THE ANALYSIS OF METHIONINE AND LEUCINE ENKEPHALINS IN RELATIONSHIP TO THE IMMUNE RESPONSE IN TRAUMA PATIENTS BY HIGH PRESSURE LIQUID CHROMATOGRAPHY AND MICROSPECTROPHOTOMETRIC TECHNIQUES

by

Ruth J. Green

Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Biological Sciences

Program

John J. Yemma Ph.D. 8-26-88

Advisor Date

Sally M. Hotchkiss August 26, 1988

Dean of the Graduate School Date

YOUNGSTOWN STATE UNIVERSITY

August, 1988

THESIS APPROVAL FORM

THESIS TITLE: The Analysis of Methionine and Leucine Enkephalins in Relationship to the Immune Response in Trauma Patients by High Pressure Liquid Chromatography and Microspectrophotometric Techniques.

AUTHOR: Ruth J. Green

DEGREE: Master of Science

ADVISOR: Dr. John J. Yemma

COMMITTEE MEMBERS:	ACCEPT	REJECT
James R. Gaylor	✓	
Paul C. Peters	✓	
John J. Yemma	✓	

DATE August 25, 1988

1 copy to accompany library copy

1 copy to advisor

1 copy to Departmental Files

ABSTRACT

THE ANALYSIS OF METHIONINE AND LEUCINE ENKEPHALINS IN RELATIONSHIP TO THE IMMUNE RESPONSE IN TRAUMA PATIENTS BY HIGH PRESSURE LIQUID CHROMATOGRAPHY AND MICROSPECTROPHOTOMETRIC TECHNIQUES

Ruth J. Green

Master of Science

Youngstown State University, 1988

Numerous reports on the affect of enkephalins (endogenous opiates) on the immune system have recently appeared in the literature. Most of these are studies which center on the site of production, effects on the nervous system, and their role in the modulation of pain. However, none of these studies report on the interaction and effect of these molecules on circulating lymphocytes. The present study presents data on serum methionine and leucine enkephalin levels and their affects on circulating lymphocytes. Twelve emergency room trauma patients were studied over a three day period. Serum enkephalin levels were assayed by means of high pressure liquid chromatography, while lymphocyte activity was accessed by measurements of DNA concentration and template activity coupled with protein synthetic activity. The latter studies were done by means of quantitative cytophotometry.

Results of this study demonstrated that as levels of methionine enkephalin increased, DNA concentration, or

synthetic activity, and protein synthesis decreased. Leucine enkephalin had the opposite effect. This suggests that methionine and leucine enkephalins bind to different receptors on the surface of circulating lymphocytes. Emergency patients' response to treatment regarding post-trauma stabilization of their condition significantly affected the levels of circulating enkephalins over the periods studied and thus the synthetic activity of circulating lymphocytes.

ACKNOWLEDGEMENTS

I would sincerely like to thank Dr. John Yemma for his guidance, dedication, and support throughout this study. Without him, this study would never have been possible.

I would like to thank Dr. Howard White, Dr. Robert Kennedy, Jackie Ferraro, and the rest of the staff at St. Elizabeth's Hospital that participated in this study, for their cooperation in drawing and handling the blood samples and their technical assistance.

Finally, I would like to thank David Waite and Wadad Jwayyed for their understanding, support, and encouragement throughout these past two years.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES - CONTINUED

LIST OF FIGURES - CONTINUED

LIST OF TABLES

LIST OF SYMBOLS

SYMBOL	DEFINITION	UNITS OF REFERENCE
A	Area	
D	Ratio of L_0/L, correction factor for an occupied space and aperture	
E	Extinction	
G	Cell cycle phase in which chromosomes are completely replicated	
I_0	Background light	
I	Light passing through the specimen	
K	Specific absorptivity constant of the chromophore at a defined wavelength	
L	Parameter equivalent to one minus the transmission (1 - T) at a defined wavelength	
M	Chromophore mass in the measured field	
R	Radius	
T	Transmission of the field	
λ	Wavelength	nm

CHAPTER I

Introduction

Methionine and leucine enkephalins, recently discovered endogenous opioid peptides, are considered to be potent immunomodulators (Murgo et al., 1986). Conflicting evidence exists, however, as to their specific function. For example, in vivo studies in a normal control group of volunteers demonstrated that methionine enkephalin increased the numbers of active T-cells in the circulation, while at the same time increasing the activity of natural killer cells (Plotnikoff, 1986). Jankovic and Maric (1987) suggest that the enkephalins have a dual effect on the humoral immune response. A large plasma concentration of enkephalins suppresses the immune reaction whereas a smaller amount enhances it. Wybran (1986) reported that there is no significant effect of the enkephalins on lymphocyte response to thymidine uptake or mitogenic activity at the site of their origin. However, data on whether enkephalins activate or depress the immune activity of circulating lymphocytes is lacking. The purpose of this study is to discover the effects of enkephalins on the activity of circulating lymphocytes in acutely traumatized patients.

As previously stated, methionine and leucine enkephalins are naturally occurring endogenous opioid peptides. They

were originally thought to be synthesized in the pituitary from a common precursor which gives rise to alpha, beta, and gamma endorphins. The precursor was identified at that time as pre-proopiomelanocortin (Cox, 1982; Smyth, 1982). Recent evidence, however, disputes this. It is presently believed that the enkephalins and their precursors are formed in the chromaffin granules of the adrenal medulla (Cox, 1982). Two enkephalin precursors have been identified: pre-proenkephalin A and pre-proenkephalin B. Pre-proenkephalin A contains six sequences of met-enkephalin and one sequence of leu-enkephalin. Pre-proenkephalin B contains four sequences of leu-enkephalin (Evans et al., 1986). The structure of methionine and leucine enkephalins are Tyr - Gly - Gly - Phe - Met and Tyr - Gly - Gly - Phe - Leu, respectively (Smyth, 1982).

The enkephalins are thought to be released in response to stressful stimuli and may be associated with the release of catecholamines (Evans et al., 1986; Murgo et al., 1986). Once released they are distributed throughout the central nervous system as well as peripheral sites. The major area of concentration is the brain and pituitary (Evans et al., 1986). Unlike catecholamines and other endorphins, the normal levels of enkephalins show no observable regular rhythm of release. There seems to be no relationship with time of day, food intake, or sleep (Shanks et al., 1981).

The exact function of the enkephalins is not yet known. It has been variously suggested that they function as

neurotransmitters, neuromodulators, or as hormones (Cox and Baizman, 1982). They have been shown to participate in a range of other activities such as analgesia, euphoria, and thermoregulation. It is generally agreed that their effects are mediated through specific membrane receptors. Cells upon which they exert their effect are thought to vary as to the type and number of receptors they have on their surface. It is also believed that the opioids vary as to their binding affinities for the different receptors (Murgo et al., 1986).

Five opioid receptors have been identified, namely, delta, epsilon, kappa, mu, and sigma (Murgo et al., 1986). Beta endorphin appears to be able to interact most specifically with delta, epsilon and mu receptors. Morphine appears to be specific for mu receptors. Enkephalins have the greatest affinity for delta receptors (Zukin and Zukin, 1981; McIntosh and Faden, 1986; Murgo et al., 1986). Plotnikoff (1986) suggests that there may be two subpopulations of delta receptors - one for met-enkephalin and one for leu-enkephalin. It has also been suggested that met-enkephalin has a high specificity for mu receptors. This would account for the observed differences in the effects of the two types of enkephalins examined in this study. Since the enkephalins are specific for fewer receptors than beta endorphin, it is thought that the enkephalin function is more specialized (Zukin and Zukin, 1981).

If, in fact, enkephalins behave as hormones, it is reasonable to assume that their activity would be relatively

long-lasting. On the other hand, rapid degradation would be expected for neurotransmitter activity. It has been reported that the half-life for enkephalins in the plasma is a few seconds to 8 - 10 minutes (Hambrook et al., 1976). However, since blood is the carrier for these opioids Roda et al. (1986) disagree and feel this hypothesis is contrary to that which is expected. Recently, three membrane-bound plasma enzymes involved in the degradation of the enkephalins have been identified: aminopeptidase, dipeptidylaminopeptidase, and enkephalinase. Their points of cleavage are shown in Figure 1 (Dickenson, 1986). The action of the aminopeptidase regarding cleavage at tyrosine is the most relevant since this step renders the peptide inactive (Roda et al., 1986). These conflicting reports present a dilemma concerning their function as reported in the literature, namely, that if enkephalins are, in fact, degraded so rapidly, how is it possible for them to exhibit their effect and how can their

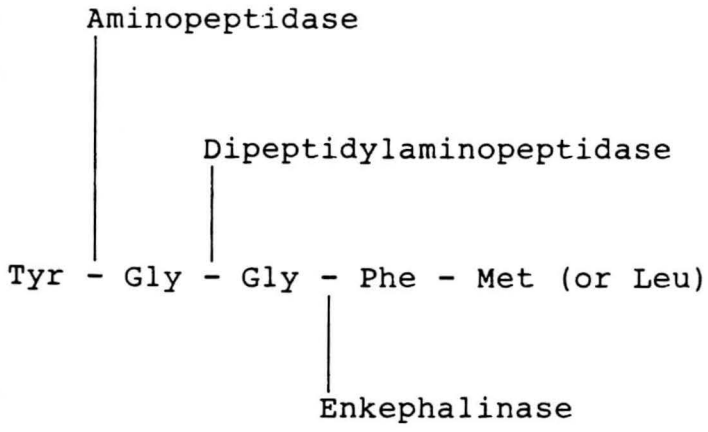


Fig. 1 Points of cleavage of the enkephalins by the three plasma enzymes.

plasma levels be measured accurately? By use of chromatography and radioimmunoassay techniques, Shanks and his co-workers (1981) had previously reported that the plasma enkephalin levels measured are intact peptides. They suggested that measurements of circulating enkephalin levels reflect continuous secretion restoring the levels being degraded in the plasma.

Roda et al. (1986) imply that there may be protective forces that prevent the enkephalins from being degraded. For example, they feel that in the adrenal gland where the enkephalins are produced, it would seem logical that the enzymes that break down the precursors would also break down the products, the enkephalins. It appears, though, that there are proteins and peptides within the chromaffin granule of the adrenal gland that the enkephalins bind to and are thereby partially protected from hydrolysis. These proteins and peptides are released into the blood at the same time as the enkephalins are and possibly protect them from degradation by plasma enzymes. Once the enkephalins are in the blood, they may similarly bind to plasma components which carry them through the blood and prevent their hydrolysis. The most recent and generally accepted mechanism of enkephalin protection in the blood proposes that there are other peptides in the plasma which compete for active sites on the degrading enzymes, therefore inhibiting their hydrolyzing effects on the enkephalins. However, most investigators agree that the turnover rate for these molecules is rather high.

The goal of this study was to identify and quantify the levels of methionine and leucine enkephalins that may be produced by cardiac and trauma patients treated by the St. Elizabeth's Hospital emergency facility. This information was then correlated with the activity of circulating lymphocytes at the same time period in order to determine the effects, if any, that the enkephalins had on these immune cells.

Lymphocyte depression following trauma has been previously reported. Bauer et al. (1978) observed a decrease in circulating numbers of both T and B lymphocytes following trauma. The extent of the depression of the lymphocytes was related to the severity of the injury. Keane and associates (1983) recognized that lymphocyte blastogenesis and lymphocyte responsiveness to mitogens were also suppressed following trauma. Infection followed as a result of a compromised immune system. Consequently, it was reported that three out of thirty-one patients died during the course of this study. O'Mahony et al. (1984) support Keane's findings of decreased lymphocyte blastogenesis and responsiveness to mitogens.

Infection due to immunosuppression following trauma is the leading cause of death in persons who survive the initial trauma (Levy et al., 1986). For this reason it is important to discover the cause of lymphocyte depression. It was formerly thought that cortisol was a major factor in immunosuppression. Numerous studies contradict this theory

(Fauci and Dale, 1974; Berenbaum et al., 1976; Keane et al., 1983; O'Mahony et al., 1984). For example, Fauci and Dale studied the effect of cortisol on the number of circulating lymphocytes by the technique of rosette formation. After a cortisol injection, the number of circulating rosette forming lymphocytes did, in fact, demonstrate a decrease, but only for a short period of time. The numbers returned to normal in less than 24 hours. This decrease was attributed to the redistribution of the lymphocytes out of the circulation and into other body compartments. This short-lived effect can not account for lymphocyte depression that could last up to 15 -20 days after trauma (Keane et al., 1983). Berenbaum et al. (1976) showed that cortisol alone could account for only a slight suppression of lymphocyte response. O'Mahony et al. (1984) stated that while lymphocyte function was suppressed following trauma, cortisol was not the effector.

In other studies, plasma beta endorphin levels were observed to increase following trauma while cortisol levels remained near normal (Shatney et al., 1985). Levels of beta endorphins have been shown to increase following trauma with a possible relationship to decreased lymphocyte responsiveness to mitogens (Levy et al., 1986).

These studies demonstrate the need for additional studies which can demonstrate molecules which may affect the immune system in medically traumatized patients. This study postulates that the enkephalins may be implicated in the previously reported effects of lymphocytic activity following

trauma.

Wybran et al. (1979) demonstrated, as previously noted, the presence of receptors on lymphocytes for morphine and enkephalins. Here it was noticed that the addition of morphine in vitro decreased the ability of the lymphocytes to form rosettes with sheep red blood cells, whereas the addition of met-enkephalin increases rosette formation. The morphine blocked the sheep red blood cells from binding to the lymphocytes whereas the met-enkephalin increased their ability to bind. This was attributed to the presence of two different receptors, delta and mu, on the surface of the lymphocytes.

Jankovic and Maric (1987) postulate that the enkephalins may exert a dual effect depending on their levels. High levels suppress whereas low levels enhance the immune system. Leu-enkephalin has been shown to increase thymidine uptake and mitogen response in developing lymphocytes, whereas met-enkephalin showed no significant effect (Wybran, 1985).

Relatively few studies have been done to correlate the levels of enkephalins with the activity of circulating lymphocytes, especially after trauma. Whereas the majority of studies carried out on lymphocyte suppression involved the numbers of circulating lymphocytes and their responsiveness to mitogens, this study attempts to examine the effects of the enkephalins on measurable DNA activity and protein synthesis in circulating lymphocytes.

CHAPTER II

Materials and Methods

Blood was drawn in green top venoject tubes at St. Elizabeth's Hospital from cardiac and trauma patients admitted to the emergency room under the supervision of Dr. Howard White. Blood samples were taken from each patient at three separate intervals: one at admission, one at 24 hours, and another at 48 hours. The blood was then quickly centrifuged in order to separate plasma from the cells. The plasma was then placed in liquid nitrogen until examination by high performance liquid chromatography (HPLC), while the lymphocytes were isolated from the blood cells, affixed to slides, and prepared for quantitative cytophotometric analysis.

Lymphocytes were separated from other blood cells in the following manner: two milliliters of the cells were layered on three milliliters of Histopaque (Sigma Chemical Company, St. Louis, Missouri). The preparation was then centrifuged at 2200 rpms for thirty minutes. The isolated lymphocytes appear as a white layer near the top of the centrifuged solution. The recovered lymphocyte layer was pipetted off into another 15 ml. centrifuge tube and washed three times with an 8.5% saline solution. Smears were made on pre-albuminized slides representative of each time period previously mentioned, for each patient. The slides were allowed to dry

and were then fixed in 10% buffered formalin for 24 hours. The slides were then washed in 70% ethanol for 24 hours. They were then removed and allowed to dry and were then placed in slide storage boxes for future quantitative measurements of nuclear DNA and protein.

Staining

DNA

Slides from each mentioned time period regarding each patient were stained for DNA measurements by means of the Feulgen nuclear reaction for quantitative cytophotometry. The stain, under certain conditions, is stochiometric for DNA (Feulgen and Rossenbeck, 1924).

The stain was prepared as follows:

10 grams of Basic Fuschin obtained from Fisher Scientific Co. (Fair Lawn, New Jersey), dye lot #42510, and 22 grams potassium meta-bisulfite were added to one liter of 1 N HCl. This solution was wrapped with aluminum foil to prevent exposure to light and was stirred mechanically for two hours. The stain was then placed in a dark cabinet overnight. Five grams of activated charcoal from Fisher Scientific were added to the stain. The stain was suction filtered until it became a colorless solution.

The staining procedure used is as follows:

Since the slides were previously hydrated in 70% ethanol, they were placed directly into distilled water for five minutes and hydrolyzed in 5 N HCl for 45

minutes (Yemma and Therrien, 1972). Acid hydrolysis removes the purines at the level of the purine-deoxyribose glucosidic bond of DNA (depurination), thus unmasking the aldehyde groups of deoxyribose, wherein lies the specificity of the reaction (DeStefano, 1948). Hydrolysis is followed by the stain for two hours. The stain reacts with the exposed aldehyde groups left by hydrolysis. Before the slides are placed in the stain, it must be fortified with 10% potassium meta-bisulfite in a ratio of 4 parts stain to 1 part potassium meta-bisulfite. Following staining, slides were rinsed in two changes of 10% potassium meta-bisulfite for five minutes each. The slides were then rinsed in distilled water for one minute and dehydrated in a graded ethanol series, consisting of 70%, 90%, and 100% ethanol for three minutes in each. Finally, the slides were cleared in xylene for 5 minutes and mounted. Controls and experimentals were stained simultaneously in order to eliminate any variation in staining.

Protein

Quantitative cytophotometric measurements for lymphocyte cytoplasmic proteins were made utilizing the methods of Deitch (1955). The procedure requires that tissues be stained with 1% Naphthol Yellow S in 1% acetic acid. Both experimentals and controls were stained simultaneously. The stain was made up as follows:

2.5 grams of Naphthol Yellow S (Sigma Chemical Company) was added to 247.5 milliliters of distilled water and 2.5 milliliters of Glacial Acetic Acid.

The following staining procedure was utilized:

The slides were dipped in boiling water to remove any formalin fixative. The slides were then put in 1% Naphthol Yellow S stain for 15 minutes followed by treatment in 1% acetic acid for 18 hours for the purpose of tissue differentiation. They were then dehydrated in an ethanol series of 50%, 70%, 95%, and 100% for five minutes each. The tissues were cleared in xylene for five minutes and mounted.

Microspectrophotometry

A Zeiss Universal Type 01 Microspectrophotometer with a 100X Planachromatic oil immersion lens was used for quantitative cytophotometric measurements of lymphocytic nuclear DNA and cytoplasmic protein. DNA measurements were made utilizing the two wavelength method of Pataw (1952). The procedure is as follows.

A maximum absorbing wavelength is selected. A second is then chosen so that

$$2E_1 = E_2$$

where:

$E_1 = \log I_o/I_s$ at λ_1 (maximum wavelength)

$E_2 = \log I_o/I_s$ at λ_2 (half maximum wavelength)

I_o is background light and I_s is light passing though the specimen. Maximum and half maximum wavelengths chosen were

560 nm and 505 nm, respectively (Yemma and Therrien, 1972).

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

$$M = KAL_1D$$

K = constant (1/e), where e is the extinction coefficient at and may be disregarded for relative measurements, and was done in this study. Transmissions (T_1 and T_2) were taken at wavelengths 1 and 2 for each nucleus. From these values, L_1 and L_2 were calculated.

$L_1 = (1 - T_1)$ = absorption where $T_1 = I_s/I_o$ at λ_1

$L_2 = (1 - T_2)$ = absorption where $T_2 = I_s/I_o$ at λ_2

$D = L_2/L_1$ (Correction factor for unoccupied space in the reading aperture)

A = area of reading aperture

M = amount of absorbing molecules in the measured area

Quantitative measurements of cytoplasmic protein were made using the plug method (Swift, 1950). In this method, the amount of dye binding to tyrosine and lysine residues is quantitatively measured.

In this study, a cytoplasmic area was measured (plug) for each lymphocyte, both experimentals and controls, so that

$$Protein_{plug} = KEA$$

K is the extinction coefficient, and was disregarded since only relative measurements were made. The wavelength utilized in the measurements was 425 nm, since this gave

maximum absorption of bound dye molecules.

E = the extinction at 425 nm

A = area of the plug

Since $A = \pi R^2$ where R is the radius of the plug, an ocular reticule, having been previously calibrated, was utilized in order to obtain this value. Care was taken to ensure that the measurements were small enough so that the rounded surface of the sphere may be considered flat. A control was run with each experimental in order to ensure that stochiometric values were obtained for all stained cells. All calculations were done using an Amdahl mainframe computer and an American Telephone and Telegraph computer.

All measurements were duplicated to ensure repeatability. In order to ensure that measurements were randomized, it is important to note that the microspectrophotometer pulses each cell 280 times per second. Thus the transmission or optical density reading obtained is actually the average of these pulses. In addition, the selection of measured cells was random.

HPLC

The measurement of methionine and leucine enkephalins was accomplished by using a Perkin Elmer series 400 chromatograph equipped with a Model LC75 variable-wavelength detector set at 280 nm.

The buffers used were A) 0.02 M phosphate buffer, pH 6.0 and B) acetonitrile. The acetonitrile and the HPLC water used to make up the phosphate buffer were obtained from

Fisher Scientific Co. The sodium phosphates, monobasic and dibasic, were obtained from Sigma Chemical Co. Before each use, the buffers were suction filtered through a 0.45 um pore size filter (Pierce Chemical Co., Rockford, Illinois), and deoxygenated with pure helium.

Blood samples were drawn in the emergency room at St. Elizabeth's Hospital from trauma and cardiac patients. After centrifugation, the serum was placed in liquid nitrogen and kept frozen until utilized. Prior to analysis, the serum samples were thawed at room temperature and filtered through a 0.45 um filter (Gelman Sciences, Ann Arbor, Michigan) into a sample vial. Twenty microliters of each sample were injected onto a C_{18} RP300 column (Pierce) using a Perkin Elmer ISS-100 injector with a 200-ul loop.

