsequent attempts demonstrated a thorough infection with concomitant visual lesions/tumors. This was verified by the fact that the animals were somewhat sluggish and numerous lesions/tumors were observed within the peritoneal cavities of both the positive control and experimental animals.

The Double-Beam spectrophotometric analysis of the plasma of the infected animals yielded acid phosphatase levels which were greatly increased in the whole cell- and toxin-treated animals when compared to the normal levels. These values are found in Table 2 which clearly demonstrates the increased levels and strongly suggests lysosomal damage. This is due to the fact that the resultant values in some cases are twice that of the normal levels, indicating that a great deal of lysosomal damage has in fact occurred. The validity of these results were then analyzed for verification through high performance liquid chromatographic analysis. This analysis also demonstrated large increases in acid phosphatase in the infected animals, thus verifying the suggested lysosomal damage. The results of this additional experimentation can be found in Figures 5-8 which clearly demonstrate a direct correlation to the results of the Double-Beam spectrophotometric analysis as well as the electrophoretic analysis which will be discussed later.

Consequently, due to such a drastic response, there is

adequate reason to believe that not only is there lysosomal damage but there is also increased DNA activity or cellular damage as well. As stated earlier, upon dissection of the now lethargic animals, numerous lesions/tumors were observed within their peritoneal cavities, thus indicating potential DNA changes as is often observed in precancerous tissues. This presumption was soon verified through microspectrophotometric analysis of renal and hepatic tissues. In fact, it was noticed that there was a quite drastic change in DNA concentration, as is observed when one views the histograms representing cellular DNA measurements in experimental tissues versus the normal controls (Figures 9-10). This shift illustrates a near total lack of cells in the infected animals at the G1 level of the normals, whereas the majority of their cells have permanently increased DNA levels with many in G2 and above, indicating the presence of many aneuploid and polyploid cells. The radical shift is a result of the partial replication of the genome DNA not followed by mitosis but instead re-entering the cell cycle at the elevated DNA level. Consequently, their G1 cells fall at approximately the S level of the normal cells, eventually resulting in polyploid cells. This, therefore, directly correlates elevated acid phosphatase levels with the drastic changes in DNA concentration, indicating severe ensuing cellular/tissue damage as is seen in precancerous and cancerous tissues (Nowell, 1976).

It was next necessary to attempt to characterize tissue changes or toxin activity associated with these reported changes.

The analysis of choice here was electrophoresis since it was suggested in the literature that the toxic agent could in fact be a polysaccharide-protein complex (Kettner et al., 1983). Our findings in this case can be observed in Figures 11-13 which demonstrate the appearance of an additional band/peak (#4) in the infected animals mid-way through the run. Here too, it is noticeable that there are increased levels of the different plasma constituents. In this case, the acid phosphatase level can be observed as the second band/peak (Laurell, C. B., 1972).

It is clearly evident that there is in fact lysosomal damage and the resultant spillage of acid phosphatase into the serum, as well as significant changes in DNA activity. Additionally, this study demonstrates that toxic material is released by *Candida albicans* into its surroundings. This is verified by the fact that infection, and all the damage associated with it, can be achieved without the presence of the whole cells. Whether the additional band/peak achieved through electrophoresis is in fact a toxic component released by *Candida albicans* cells requires further investigation.

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