The mobile phase was delivered as a linear gradient of 100% A to 50 % A and 50 % B in 20 minutes at a flow rate of 1.5 ml/min. Following each run, the column was flushed with HPLC water for 10 minutes. An oven supporting the column was utilized in order to keep the system at a constant temperature of 30 degrees centigrade.

Methionine and leucine enkephalin standards were obtained from Sigma Chemical Co. and were reconstituted to a concentration of 1 mg/ml. The standards were utilized as external standards in order to detect the serum enkephalin levels of experimental patients' samples. A Perkin Elmer 7500 Professional Computer was used to record and analyze the data.

CHAPTER III

Results

Levels of serum methionine and leucine enkephalin were determined for 7 cardiac and 5 trauma patients admitted to St. Elizabeth's Hospital emergency room, by the technique of high performance liquid chromatography. This was coupled with quantitative cytophotometric measurements of DNA and protein concentrations of circulating lymphocytes for each patient. Results are shown in Figures 2 - 33. HPLC data is presented as graphs upon which are plotted retention time versus voltage. Cytophotometric data is illustrated by means of histograms upon which are plotted number of cells analyzed versus DNA or protein concentration. All are displayed in relative units. A summary of the data is listed in Table 1.

Controls were prepared from pooled serum obtained from normal, healthy individuals (Figs. 2-4). Enkephalin levels of patients were compared first to a pure standard purchased from Sigma Chemical Co. in order to pinpoint their location as peaks produced by the HPLC. Chromatographic analyses of experimental serums were then compared to the normal control in order to determine their retention time or relative area. Enkephalin levels are reported in arbitrary units and represent the amount present when compared to normal levels in the pooled control.

DNA measurements were performed by stochiometrically staining the lymphocytes by means of the Feulgen reaction coupled with quantitative cytophotometric measurements and reported in arbitrary units of concentrations. Histograms were plotted for each patient showing the distribution of the measured nuclear DNA values. The mean, standard deviation and standard error are reported for each histogram. The results over a three-day span is shown for each patient studied. It is important to note that all histograms exhibit a unimodal distribution of DNA concentrations as would be expected for a non-dividing cellular population. Since the lymphocytes studied were circulating lymphocytes, no mitosis was seen. These lymphocytes may, however, exhibit an increase or decrease in template activity through an increase or decrease in diffuse DNA, resulting in a differential in dye binding which is measurable. This change should be reflected in slight increases or decreases in cellular DNA and thus protein levels. However, cells with a DNA concentration equal to a doubling of DNA, as would be expected in a dividing cellular population reflecting the presence of a synthetic and G_2 phase of the cell cycle prior to mitosis, should not normally be seen. The data presented here does, in fact, demonstrate slight changes in template activity, regarding some cells within the experimental population but not equivalent to that which would be expected of synthetic or G_2 phase cells. The DNA mean obtained from the pooled control was 1.398. Controls along with

experimentals are presented in Figs. 3, 6, 8, 10, 13, 15, 18, 21, 24, 26, 29, 31, and 33.

Those patients for whom cytoplasmic protein measurements of the lymphocytes were made will serve to emphasize either an increase or decrease in DNA template activity. The lymphocytes were stained with Naphthol Yellow S and concentrations were determined by quantitative cytophotometric measurements. The correlation between DNA and protein synthesis was observed to be consistent in the patients studied. The mean for the relative protein concentration of the pooled control was 5.928 and is presented along with experimentals in Figs. 4, 11, 16, 19, 22, and 27.

Patient Tr - 97

Serum samples for this patient were taken on days 1, 2, and 5. (Figs. 5 and 6) Days 1 and 2 show high levels of enkephalins which become undetectable by day 5. The concentration of DNA was slightly above normal upon admission but dropped by the second day. It then returned to normal by the fifth day. Notice the shift to the left of the histogram on day 2, as compared to that of the control, signifying a reduction in DNA template activity. This data indicates that DNA responses to enkephalin levels may be time dependent. In addition, this data appears to support the contention of Jankovic and Maric (1987) which states that high levels of enkephalins may suppress the immune response, whereas lower levels may have the opposite effect.

Patient Cr - 01

This patient had serum samples drawn on days 1, 3, and 4. Elevated levels of met-enkephalin are seen upon admission (5.44 times normal) (Fig. 7) while the concentration of DNA demonstrated lower template activity during this period (0.617) (Fig. 8). Leu-enkephalin is 1.55 times normal. Days 3 and 4 show undetectable levels of met-enkephalin. Both leu-enkephalin and the DNA concentration have increased over this two-day period.

Patient Cr - 59

Patient Cr - 59 had measurable DNA template activity near normal on the first day which subsequently dropped to almost half the normal value by the second day. (Fig. 10) The third day demonstrated a return to near normal levels. Protein synthetic activity decreased on the second but increased by the third day. (Fig. 11) Serum methionine and leucine enkephalin levels were relatively high on the first day, 5.03 and 1.80 times normal, respectively, and dropped to undetectable levels by the third day. (Fig. 9) A time dependent relationship between enkephalin levels and DNA synthetic activity appears operative.

Patient Tr - 41

High levels of serum met-enkephalin levels were recorded on day one, 5.28 times normal. This slowly decreased to 3.26 times normal by the third day. Leu-enkephalin appeared only in day 2. Mean DNA levels dropped from 0.898 to 0.620 over

the three day period. This drop is reflected by the shift to the left of the histograms when compared with controls. (Figs. 12 and 13)

Patient Cr - 90

Patient Cr - 90 demonstrated undetectable serum levels of met-enkephalin for each of the three day serum samples. (Fig. 14) The level of leu-enkephalin upon admission was 3.85 times normal. This level increased by the second day.. On the third day a slight decrease was observed, however, levels were still 2.20 times that of normal. DNA and protein concentrations were below normal on admission and correlated well with leu-enkephalin levels, demonstrating a decrease in synthetic activity. (Figs. 15 and 16)

Patient Cr - 55

Met-enkephalin levels were detectable only on day 2 when the DNA activity was recorded at the lowest level for the period measured. Leu-enkephalin was detectable at this time as well as the third day. (Fig. 17) DNA measurements of activity were near normal by the third day. Protein synthetic activity correlated well with this pattern of activity. (Figs. 18 and 19)

Patient Cr - 02

Levels of serum met-enkephalin were below detectable levels in the three serum samples that were taken. However, the level of leu-enkephalin was 2.32 times normal on day 1 and stabilized to 1.61 and 1.62 times normal for day 2 and 3,

respectively. (Fig. 20) DNA concentration, or activity, was low on the first day (0.583) but increased to a steady level for the second and third day (0.609 and 0.605). (Fig. 21) The protein concentrations demonstrated a similar pattern of correlation. (Fig. 22)

Patient Cr - 16

Serum met-enkephalin levels were undetectable until the third day sample collection at which time it measured 6.26 times normal. (Fig. 23) DNA activity gradually increased to near normal levels by the third day. (Fig. 24)

Patient Cr - 94

Patient Cr - 94 demonstrated undetectable serum enkephalin levels until the third day. Levels on this day were 4.39 times normal for met-enkephalin and 1.48 times normal for leu-enkephalin. (Fig. 25) The DNA concentrations steadily decreased, demonstrating decreased synthetic activity, over the course on the three days. (Figs. 26 and 27) Note that the DNA concentration is lowest when the enkephalin levels are highest.

Patient Tr - 69

Serum enkephalin levels were below detectable levels in this patient for each of the three day serum samples. (Fig. 28) However, the concentration of DNA was almost half that of the normal on the first day, reflecting a decrease in synthetic activity, but returned to normal by the third day. (Fig. 29)

Patient Tr - 14

No measurable levels of serum enkephalins were detected for any of the three days sampled. (Fig. 30) DNA activity was initially low and steadily increased over the three days. (Fig. 31)

Patient Tr - 89

This patient was recorded as having suffered extensive injury to the brain and spinal cord, the result of a suicide attempt. Relatively no serum enkephalins were detectable. (Fig. 32) DNA activity as well as the number of lymphocytes dropped dramatically by the third day. (Fig. 33) This patient died soon after admission to the hospital.

It is quite interesting to note that the results demonstrate good correlation between enkephalin levels, DNA template activity, and protein synthesis. This is supported by evidence that when serum met-enkephalins levels are above normal, DNA template activity and protein synthesis are below normal. In addition, when leu-enkephalin levels increase, DNA activity and protein synthesis correspondingly increase. These observations suggest that methionine and leucine enkephalins have opposite roles in regard to their effects on the lymphocytes and that this relationship appears to be time dependent.

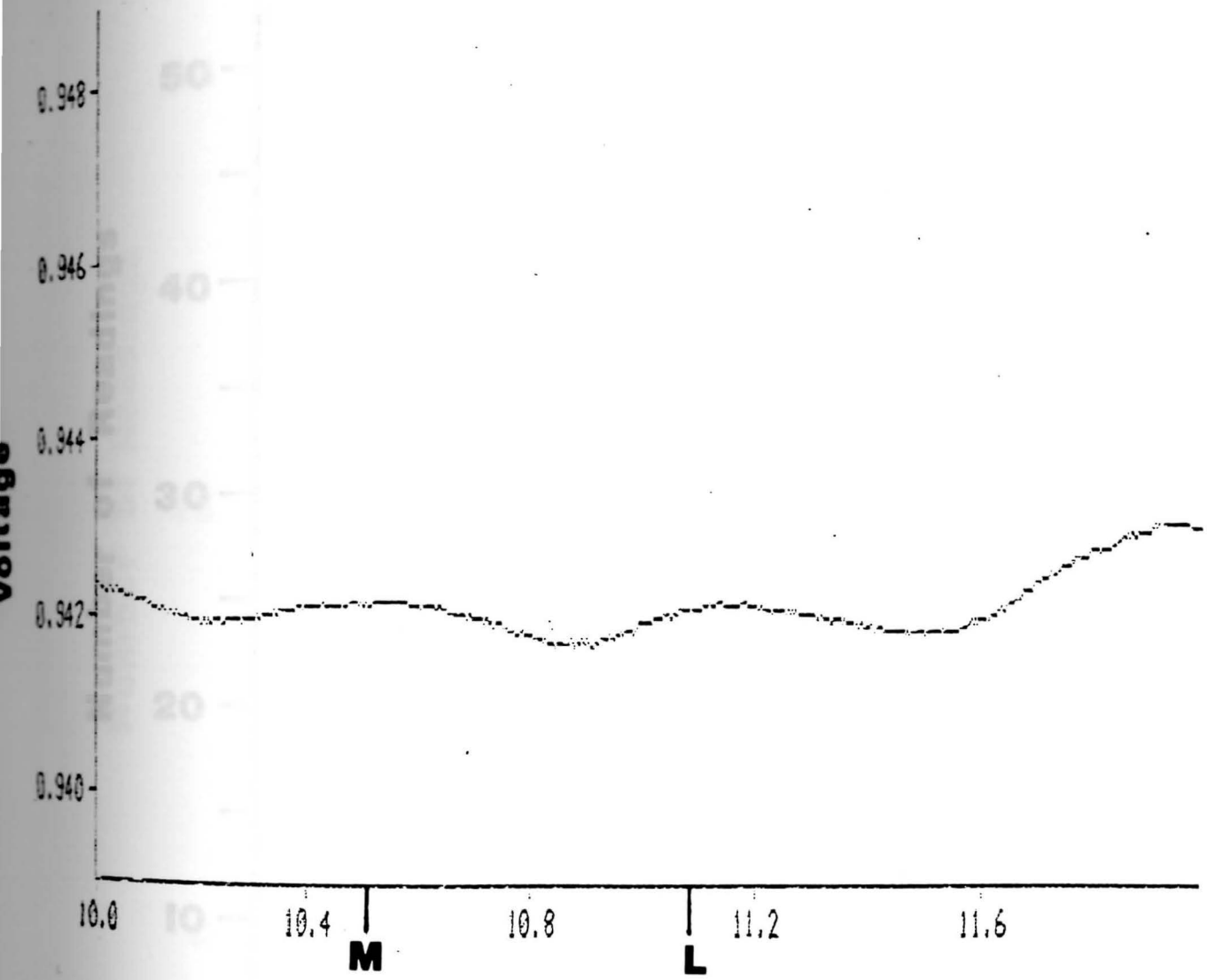
Figures 2 - 4

Enkephalin, DNA, and Protein Data

for Pooled Control

Chromatographic Analysis of the Enkephalins

Figure 2

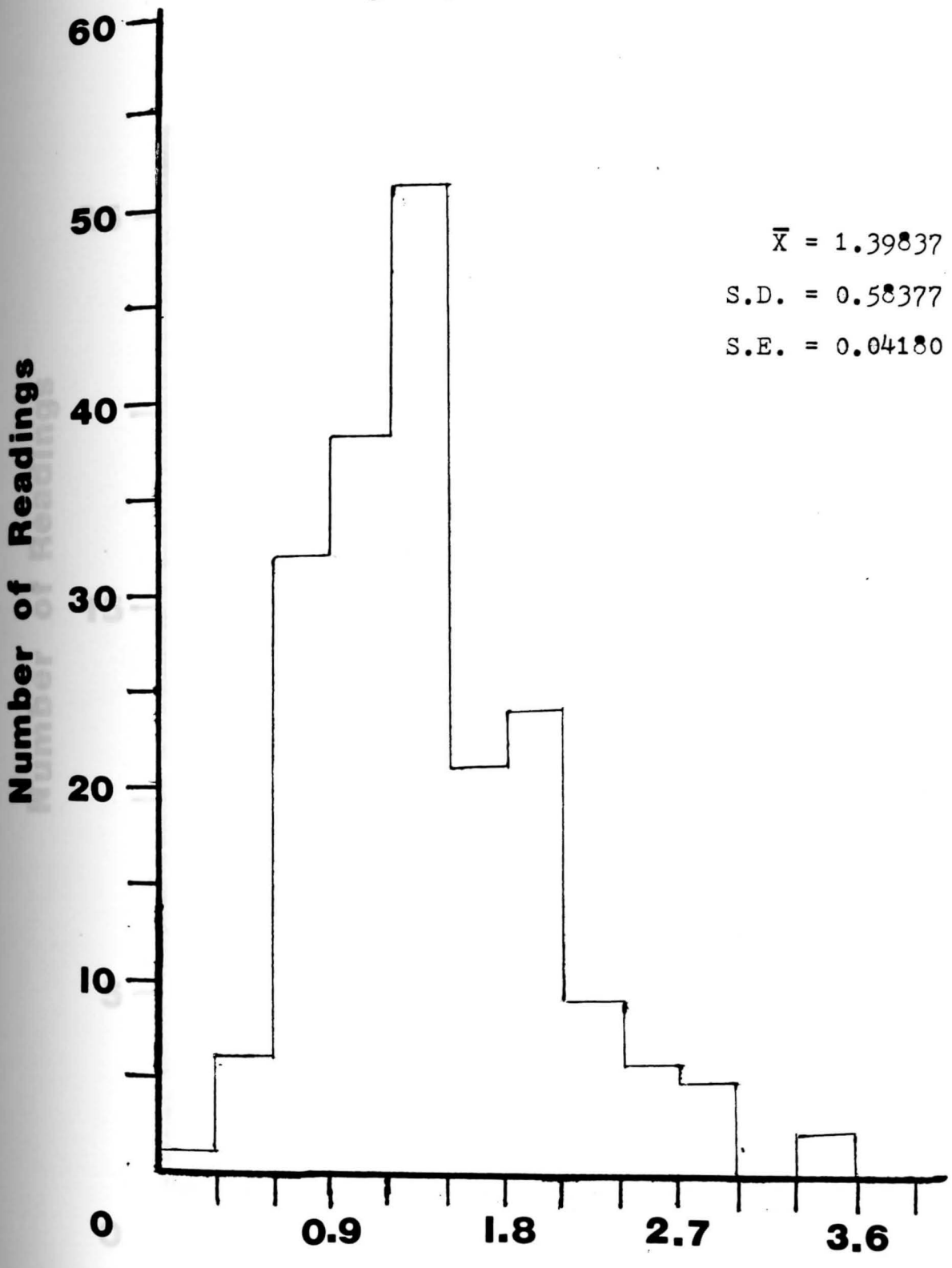


M = time where met-enkephalin should appear

L = time where leu-enkephalin should appear

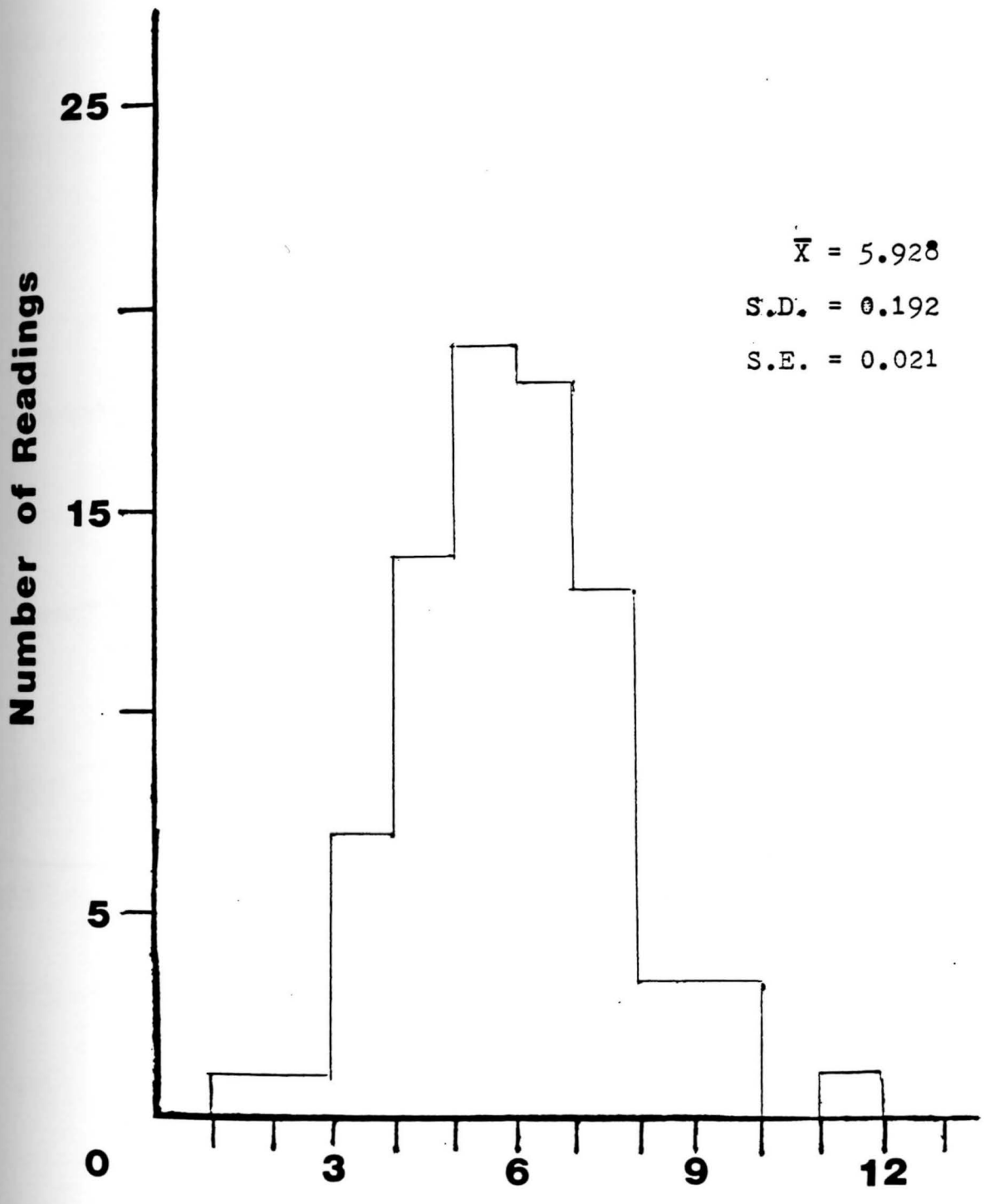
Histogram of Relative DNA Concentrations
0.3 Arbitrary Units / Division

Figure 3



Histogram of Relative Protein Concentrations
1.0 Arbitrary Unit / Division

Figure 4



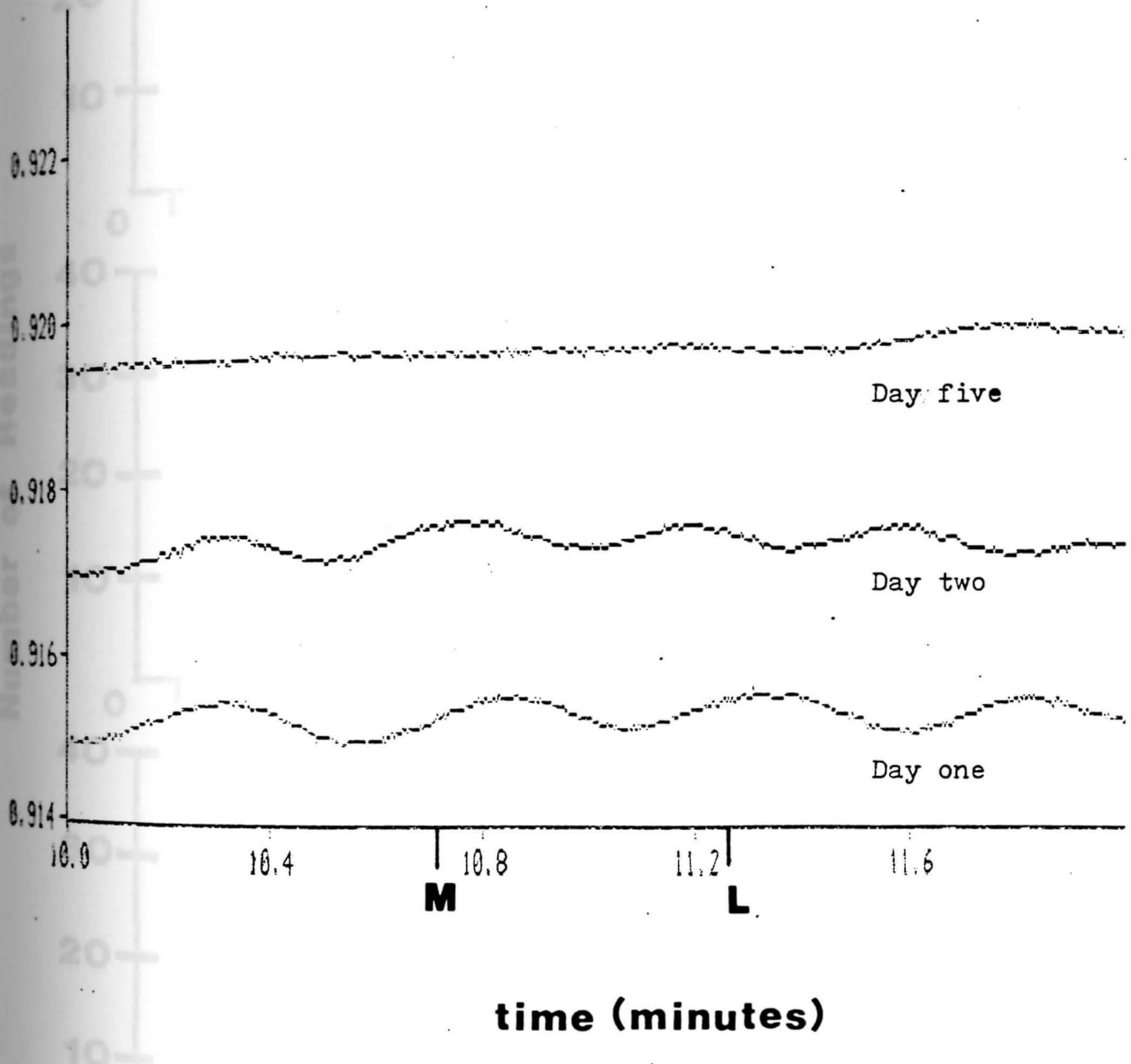
Figures 5 and 6

Enkephalin and DNA Data

for Patient Tr - 97

Chromatographic Analysis of the Enkephalins

Figure 5



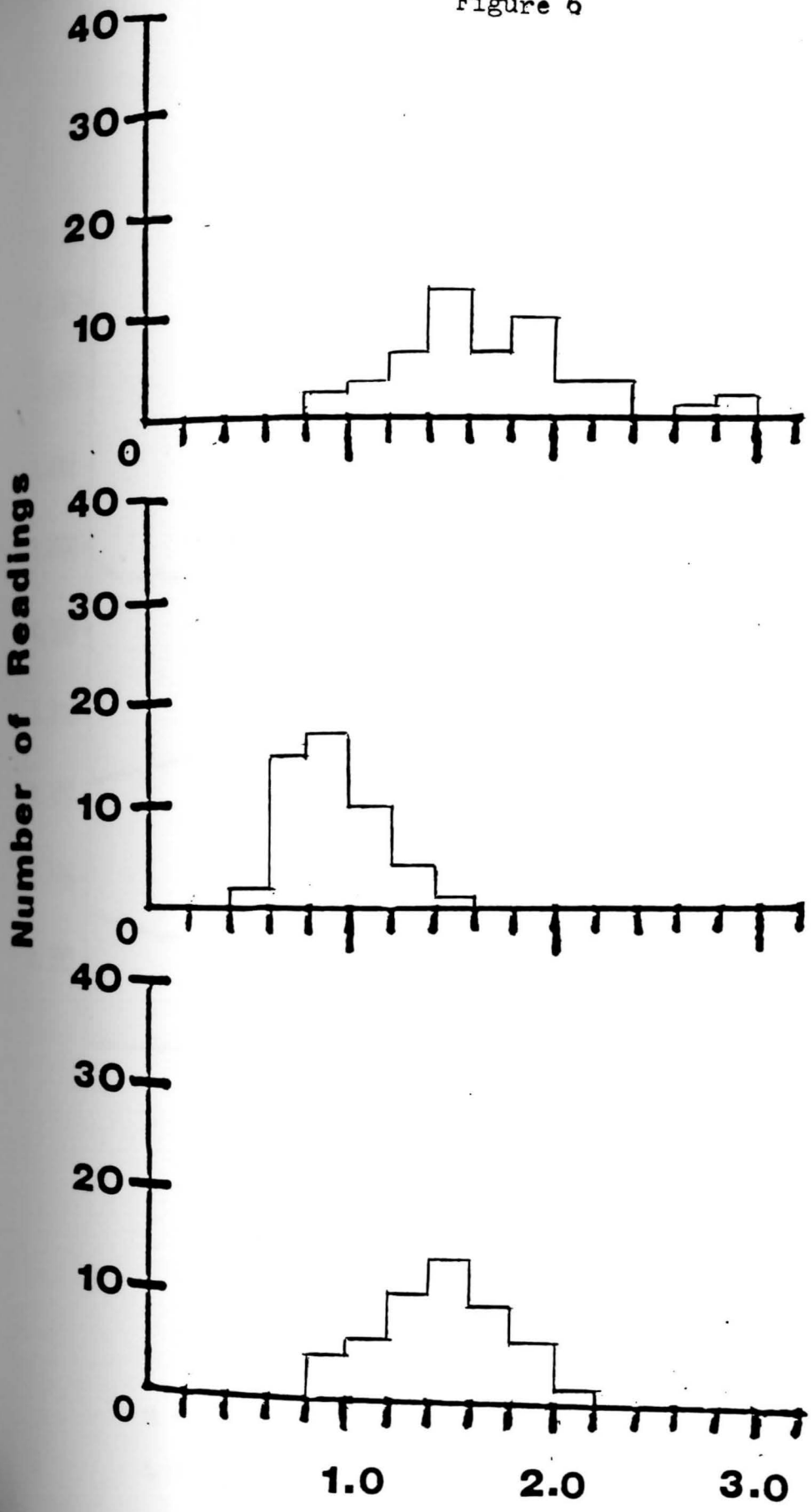
M = time where met-enkephalin should appear

L = time where leu-enkephalin should appear

Histograms of Relative DNA Concentrations

0.2 Arbitrary Units / Division

Figure 6



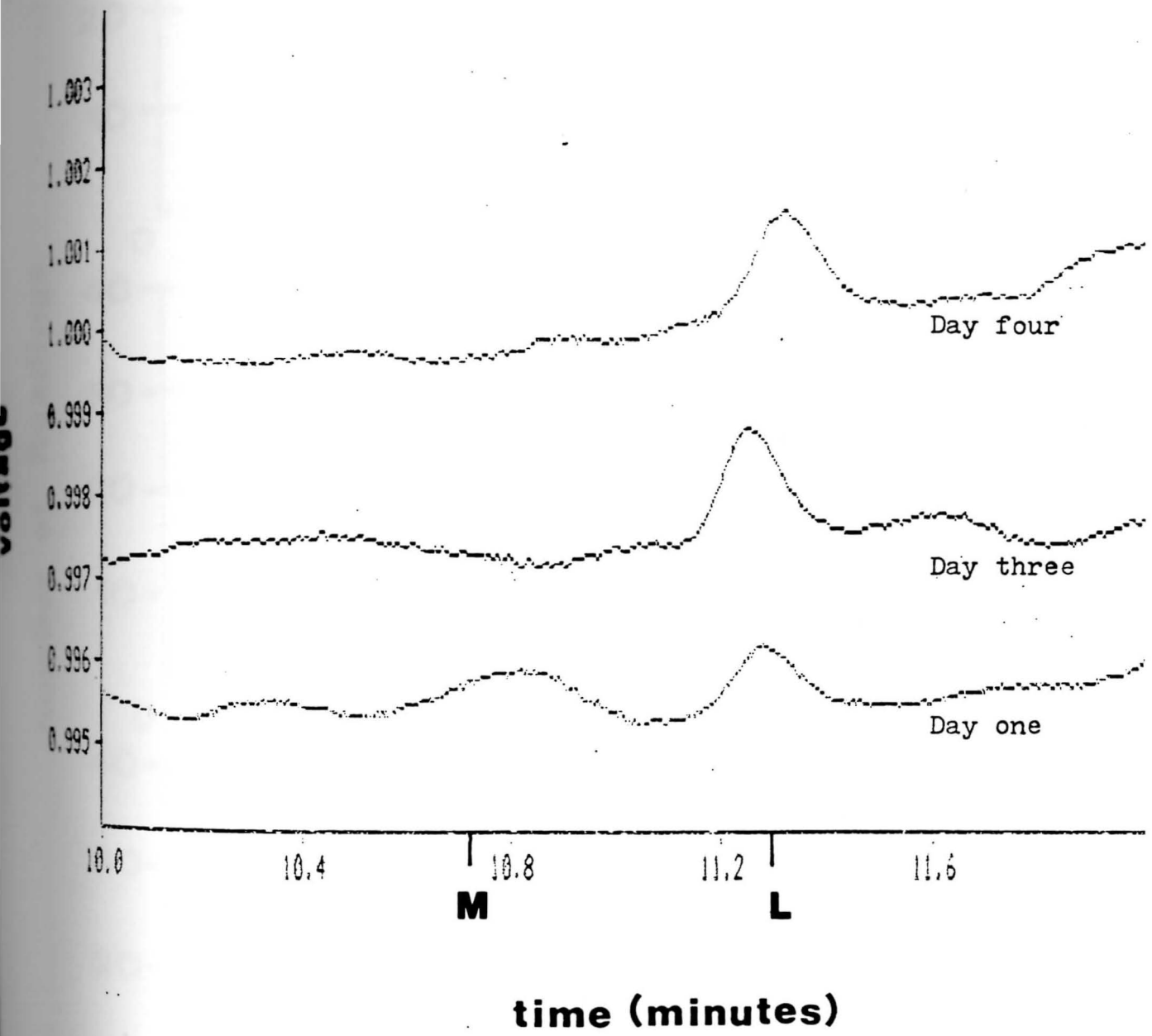
Figures 7 and 8

Enkephalin and DNA Data

for Patient Cr - 01

Chromatographic Analysis of the Enkephalins

Figure 7



M = time where met-enkephalin should appear

L = time where leu-enkephalin should appear

Figures 9 - 11

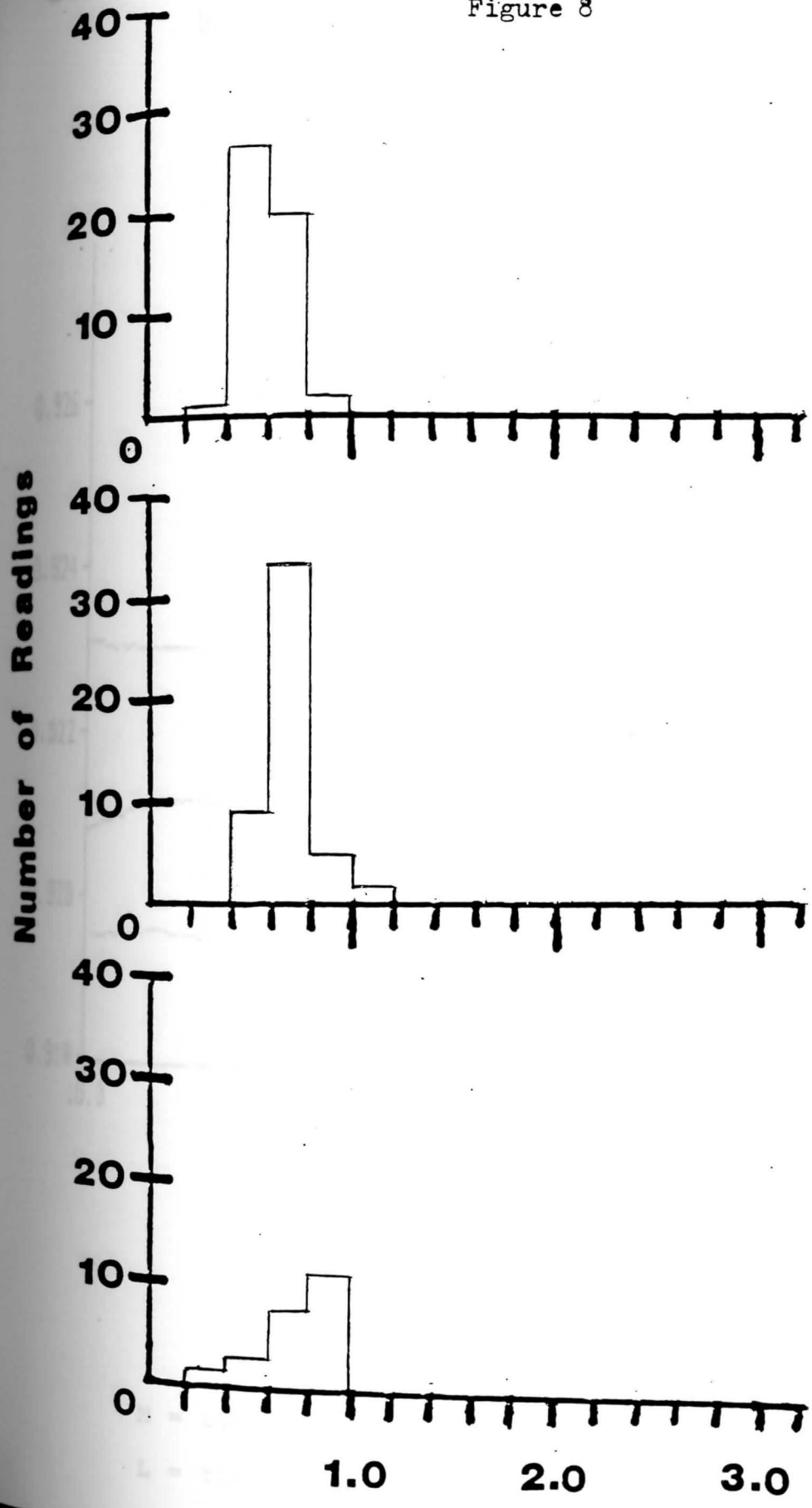
Enkephalin, DNA, and Protein Data

for Patient Cr - 59

Histograms of Relative DNA Concentrations

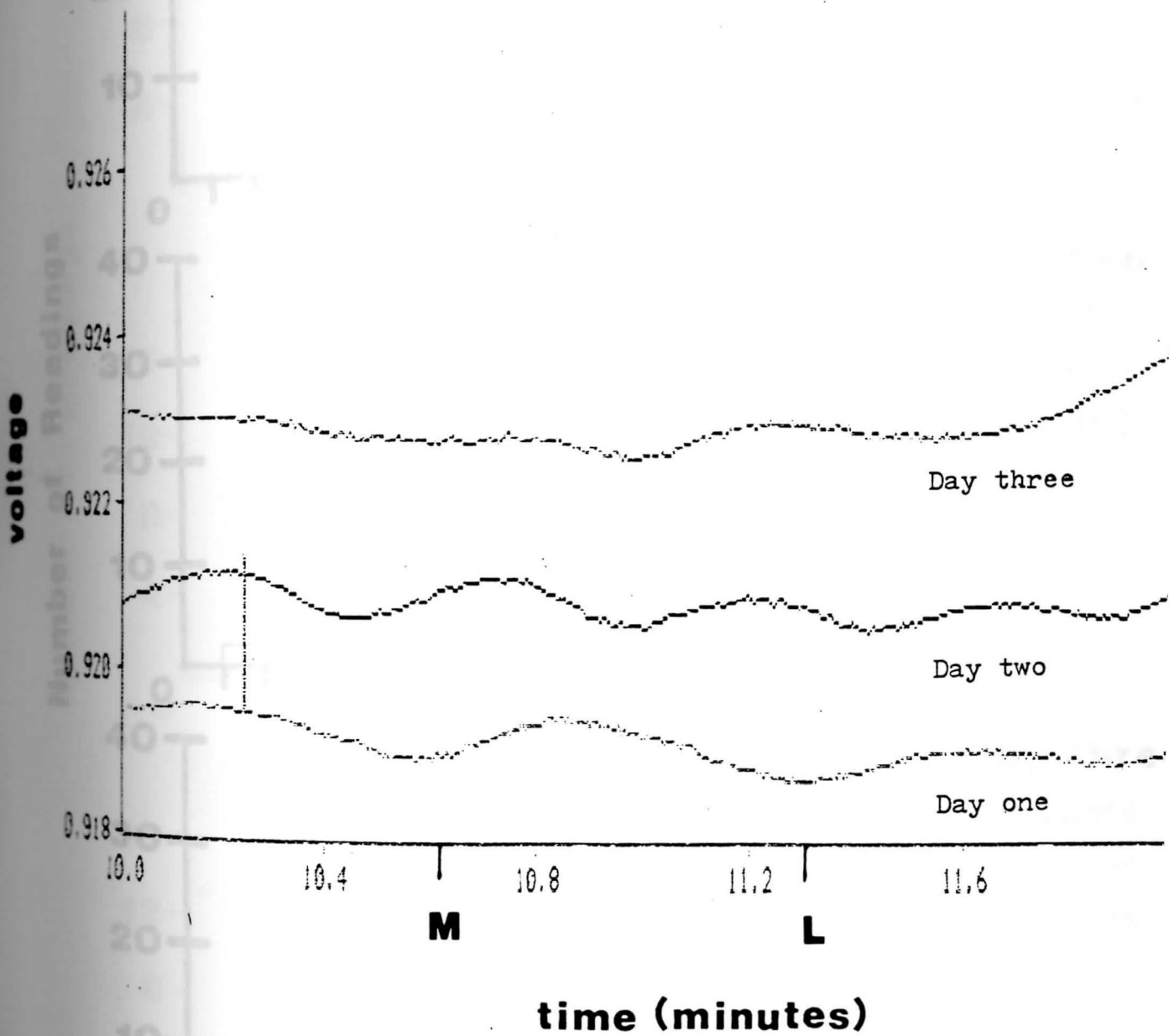
0.2 Arbitrary Units / Division

Figure 8



Chromatographic Analysis of the Enkephalins

Figure 9



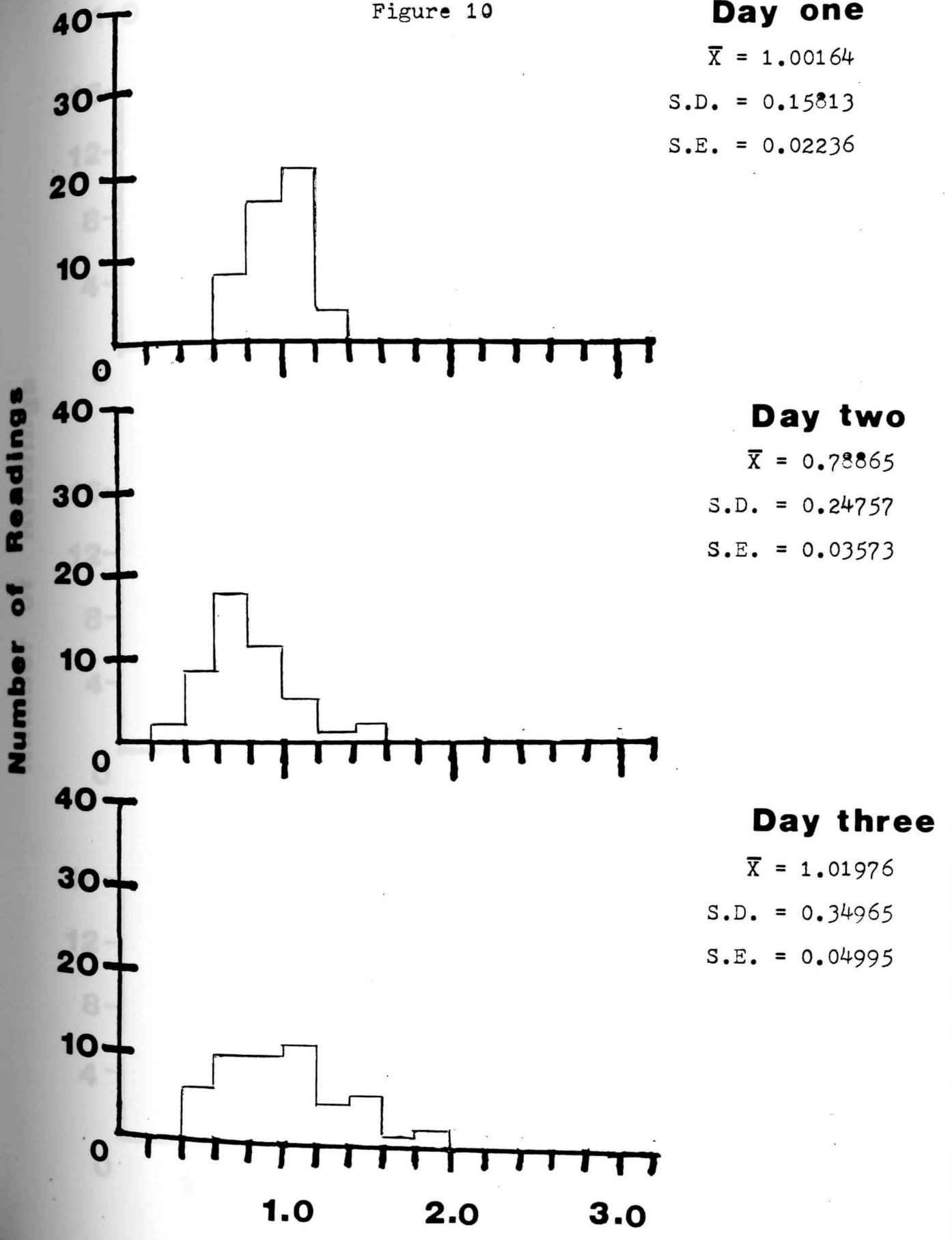
M = time where met-enkephalin should appear

L = time where leu-enkephalin should appear

Histograms of Relative DNA Concentrations

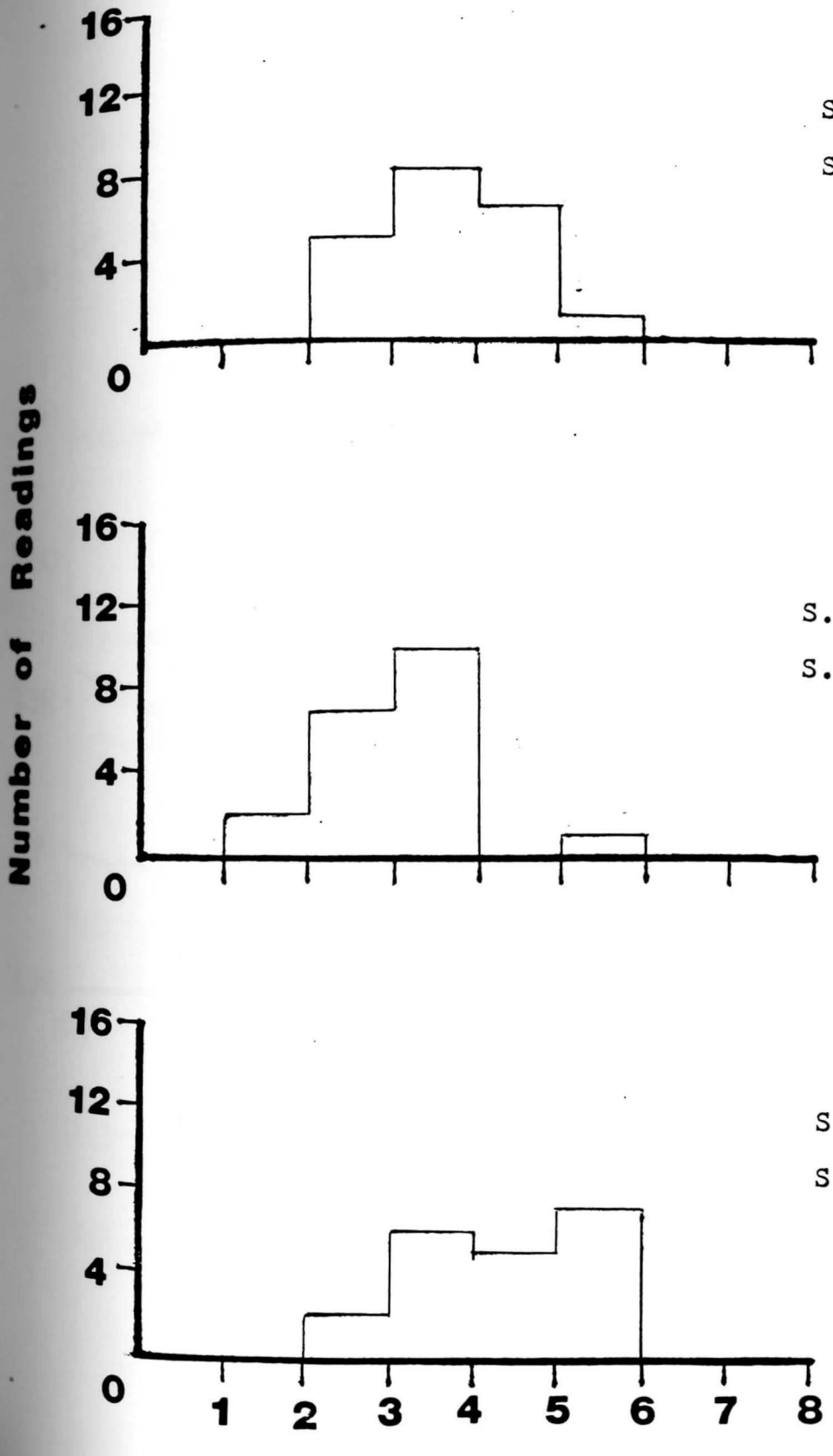
0.2 Arbitrary Units / Division

Figure 10



Histograms of Relative Protein Concentrations
1.0 Arbitrary Unit / Division

Figure 11



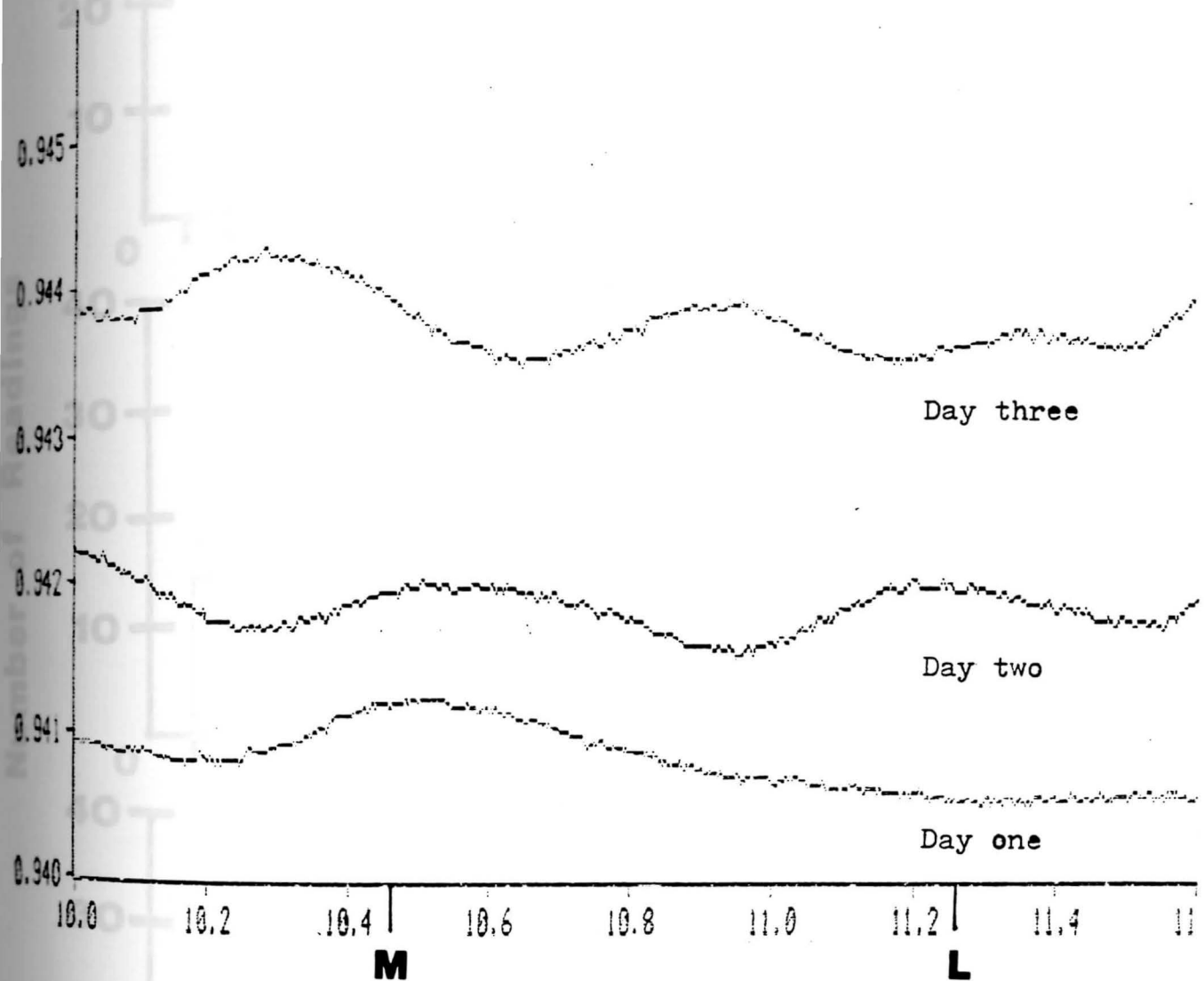
Figures 12 and 13

Enkephalin and DNA Data

for Patient Tr - 41

Chromatographic Analysis of the Enkephalins

Figure 12



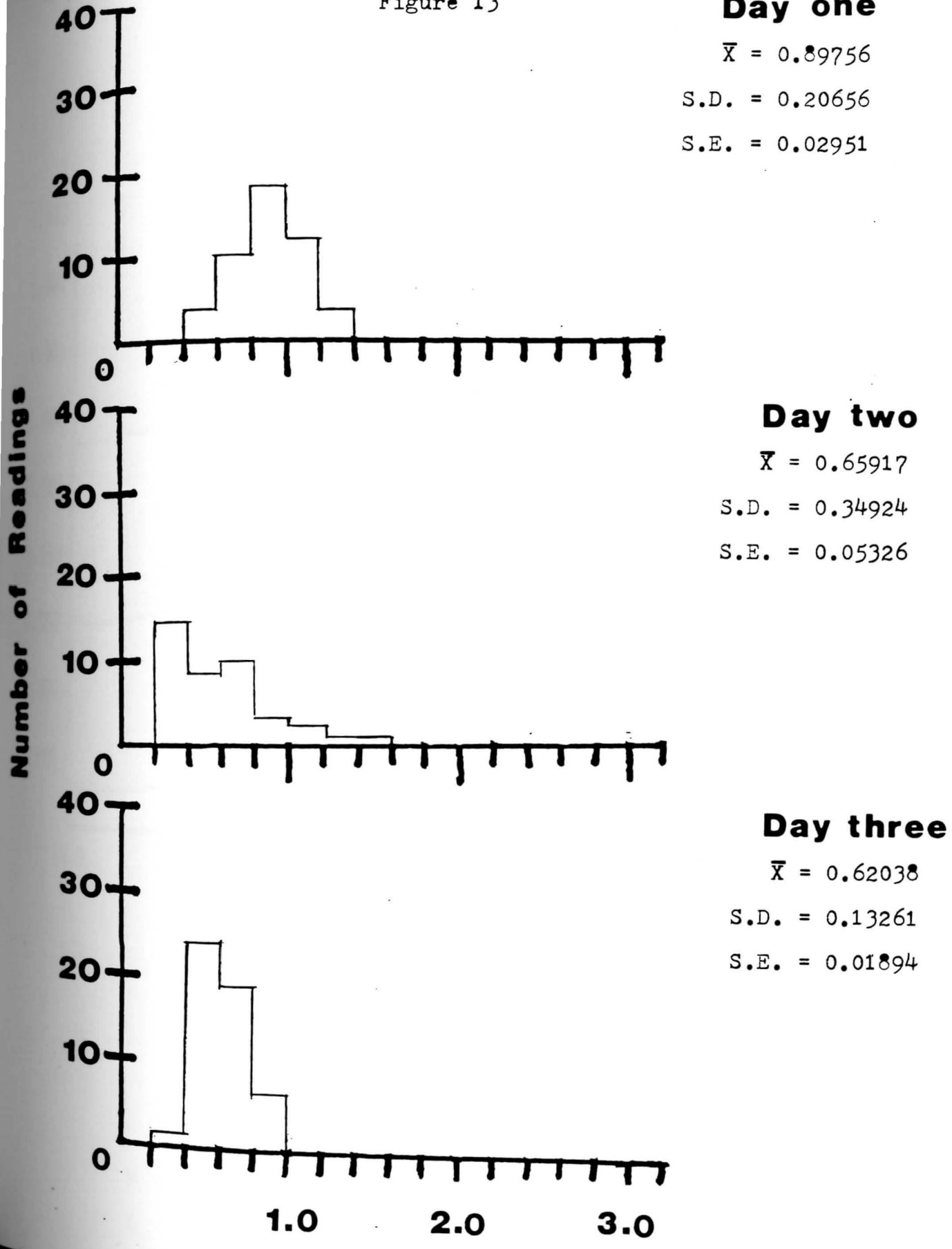
M = time where met-enkephalin should appear

L = time where leu-enkephalin should appear

Histograms of Relative DNA Concentrations

0.2 Arbitrary Units / Division

Figure 13



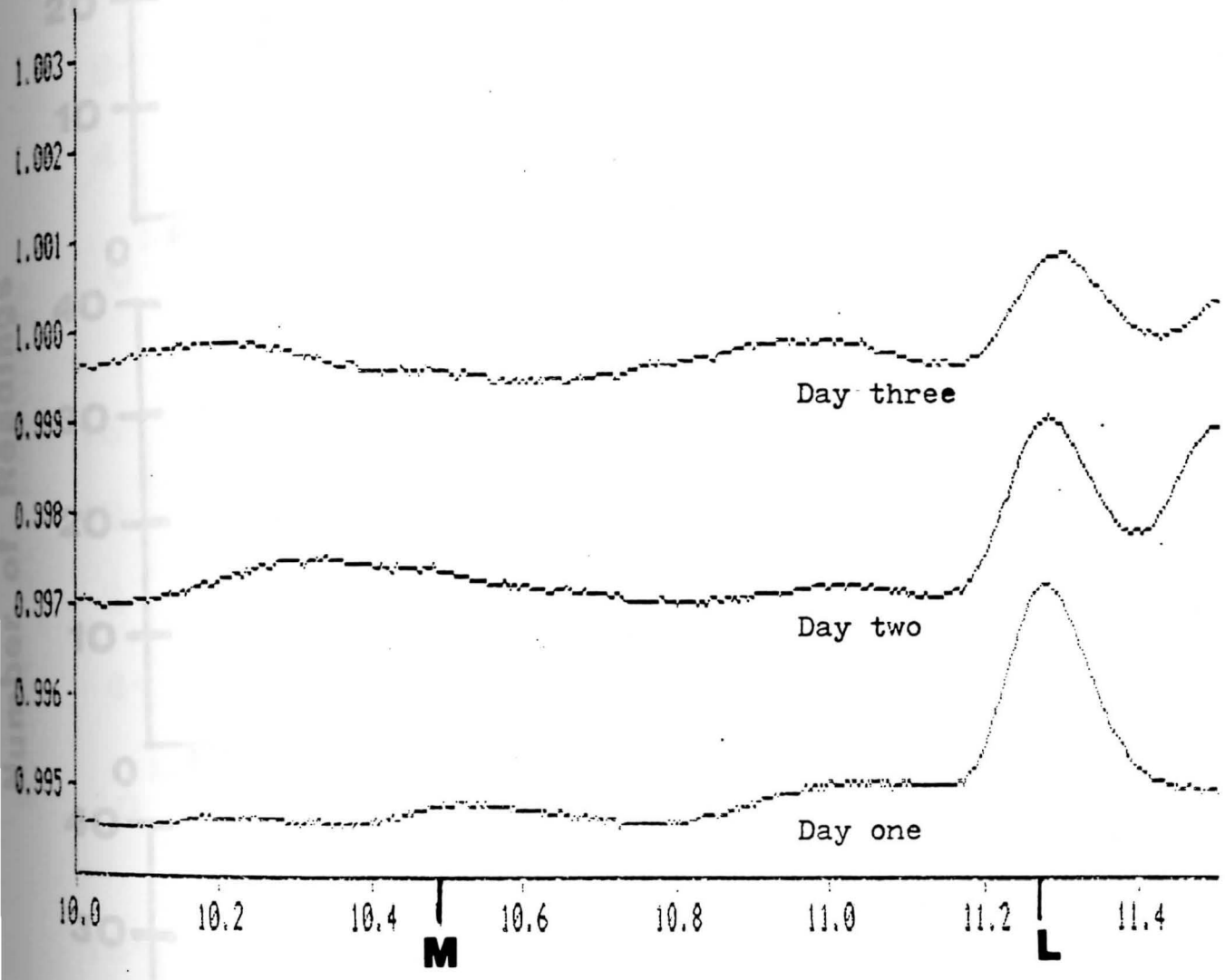
Figures 14 - 16

Enkephalin, DNA, and Protein Data

for Patient Cr - 90

Chromatographic Analysis of the Enkephalins

Figure 14



time (minutes)

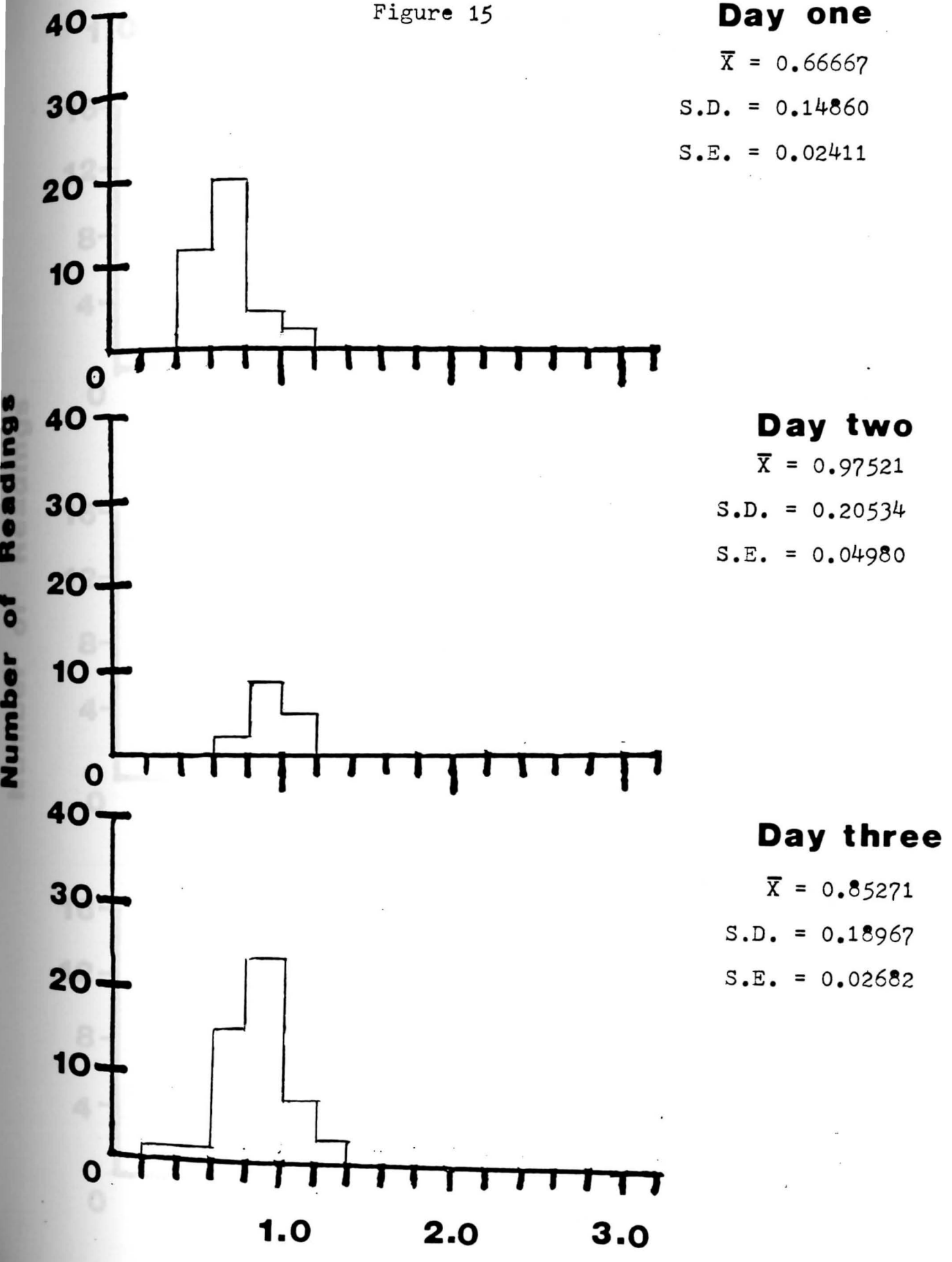
M = time where met-enkephalin should appear

L = time where leu-enkephalin should appear

Histograms of Relative DNA Concentrations

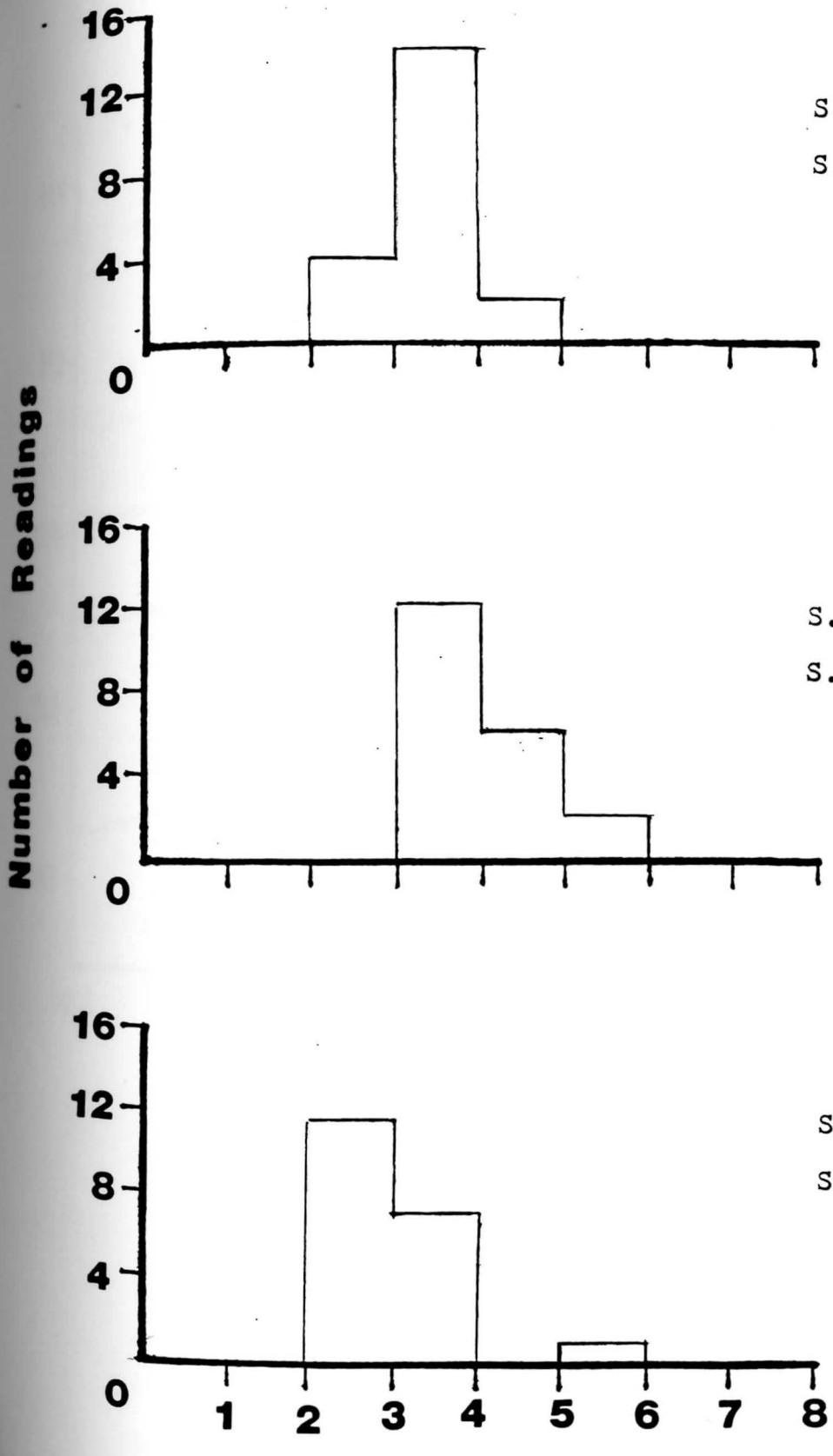
0.2 Arbitrary Units / Division

Figure 15



Histograms of Relative Protein Concentrations
1.0 Arbitrary Unit / Division

Figure 16



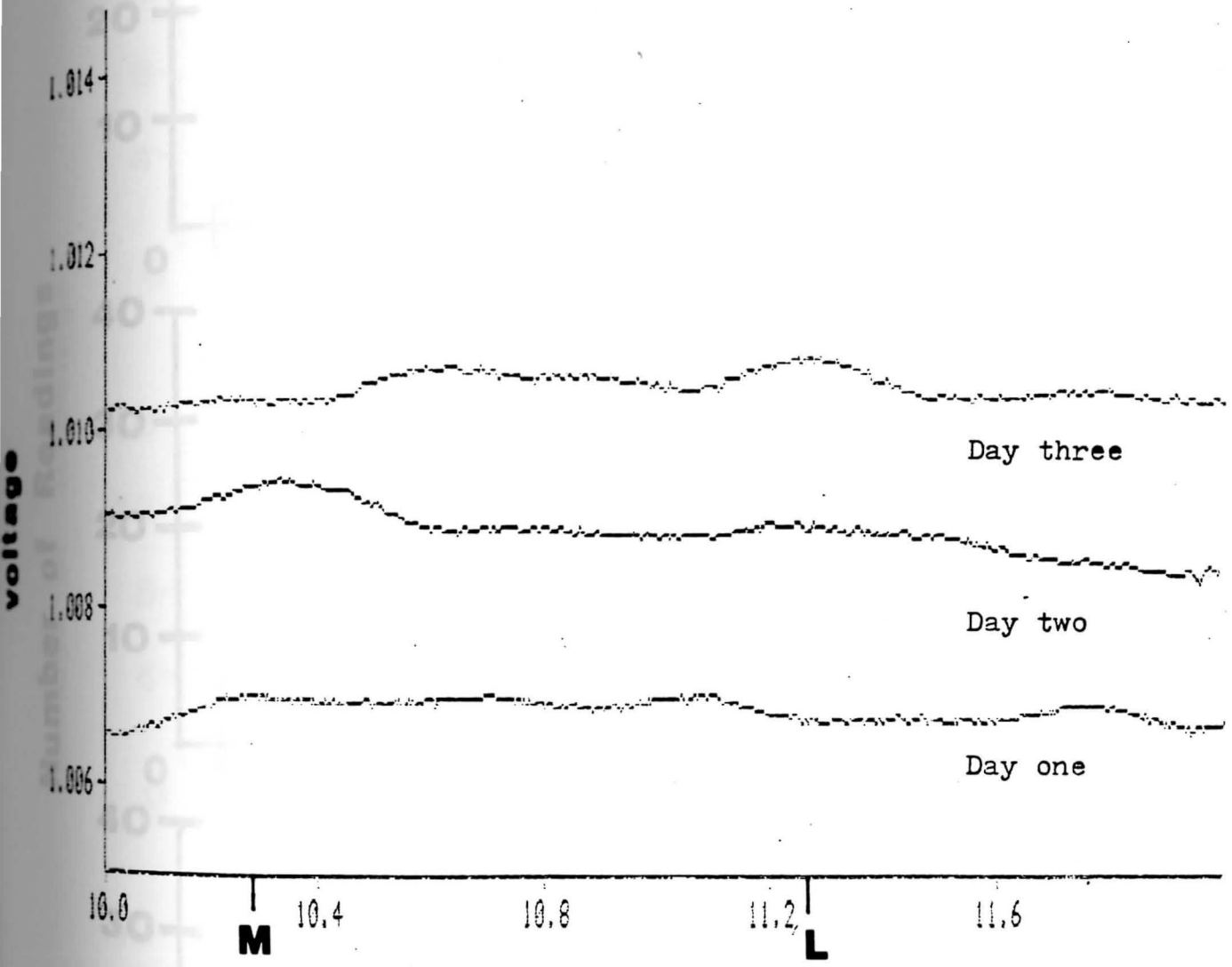
Figures 17 - 19

Enkephalin, DNA, and Protein Data

for Patient Cr - 55

Chromatographic Analysis of the Enkephalins

Figure 17



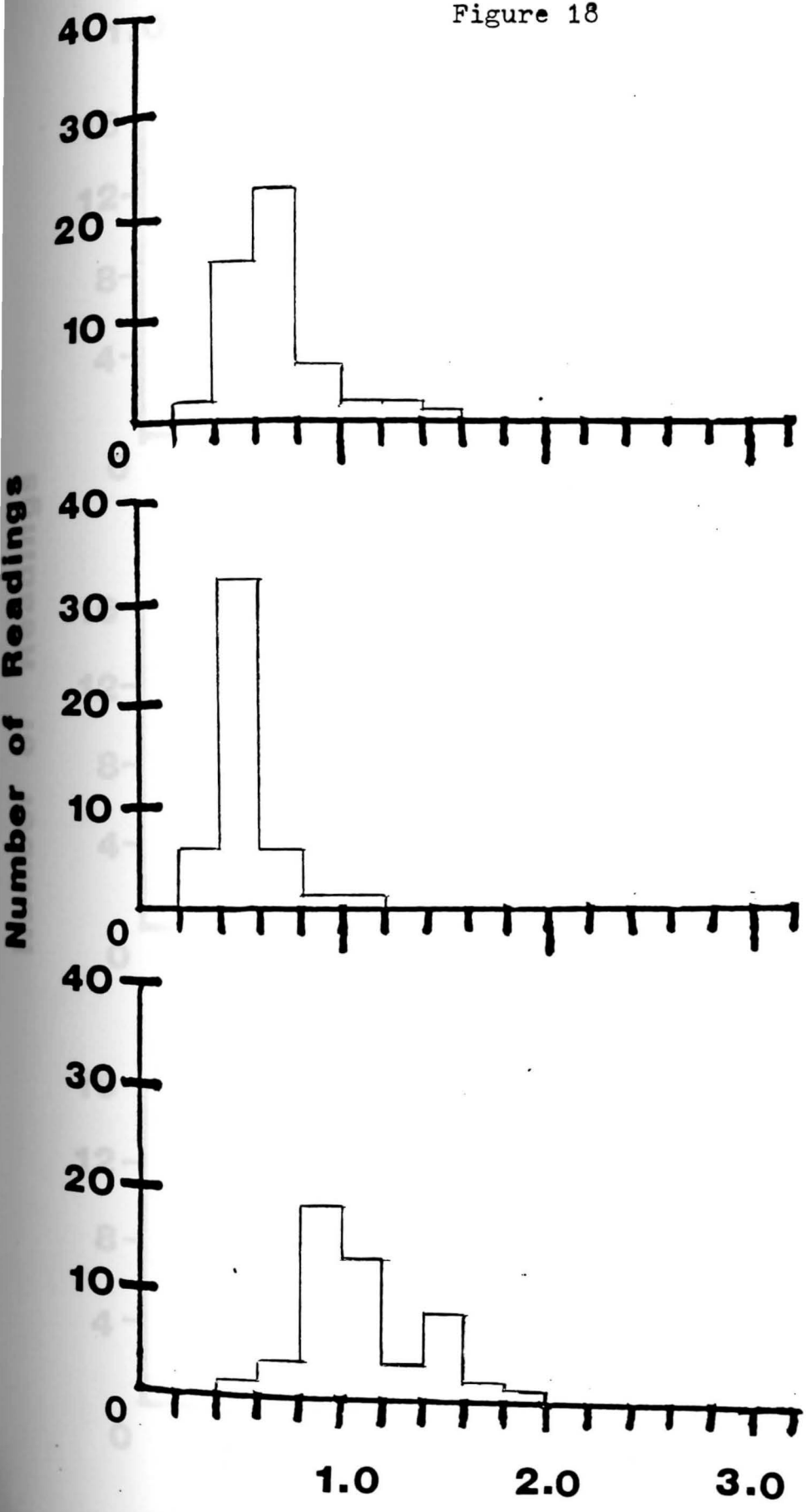
M = time where met-enkephalin should appear

L = time where leu-enkephalin should appear

Histograms of Relative DNA Concentrations

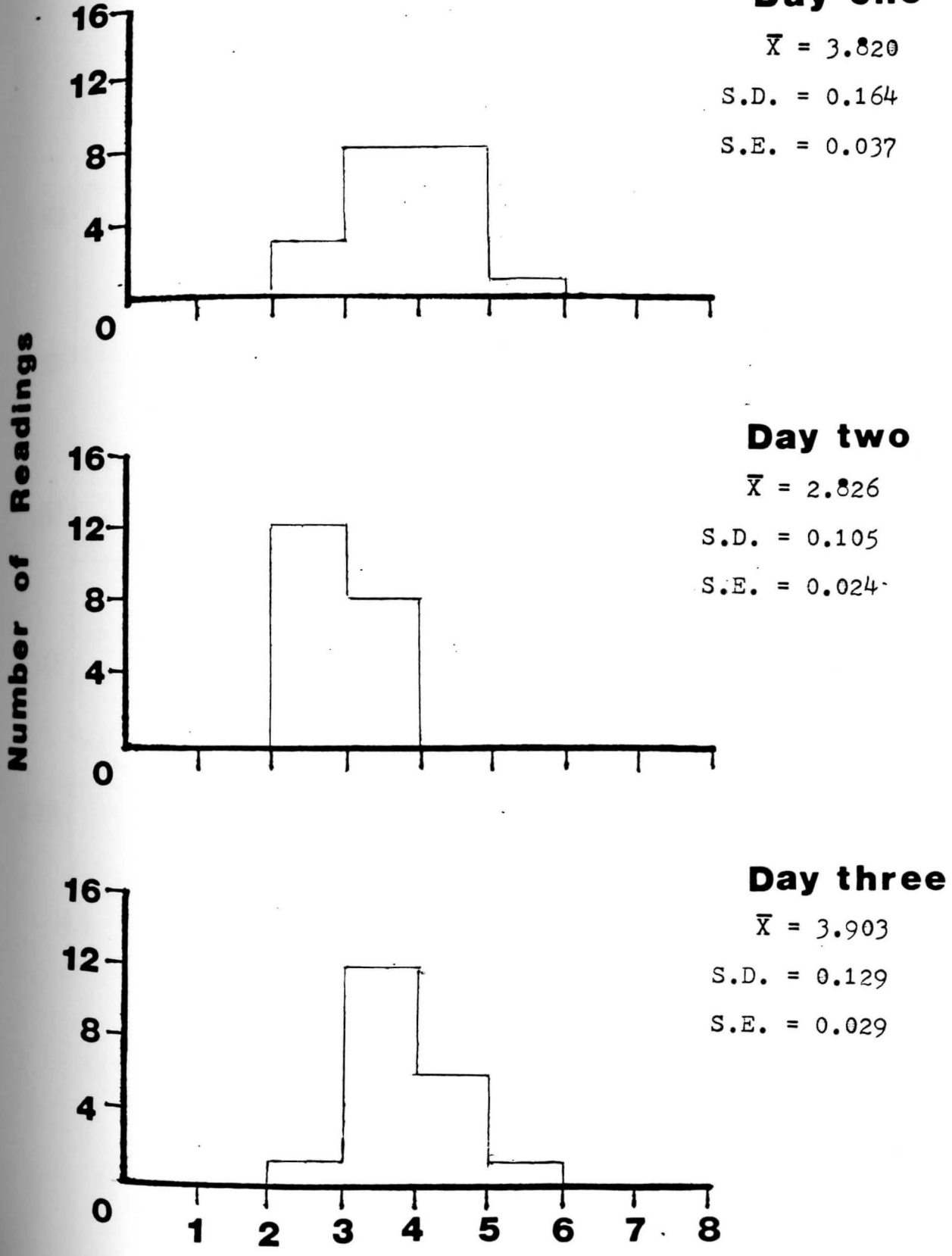
0.2 Arbitrary Units / Division

Figure 18



Histograms of Relative Protein Concentrations
1.0 Arbitrary Unit / Division

Figure 19



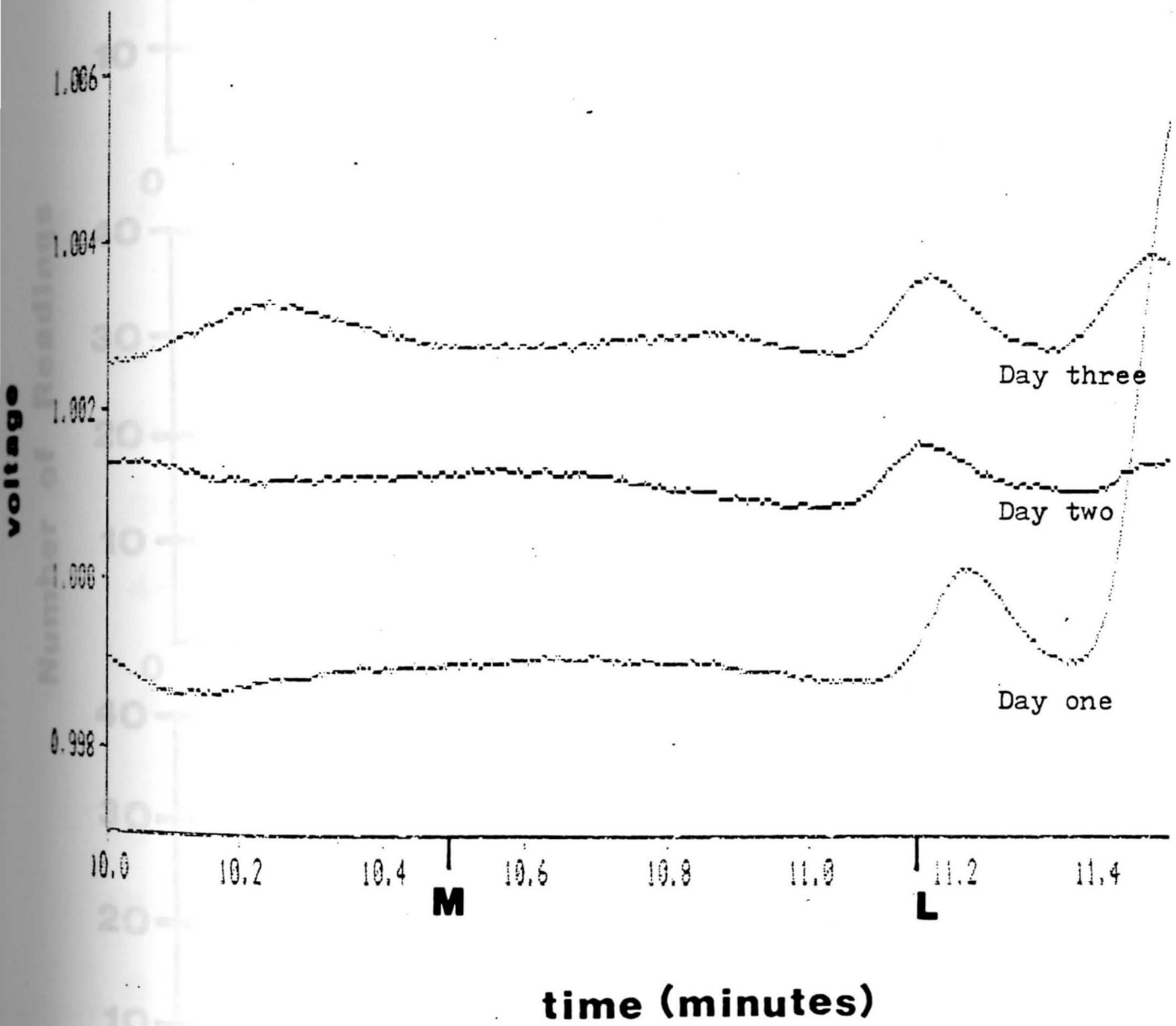
Figures 20 - 22

Enkephalin, DNA, and Protein Data

for Patient Cr - 02

Chromatographic Analysis of the Enkephalins

Figure 20



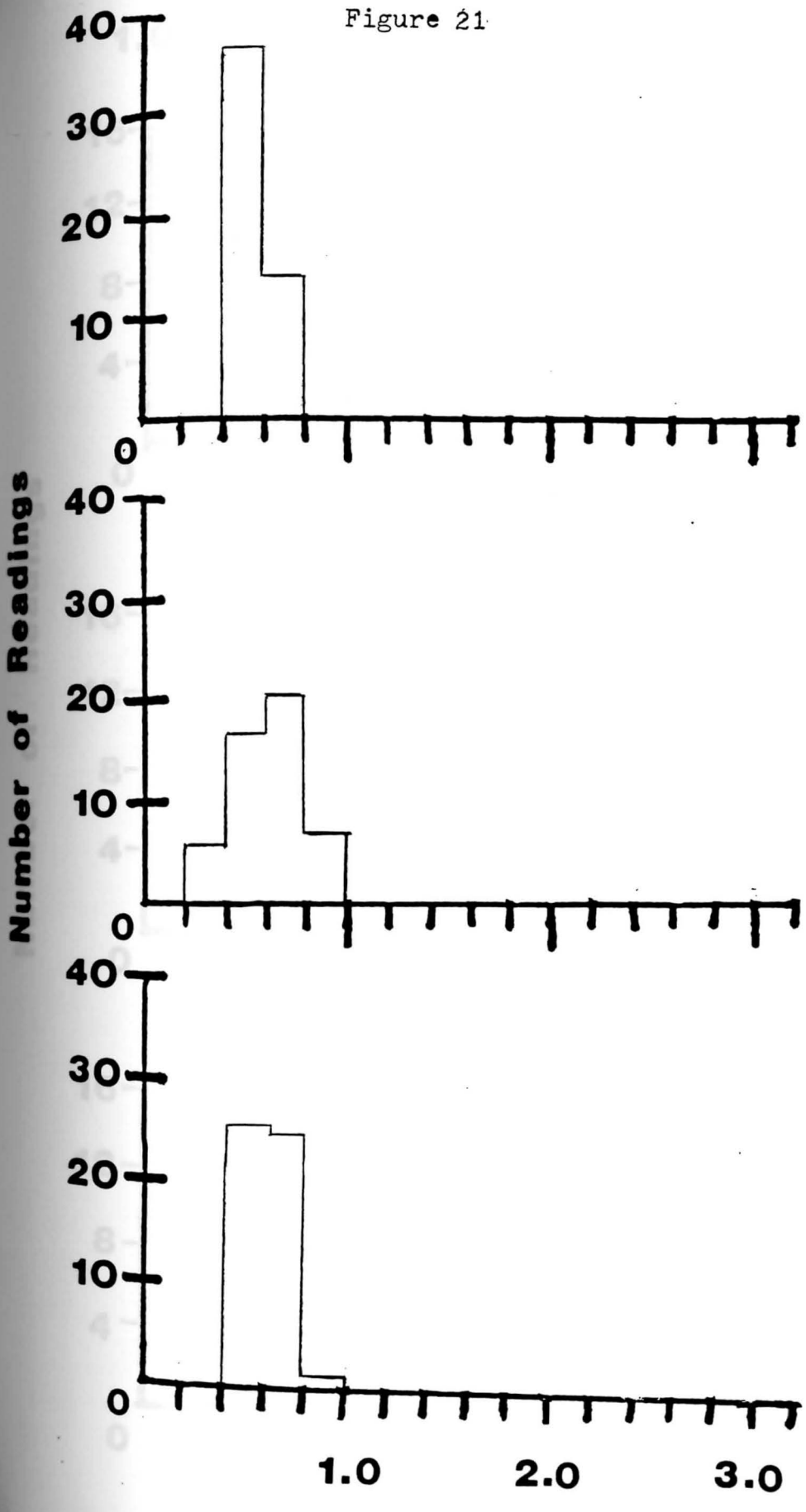
M = time where met-enkephalin should appear

L = time where leu-enkephalin should appear

Histograms of Relative DNA Concentrations

0.2 Arbitrary Units / Division

Figure 21



Day one

$\bar{X}$ = 0.58320

S.D. = 0.14700

S.E. = 0.02079

Day two

$\bar{X}$ = 0.60852

S.D. = 0.16815

S.E. = 0.02427

Day three

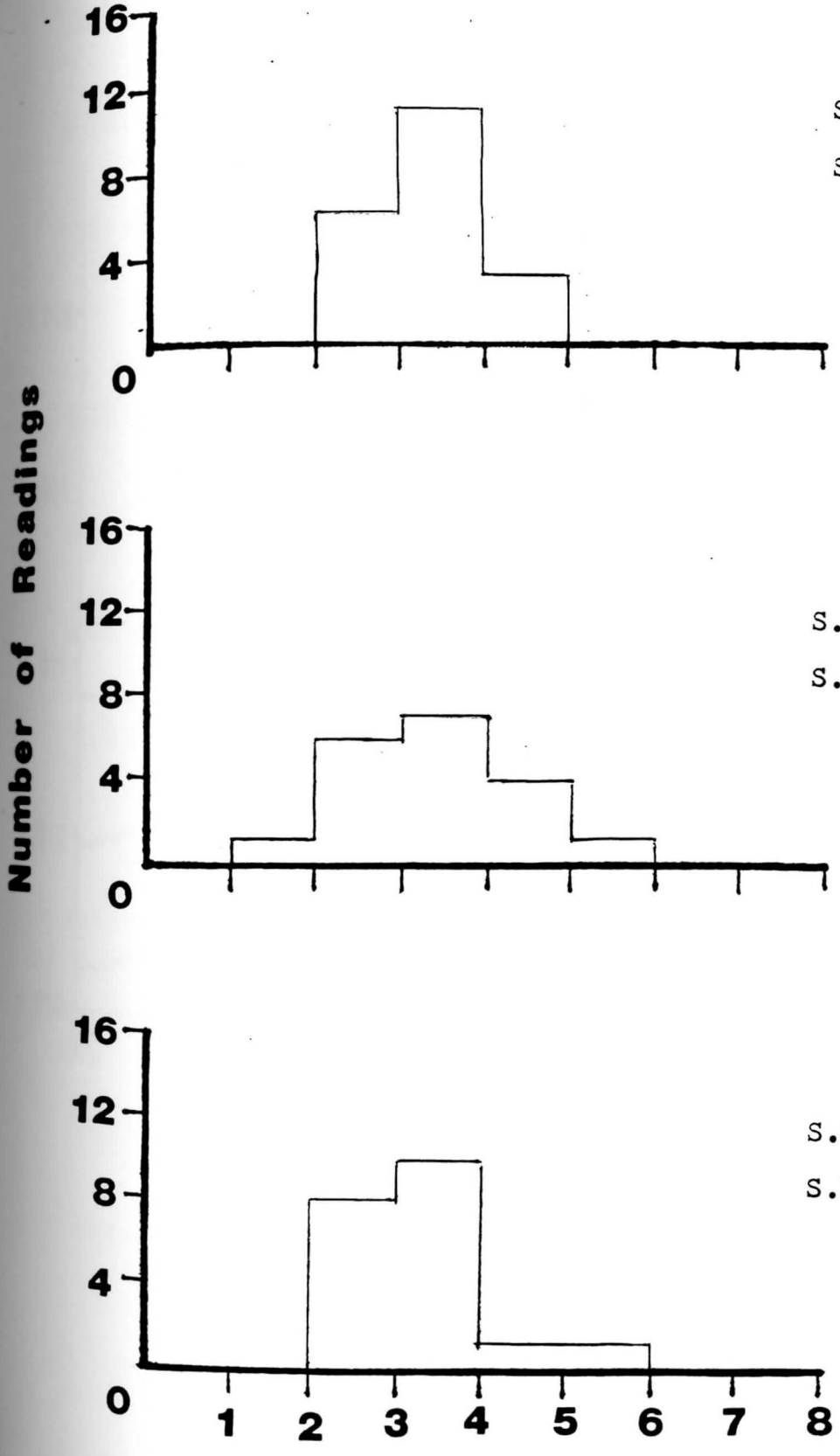
$\bar{X}$ = 0.60523

S.D. = 0.09980

S.E. = 0.01411

Histograms of Relative Protein Concentrations
1.0 Arbitrary Unit / Division

Figure 22



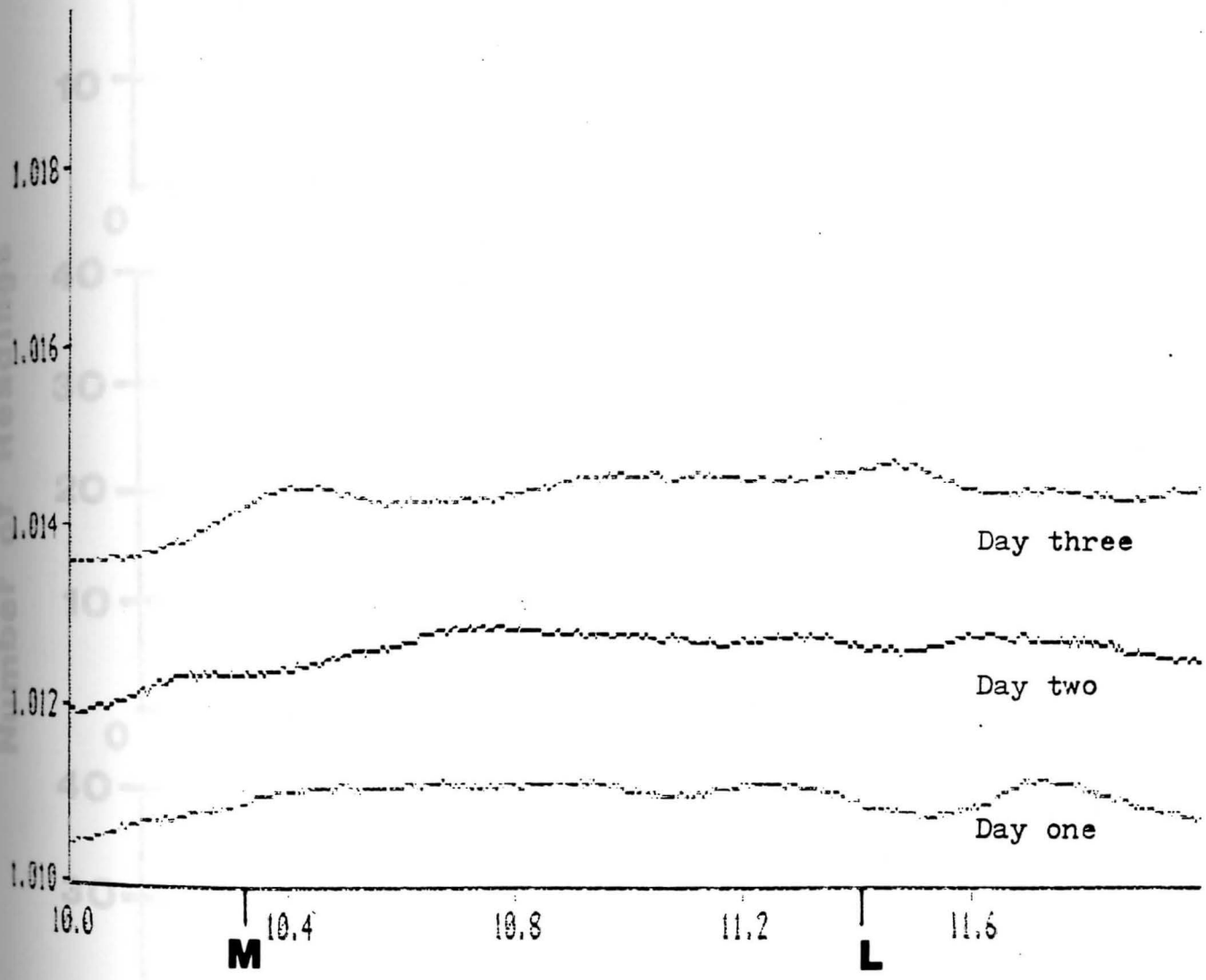
Figures 23 and 24

Enkephalin and DNA Data

for Patient Cr - 16

Chromatographic Analysis of the Enkephalins

Figure 23



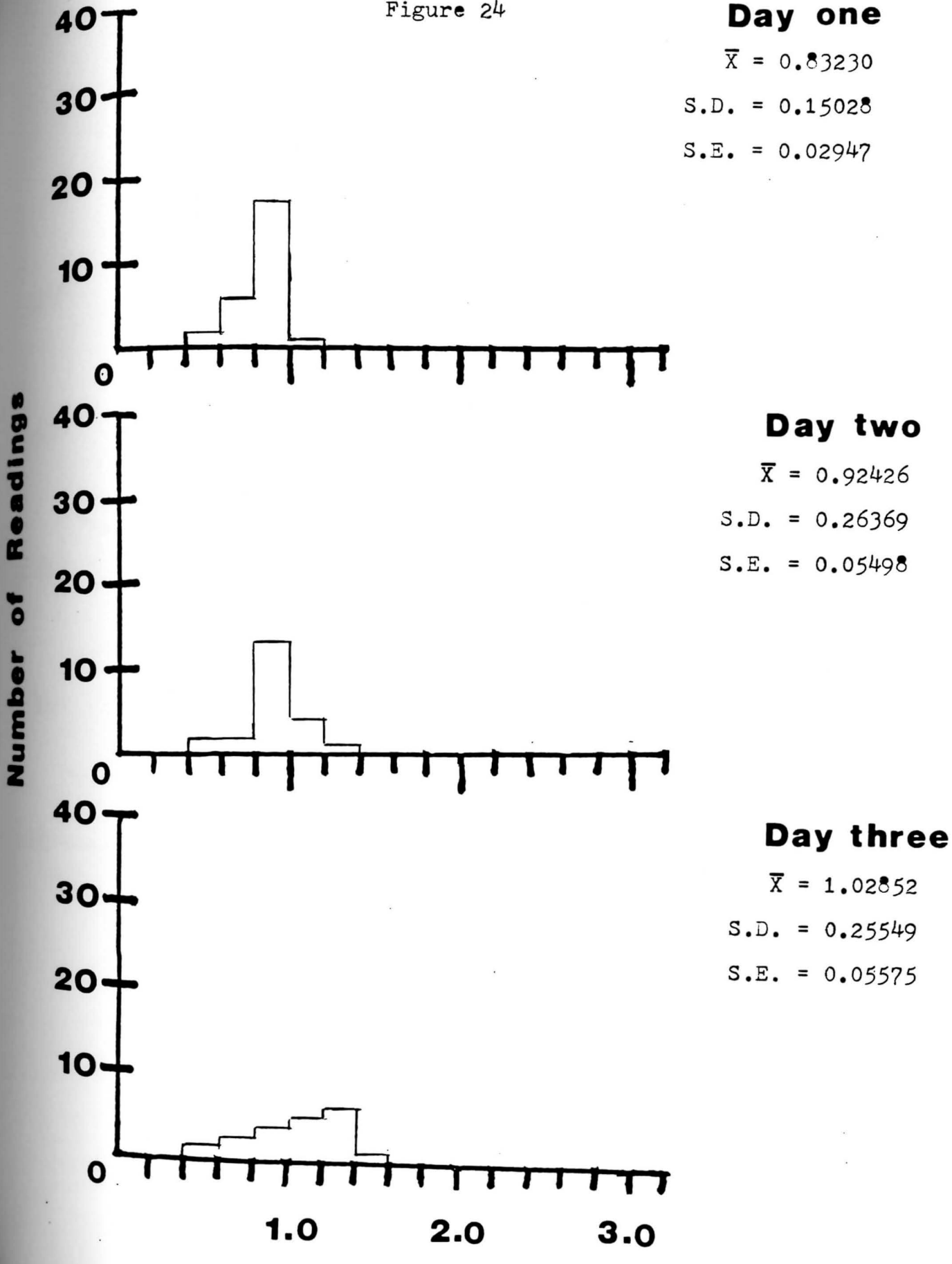
M = time where met-enkephalin should appear

L = time where leu-enkephalin should appear

Histograms of Relative DNA Concentrations

0.2 Arbitrary Units / Division

Figure 24



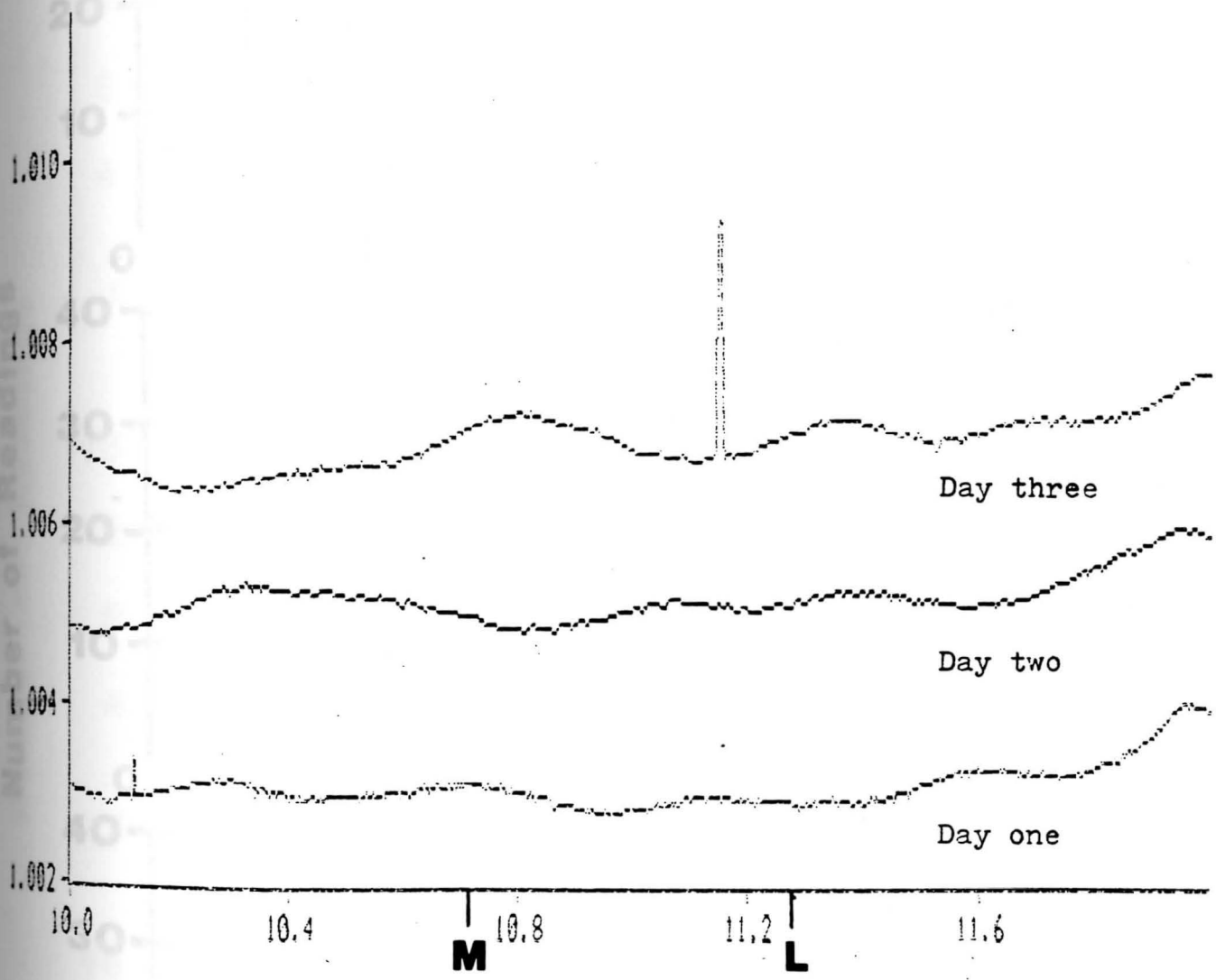
Figures 25 - 27

Enkephalin, DNA, and Protein Data

for Patient Cr - 94

Chromatographic Analysis of the Enkephalins

Figure 25



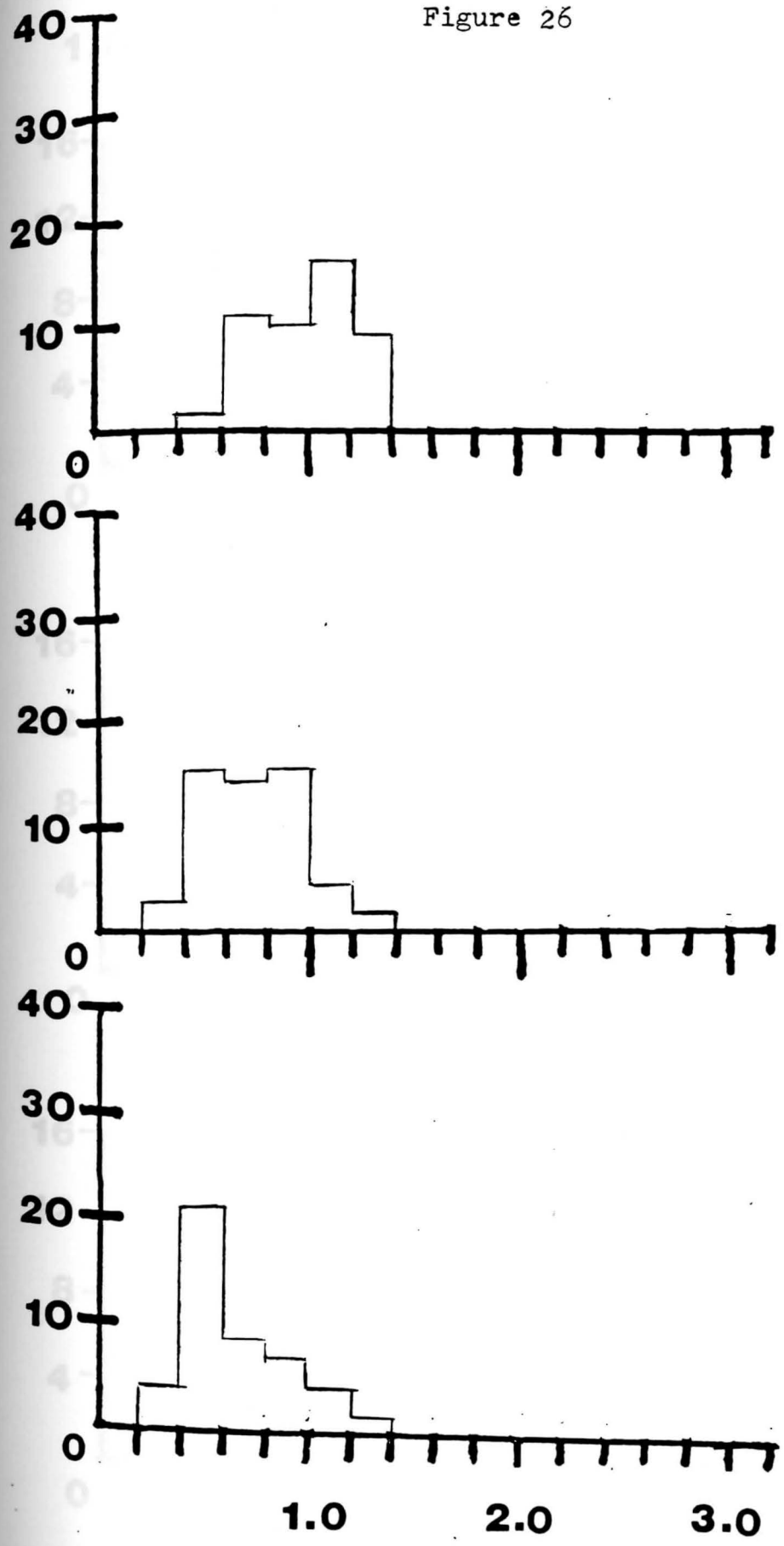
M = time where met-enkephalin should appear

L = time where leu-enkephalin should appear

Histograms of Relative DNA Concentrations

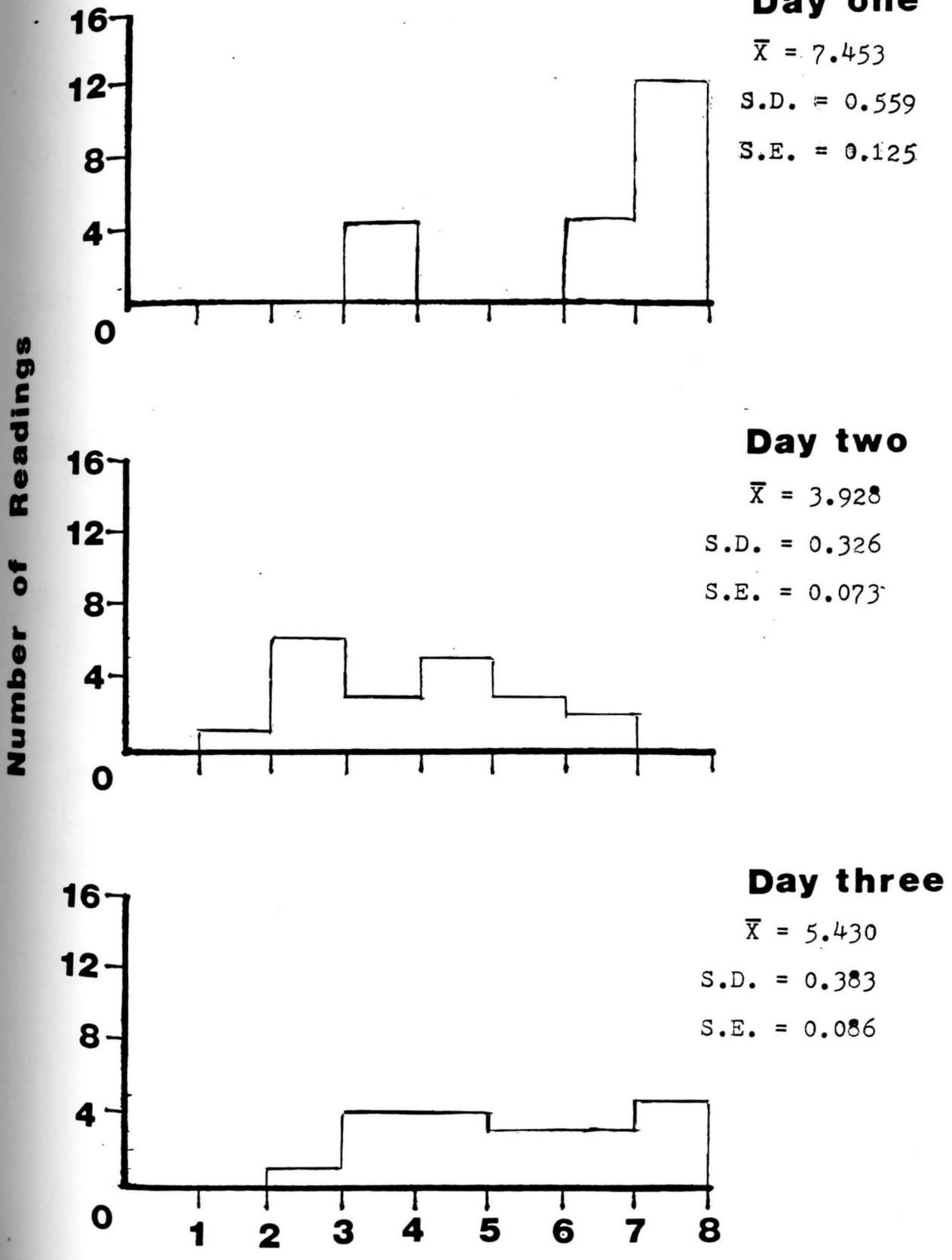
0.2 Arbitrary Units / Division

Figure 26



Histograms of Relative Protein Concentrations
1.0 Arbitrary Unit / Division

Figure 27



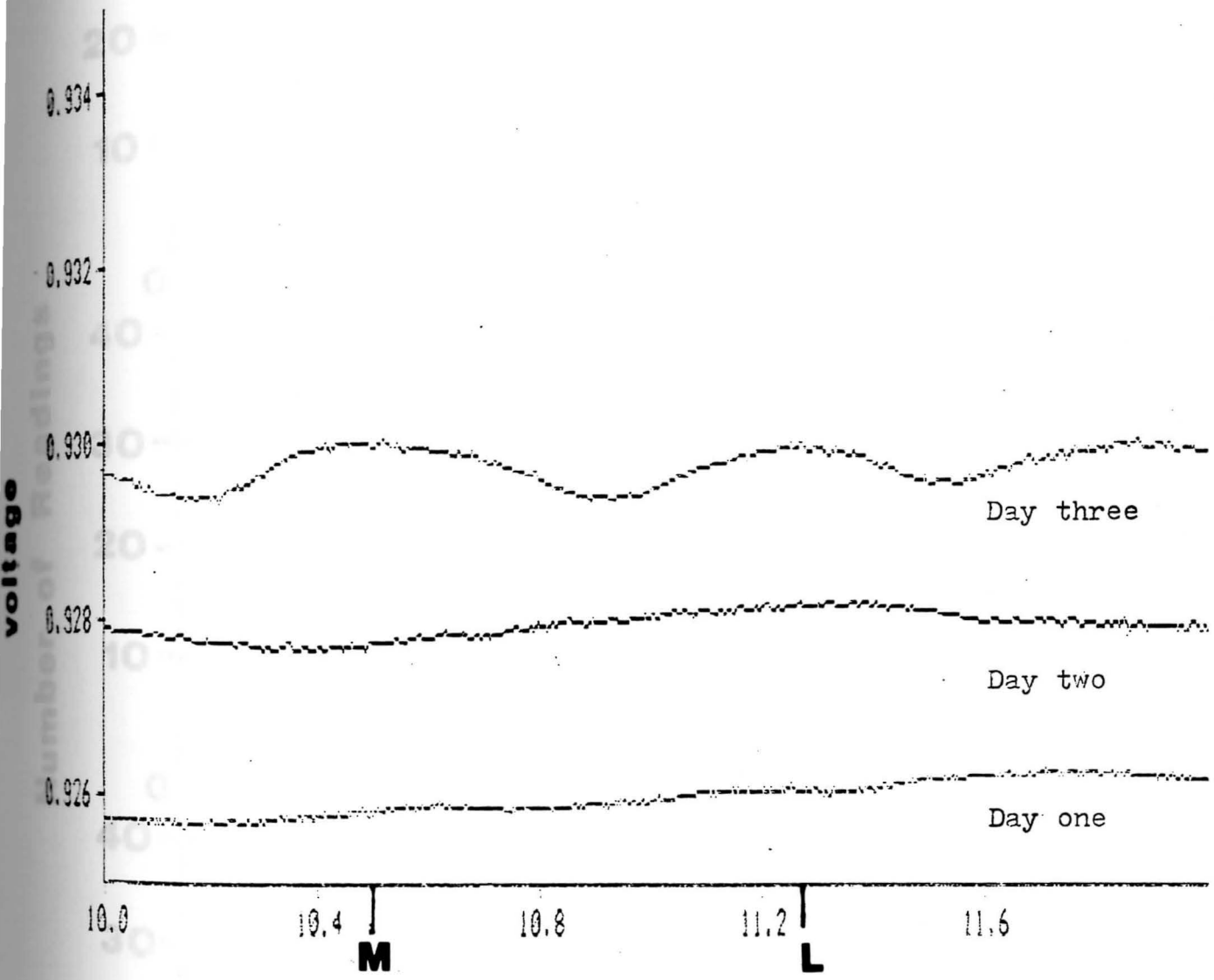
Figures 28 and 29

Enkephalin and DNA Data

for Patient Tr - 69

Chromatographic Analysis of the Enkephalins

Figure 28



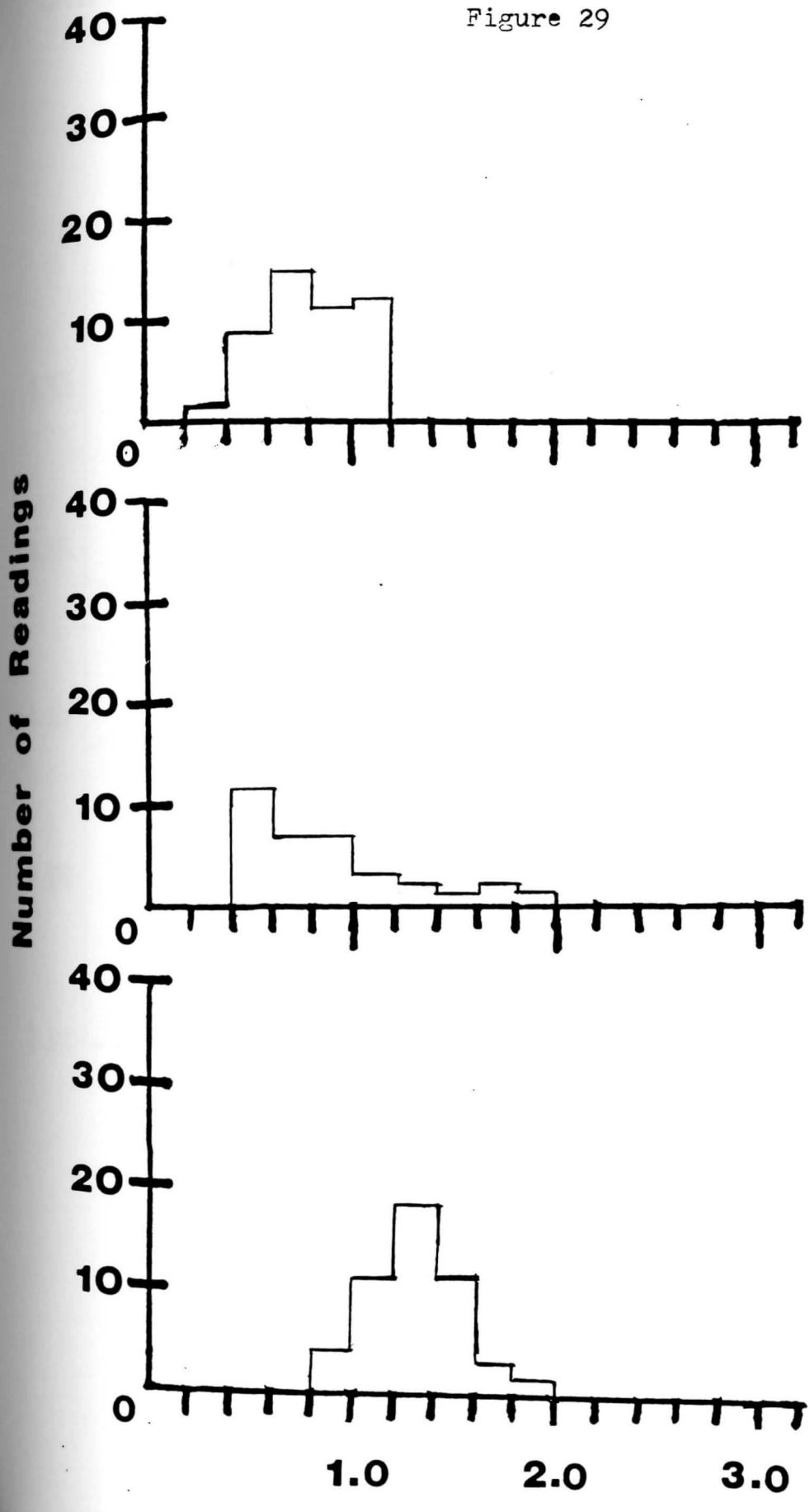
M = time where met-enkephalin should appear

L = time where leu-enkephalin should appear

Histograms of Relative DNA Concentrations

0.2 Arbitrary Units / Division

Figure 29



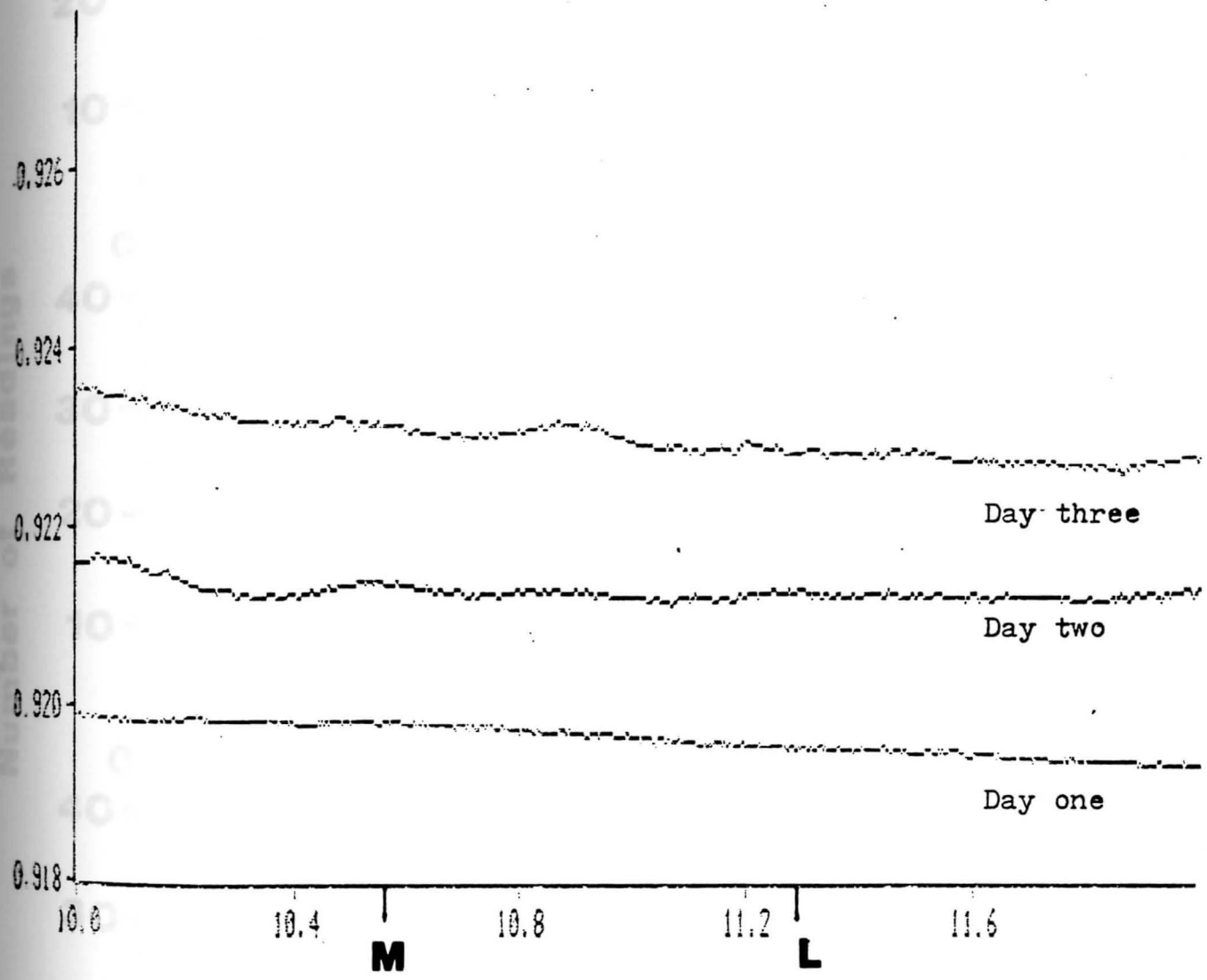
Figures 30 and 31

Enkephalin and DNA Data

for Patient Tr - 14

Chromatographic Analysis of the Enkephalins

Figure 30



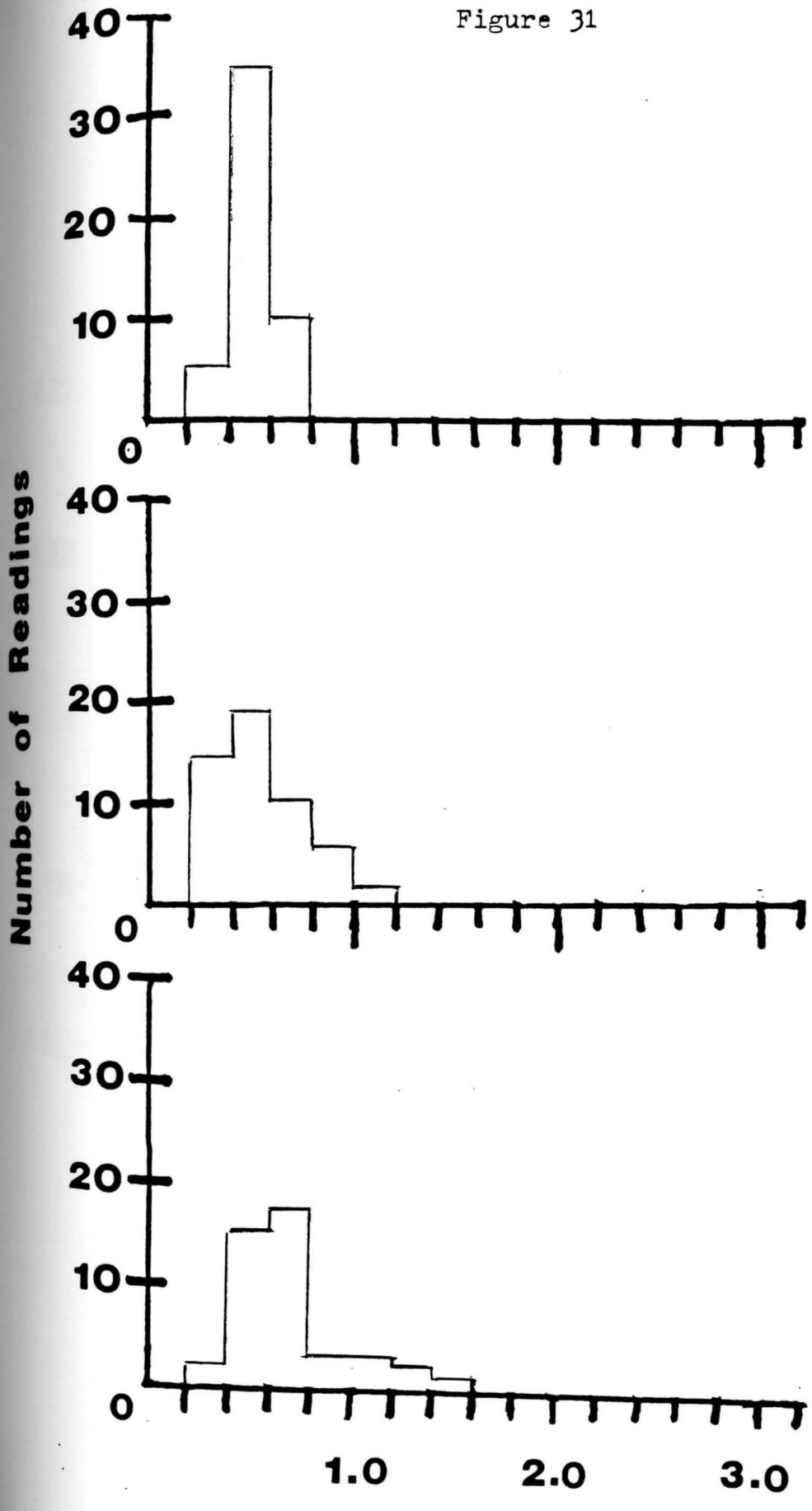
M = time where met-enkephalin should appear

L = time where leu-enkephalin should appear

Histograms of Relative DNA Concentrations

0.2 Arbitrary Units / Division

Figure 31



Day one

$\bar{X}$ = 0.52720

S.D. = 0.08498

S.E. = 0.01214

Day two

$\bar{X}$ = 0.55200

S.D. = 0.19648

S.E. = 0.02807

Day three

$\bar{X}$ = 0.70342

S.D. = 0.25253

S.E. = 0.03851

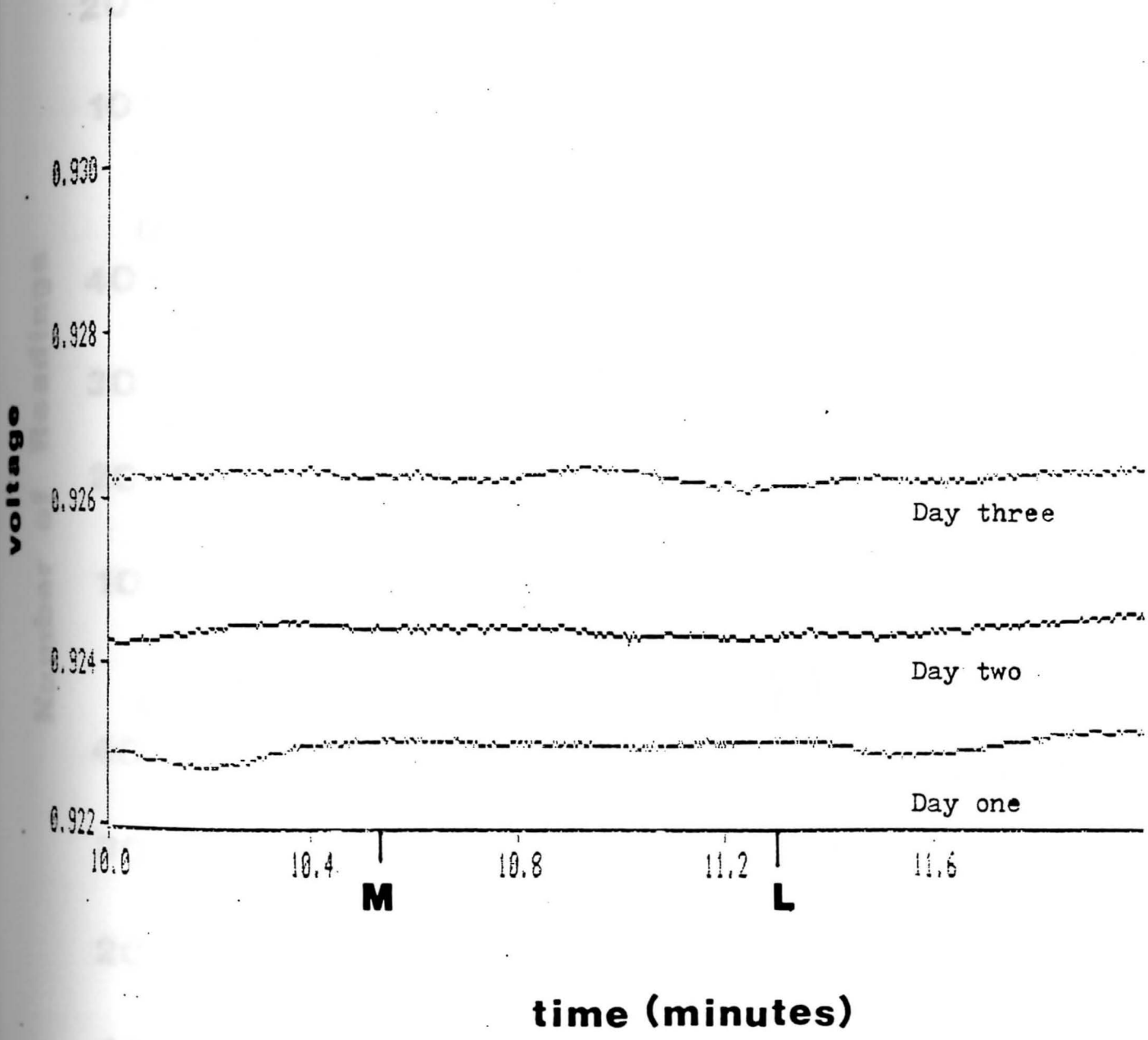
Figures 32 and 33

Enkephalin and DNA Data

for Patient Tr - 89

Chromatographic Analysis of the Enkephalins

Figure 32



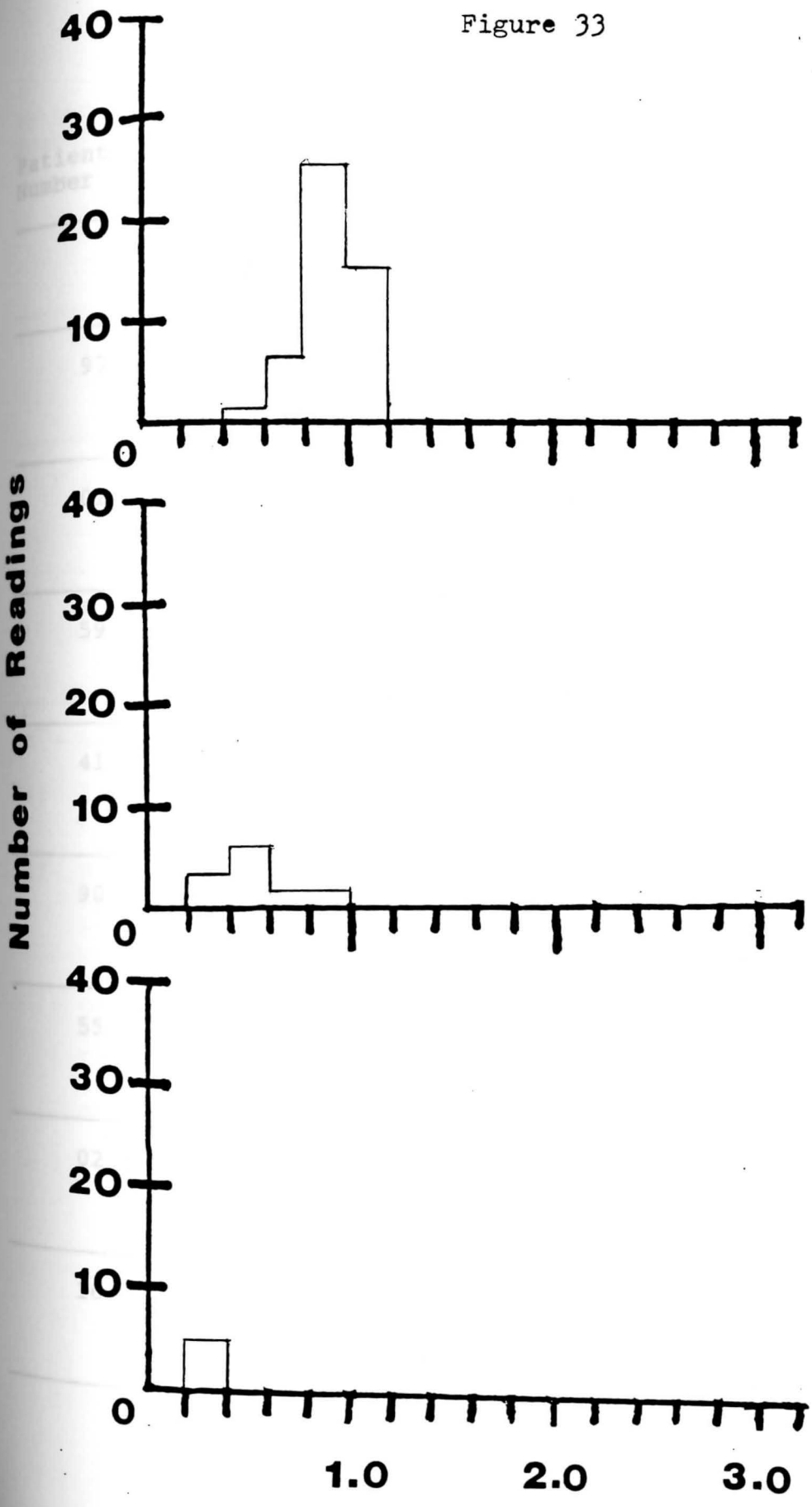
M = time where met-enkephalin should appear

L = time where leu-enkephalin should appear

Histograms of Relative DNA Concentrations

0.2 Arbitrary Units / Division

Figure 33



Day one

$\bar{X}$ = 0.95028

S.D. = 0.17450

S.E. = 0.02519

Day two

$\bar{X}$ = 0.47187

S.D. = 0.17436

S.E. = 0.05514

Day three

$\bar{X}$ = 0.27716

S.D. = 0.03859

S.E. = 0.01929

Table 1

SUMMARY OF DATA

Patient Number	Patient Type[1]	Age	Day	Relative Enkephalin Levels[2]	Relative DNA Concs.	Relative Protein Concs.
	Pooled Control			1.00,1.00	1.398±0.584	5.928±0.192
97	T	*	1	5.46,2.76	1.688±0.455	
			2	7.06,2.23	0.900±0.222	
			5	0,0	1.494±0.413	
01	C	69	1	5.44,1.55	0.617±0.245	
			2	0,4.20	0.686±0.129	
			4	0,6.72	0.767±0.153	
59	C	51	1	5.03,1.80	1.002±0.158	3.522±0.199
			2	4.35,1.11	0.789±0.248	3.001±0.183
			3	0,0	1.020±0.350	4.328±0.207
41	T	*	1	5.28,0	0.898±0.207	
			2	4.09,2.11	0.659±0.349	
			3	3.26,0	0.620±0.133	
90	C	69	1	0,3.85	0.667±0.149	3.368±0.130
			2	0,3.93	0.975±0.205	3.953±0.152
			3	0,2.20	0.853±0.190	2.994±0.145
55	C	75	1	0,0	0.693±0.216	3.820±0.164
			2	1.29,1.61	0.520±0.140	2.826±0.105
			3	0,1.11	1.099±0.299	3.903±0.129
02	C	96	1	0,2.32	0.583±0.147	3.328±0.126
			2	0,1.61	0.609±0.168	3.480±0.201
			3	0,1.62	0.605±0.100	3.255±0.164
16	C	65	1	0,.59	0.832±0.150	
			2	0,0	0.924±0.264	
			3	6.26,.52	1.029±0.255	

Table 1 Cont.

Patient Number	Patient Type[1]	Age	Day	Relative Enkephalin Levels[2]	Relative DNA Concs.	Relative Protein Concs.
94	C	*	1	0,0	0.964±0.231	7.453±0.559
			2	0,0	0.728±0.222	3.928±0.326
			3	4.39,1.48	0.685±0.346	5.430±0.383
69	T	*	1	0,0	0.787±0.224	
			2	0,0	0.929±0.662	
			3	0,0	1.296±0.213	
14	T	*	1	0,0	0.527±0.085	
			2	0,0	0.552±0.196	
			3	0,0	0.703±0.253	
89	T	*	1	0,.24	0.950±0.175	
			2	0.0	0.472±0.174	
			3	0,0	0.228±0.039	

* - Data not available

(1) T = trauma patient
C = cardiac patient

(2) Enkephalin levels were determined by comparing the relative areas of the enkephalins in the patient serum to the area in a pooled normal control. Thus, if a patient has a level of 5.00, this indicates that the area produced by that enkephalin is 5.00 times greater than that in the pooled control. A level of 0 means that there were no detectable enkephalins in that sample.
The first number in the pair for each day represents the level of met-enkephalin. The second represents the level of leu-enkephalin.

CHAPTER IV

Discussion

Impaired lymphocytic responsiveness as a result of trauma has been reported by various researchers (Bauer et al., 1978; Keane et al., 1983; O'Mahoney et al., 1984). This impairment leads to an increased susceptibility to infection, which is one of the major causes of death in traumatized patients (Bauer et al., 1978; Levy et al., 1986). These facts serve to illustrate the need for studies which can increase our understanding of the causes of lymphocyte depression and, just as importantly, the relative competency of circulating lymphocytes in the presence of high serum enkephalin levels.

At various times, cortisol, beta-endorphins, and enkephalins have all been implicated as having some role in immunomodulation (Berenbaum et al., 1976; Shatney et al., 1985; Wybran, 1985; Levy et al., 1986). However, studies have shown that plasma cortisol levels have no effect on immunosuppression (Fauci and Dale, 1974; Shatney et al., 1985). Plasma beta-endorphin levels increase following traumatic injury, but no specific receptors for this opioid on circulating lymphocytes have ever been identified (Shatney et al., 1985; Wybran et al., 1985; Levy et al., 1986).

On the other hand, receptors for enkephalins on circulating lymphocytes have been well demonstrated (Wybran et al., 1979). This would indicate that the enkephalins have a direct effect on these immune cells and are potential mediators of immunomodulation (Murgo et al., 1986). It has also been suggested that the function of the enkephalins is more specialized than that of beta-endorphin (Zukin and Zukin, 1981). The objective of this study was to determine if there is evidence of a correlation between serum enkephalin levels produced as a result of trauma, DNA template activity, and thus protein synthetic activity in circulating lymphocytes. These studies were carried out on 7 cardiac and 5 trauma patients over a three-day interval. Patients are discussed in a random order after their receipt.

Patient Tr - 97

High levels of met-enkephalin are observed on the first day, which increased during the second day. A decrease in DNA template activity was observed to occur on the second day. This can be explained by a delayed response to met-enkephalin. It can be surmised from this data that a change in met-enkephalin levels is followed by a delayed response in the immune cells, i.e., the response exhibits a time dependent relationship or effect. A fifth day sample readily supports this contention and predicts that a third day sample would show a further depression in lymphocytic activity with an increase in met-enkephalin levels. It should also be noted that leu-enkephalin decreases as DNA activity decreases

showing an opposite effect to that of met-enkephalin.

Patient Cr - 01

High levels of met-enkephalin on the first day are observed and can be associated with depressed lymphocyte activity. On the third and fourth day, met-enkephalin levels are undetectable. However, leu-enkephalin levels are observed to increase and are accompanied by increased DNA template activity. This appears to indicate that leu-enkephalin is able to exert a noticeable effect on immune cells in the absence of met-enkephalin.

Patient Cr - 59

High levels of met-enkephalin on days 1 and 2 with a decrease in lymphocyte DNA template activity and protein synthesis on day 2 once again demonstrates and reinforces the hypothesis, previously stated, that a delayed response of lymphocytic DNA activity and protein synthesis to the presence of met-enkephalin does, in fact, occur. The correlation of leu-enkephalin with increased DNA activity on the third day can readily be observed. This data permits the observation that these two parameters are related. When met-enkephalin levels increase, DNA template activity decreases. When leu-enkephalin levels are elevated, DNA activity regarding synthetic activity also increases.

Patient Tr - 41

Even though met-enkephalin levels in this patient are shown to decrease over a three-day period, they are still

able to exert a suppressive effect, which is reflected in a steady decrease in DNA template activity. In addition, it appears that once the levels of met-enkephalin reach normal levels, the synthetic activity of the lymphocytes can also be expected to return to normal.

Patient Cr - 90

Lymphocytic DNA template activity appeared to be half that of normal values upon admission, when compared to controls. However, this can possibly be attributed to patient delay in seeking treatment, which is common to many cardiac patients. It was observed that levels of leu-enkephalin subsequently increased, as did DNA template activity and protein synthesis in later observations. This is suggestive of a leu-enkephalin stimulatory effect on circulating lymphocytes.

Patient Cr - 55

Initial depressed DNA template activity is observed with low or undetectable enkephalin levels, which is possibly due to patient delay in seeking treatment, as previously mentioned. Increased levels of enkephalins on the second day with accompanying further depression of DNA template activity are observed. This patient stabilized by the third day as shown by normal levels of enkephalins and DNA activity.

Protein synthesis followed the pattern of DNA template activity and demonstrated suppression as was expected.

patient Cr - 02

Met-enkephalin levels are shown to be below detectable amounts whereas low DNA activity is observed, and serves to reinforce the observation previously discussed which suggests that patient delay in treatment produces this affect. On day 2 and 3 no met-enkephalin levels were evident. However, elevated levels of leu-enkephalin remained essentially the same over these two periods. DNA activity also remained consistent with previously reported findings.

Patient Cr - 16

As observed in preceding patients, this patient appeared to delay treatment during the initial period of trauma. Initial lymphocyte suppression is observed which, as previously presented data indicates, normally reflects the interaction of enkephalins, especially met-enkephalin. No substantial elevated levels of enkephalins were observed on the first two days and activity of the lymphocytes began to return to normal due to stabilization of the patient. The high level of met-enkephalin produced on the third day indicates that destabilization occurred as a result of possible reinfarction, or a worsening of the patient's condition. It is reasonable to assume on the basis of previous data presented that depression of DNA activity would have continued in this case.

Patient Cr - 94

Observation of DNA concentration, or synthetic activity, of lymphocytes indicates that there is a steady decrease of activity over the three-day period accompanied by undetectable levels of enkephalins during the first two days. This can be explained by the fact that enkephalins degrade quickly and readily demonstrates the necessity of rapid freezing of samples prior to analysis. In addition, high levels of met-enkephalin demonstrated on the third day supports this contention and indicates that prior samples did, in fact, contain elevated enkephalin levels but were possibly degraded prior to analysis. It should be noted that the activity of lymphocytic DNA exhibits the highest level of depression when met-enkephalin levels are the most elevated. Here it appears evident that met-enkephalin is implicated in the immunosuppression of these cells.

Patient Tr - 69

Measurements of lymphocytic DNA activity were seen to be, in this case, depressed in the absence of detectable enkephalin levels. However, it was assumed that there were enkephalin levels present before admission, but that they were not detectable at the time the patient's serum was analyzed. Since it was not possible in all cases to obtain a blood sample immediately following the trauma, it can be postulated that this patient was possibly stabilized before admission to the hospital. DNA changes due to activity or inactivity, as the case may be, are long lasting whereas

enkephalins are not so (Hambrook, 1976). These facts would explain undetectable enkephalin levels and depressed DNA activity upon admission. It is important to note that enkephalins were not produced over the three-day period as DNA activity returned to near normal.

Patient Tr - 14

As observed in patient Tr - 69 the presence of undetectable enkephalin levels with initial DNA suppression once again tends to support the hypothesis that a delay in treatment, which resulted in a delay in the taking of the first serum sample, accounts for an initial unobserved elevation in enkephalin levels, particularly that of met-enkephalin because of a high turnover rate in these molecules, especially when patient stabilization occurs. The fact that the DNA activity increased over the three-day period to normal levels, as a result of lack of stimulation from the enkephalins, attest to the fact that patient stabilization had, in fact, occurred.

Patient Tr - 89

This patient was determined by hospital personnel to be brain dead. It was observed, as expected, that no appreciable production of enkephalins took place over a three-day period. This data serves to demonstrate the importance of the cerebral function in the regulation of enkephalin production.

From the evidence obtained in this study, a correlation between the amount of enkephalins produced in cardiac and trauma patients and their effects on the activity of circulating lymphocytes can be postulated. Both methionine and leucine enkephalin appear to play a role in regard to effects on the immune system. When present at high levels, met-enkephalin appears to be a dominating force causing immunosuppression, similar to the effects produced by morphine. Leu-enkephalin, on the other hand, when present alone, appears to enhance the activity of lymphocytes. This data is supported by Wybran's (1979) findings regarding delta and mu receptors present on the lymphocytic cell surface. His studies have demonstrated that morphine is specific for mu receptors, whereas leu-enkephalin has specificity for delta receptors. Met-enkephalin has high affinity for both delta and mu receptors (Zukin and Zukin, 1981). If met-enkephalin and leu-enkephalin bind to different receptors on the surface of circulating lymphocytes, this would explain the effects produced by met-enkephalin that are similar to morphine and the opposite effects reported in this study regarding leu-enkephalin.

The findings in this study provide insight as to the relationship of the enkephalins to the immune system. However, it is evident that more research is needed in this area. It is interesting to speculate, for example, on a possible extension of this study, i.e., what is the mode of genetic activity or repression when enkephalin - receptor

interaction takes place, regarding immune cells? Hopefully, with more knowledge of enkephalin function, immunosuppression following trauma will be better understood.

BIBLIOGRAPHY

Bauer, A. R., C. McNeil, E. Trentelman, S. A. Swift, and J. D. Mason. 1978. "The depression of T lymphocytes after trauma." Am. J. Surg. 136:674 - 680.

Berenbaum, M. C., W. A. Cope, and R. V. Bundick. 1976. "Synergistic effect of cortisol and prostaglandin E on the PHA response: Relation to immunosuppression induced by trauma." Clin. Exp. Immunol. 26:534 - 541.

Cox, B. M. 1982. "Endogenous opioid peptides: A guide to structures and terminology." Life Sci. 31(16 & 17): 1645 - 1658.

Cox, B. M. and E. R. Baizman. 1982. "Physiological functions of the endorphins." In Endorphins: Chemistry, Physiology, Pharmacology, and Clinical Relevance. Vol. 20. J. B. Malick and R. M. S. Bell, Eds:113 - 196. Marcel Dekker, Inc. New York.

Deitch, A. D. 1955. "Microspectrophotometric study of the binding of the anionic dye, naphthol yellow S, by tissue sections and by purified proteins." Lab. Invest. 4:324 - 351.

DeStefano, H. S. 1948. "A cytochemical study of the Feulgen nucleal reaction." Chromosoma 3:282.

Dickenson, A. H. 1986. "Enkephalins: A new approach to pain relief?" Nature 320:681 - 682.

Evans, C. J., E. Erdelyi, and J. D. Barchas. 1986. "Candidate opioid peptides for interaction with the immune system." In Enkephalins and Endorphins: Stress and the Immune System. N. P. Plotnikoff, R. E. Faith, A. J. Murgo, and R. A. Good, Eds:3 - 16. Plenum Press. New York.

Fauci, A. S. and D. C. Dale. 1974. "The effect of In Vivo hydrocortisone on subpopulations of human lymphocytes." J. Clin. Invest. 53:240 - 246.

Feulgen, R. and H. Rossenbeck. 1924. "Microskoposch-Chemeschen Nachweis einer Nucleinsaure von Typus der Thymo nucleinsaure und die darauf dirunkencle elektive Farburg von Zellkernen in Mikroskopischen Praparaten." Z. Physiol. Chem. 135:203 - 248.

Hambrook, J. M., B. A. Morgan, M. J. Rance, and C. F. C. Smith. 1976. "Mode of deactivation of the enkephalins by rat and human plasma and rat brain homogenates." Nature 262:782 - 783.

Jankovic, B. D. and D. Maric. 1987. "Enkephalins and immunity I: In Vivo suppression and potentiation on humoral immune response." Ann. N. Y. Acad. Sci. 469:115 - 125.

Keane, R. M., W. Birmingham, C. M. Shatney, R. A. Winchurch, and A. M. Munster. 1983. "Prediction of sepsis in the multitraumatic patient by assays of lymphocyte responsiveness." Surg. Gynecol. Obstet. 156:163 - 167.

Levy, E. M., T. McIntosh, and P. H. Black. 1986. "Elevation of circulating B-endorphin levels with concomitant depression of immune parameters after traumatic injury." J. Trauma 26(3):246 - 249.

McIntosh, T. K. and A. I. Faden. 1986. "Opiate antagonists in traumatic shock." Ann. Emerg. Med. 15:1462 -1465.

Murgo, A. J., R. E. Faith, and N. P. Plotnikoff. 1986. "Enkephalins: Mediators of stress-induced immunomodulation." In Enkephalins and Endorphins: Stress and the Immune System. N. P. Plotnikoff, R. E. Faith, A. J. Murgo, and R. A. Good, Eds:221 - 240. Plenum Press. New York.

O'Mahony, J. B., S. B. Palder, J. J. Wood, A. McIrvine, M. L. Rodrick, R. H. Demling, and J. A. Mannick. 1984. "Depression of cellular immunity after multiple trauma in the absence of sepsis." J. Trauma 24(10):869 - 875.

Patau, K. 1952. "Absorption microspectrophotometry of irregular shaped objects." Chromosoma 5:341 - 362.

Plotnikoff, N. P., G. C. Miller, S. Solomon, R. E. Faith, L. Edwards, and A. J. Murgo. 1986. "Methionine enkephalin: Immunomodulator in normal volunteers (In Vivo)." In Enkephalins and Endorphins: Stress and the Immune System. N. P. Plotnikoff, R. E. Faith, A. J. Murgo, and R. A. Good, Eds:399 - 405. Plenum Press. New York.

Roda, L. G., G. Roscetti, R. Possenti, F. Venturelli, and F. Vita. 1986. "Control mechanisms in the enzyme hydrolysis of adrenal-released enkephalins." In Enkephalins and Endorphins: Stress and the Immune System. N. P. Plotnikoff, R. E. Faith, A. J. Murgo, and R. A. Good, Eds:17 - 34. Plenum Press. New York.

Shanks, M. F., V. Clement-Jones, C. J. Linsell, P. E. Mullen, L. H. Rees, and G. M. Besser. 1981. "A study of 24-hour profiles of plasma met-enkephalin in man." Brain Res. 212:403 - 409.

Shatney, C. H., R. M. Cohen, M. R. Cohen, and D. K. Imagawa. 1985. "Endogenous opioid activity in clinical hemorrhagic shock." Surg. Gynecol. Obstet. 160:547 - 551.

Smyth, D. G. 1982. "Structural relationships of the potent endorphins." In Endorphins: Chemistry, Physiology, Pharmacology, and Clinical Relevance. Vol. 20. J. B. Malick and R. M. S. Bell, Eds:9 - 22. Marcel Dekker, Inc. New York.

Swift, H. 1950. "Deoxyribose nucleic acid content of animal nuclei." Physiol. Zool. 23:169 - 198.

Wybran, J., T. Appelboom, J. P. Famaey, and A. Govaerts. 1979. "Suggestive evidence for receptors for morphine and methionine-enkephalin on normal human blood T lymphocytes." J. Immuno. 123(3):1068 - 1070.

Wybran, J. 1985. "Enkephalins and endorphins as modifiers of the immune system: present and future." Fed. Proc. 44(1):92 - 94.

Wybran, J. 1986. "Enkephalins as molecules of lymphocyte activation and modifiers of the biological response." In Enkephalins and Endorphins: Stress and the Immune System. N. P. Plotnikoff, R. E. Faith, A. J. Murgo, and R. A. Good, Eds:253 - 262. Plenum Press. New York.

Yemma, J. J. and C. D. Therrien. 1972. "Quantitative microspectrophotometry of nuclear DNA in selfing strains of the myxomycete Didymium iridis." Amer. J. Bot. 59:828 - 835.

Zukin, R. S. and S. R. Zukin. 1981. "Minireview. Multiple opiate receptors: Emerging concepts." Life Sci. 29(26):2681 - 2690.