1982

TRICYCLIC ANTIDEPRESSANTS AND SERUM IONIZED CALCIUM

Girolamo A. Ortolano
University of Rhode Island

TRICYCLIC ANTIDEPRESSANTS AND SERUM IONIZED CALCIUM

BY

GIROLAMO A. ORTOLANO

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

PHARMACOLOGY AND TOXICOLOGY

UNIVERSITY OF RHODE ISLAND

1982

DOCTOR OF PHILOSOPHY DISSERTATION

OF

GIROLAMO A. ORTOLANO

Approved:

Dissertation Committee

Major Professor _____

_____

_____

_____

_____

Dean of the Graduate School

UNIVERSITY OF RHODE ISLAND

1982

ABSTRACT

Serum ionized calcium has long been viewed as the physiologically active form of calcium, yet the paucity of studies concerned with this response measure is apparent and may be related to the high cost of modern potentiometric instrumentation. Reducing the cost factor was approached by integrating an inexpensive static-type calcium ion-specific electrode into a fabricated plastic electrode chamber. Characterization of the electrode system was performed by elucidating the optimum parameters for electrode use in serum samples. Conformance to the extended Nicolsky-Eisenman equation was used as a criterion for acceptable electrode system performance. Negligable effects of proteins on the performance of the electrode was established by comparing calcium binding kinetics and pH dependence data obtained with the system with values obtained from the literature. The electrode system was shown to correlate well when compared with the Orion SS-20 serum ionized calcium instrument with over 97% of the variance accounted for by regression. The administration of calcium chloride or the divalent cation chelator, ethylene diamine tetraacetic acid, to rats resulted in predictable responses in serum ionized calcium.

The serum ionized calcium electrode system was employed in tests of hypotheses concerning serum ionized calcium and

antidepressant activity of the tricyclic antidepressant, protriptyline and the thyroid hormone, tri-iodothyronine. Rats treated with protriptyline (10mg/kg) for 1, 6 or 18 days and tested 2, 6, 12 and 24 hours after the last dose failed to show significant changes in serum ionized calcium. Similarly, animals treated with tri-iodothyronine at varying doses for 1, 5 or 10 days and tested 1, 4 or 24 hours after the last dose also failed to show changes in this response measure. Concomitant thyroid hormone at varying doses and protriptyline (5mg/kg) did not result in changes in serum ionized calcium or in any response measures studied which are either known or are suspected to be reflective of changes in calcium homeostasis. The results failed to support a calcium hypothesis of antidepressant activity.

The results are at variance with those of clinical studies among depressives reported in the literature. Discussion offers support for viewing the rat as a poor animal model for studying alterations in calcium homeostasis.

ACKNOWLEDGEMENTS

I would like to chronologically acknowledge so many people for the numerous ways my life and this work has been affected by their very existence. I thank all of you for what you have given me. Mom, dad, brother Len, Peter J. Ferrara, Arnie Heitner, Bob and Delores Olander, Gary Carlson, Al Swonger, John DeFeo, Ray Scienza, Barbara Streppone, Craig Smith, John and Lynn Muro, Ed Kaiser, Ron Stuart, Ken Wunschel, Jr., Rupert P. Hammond and last but not least my childern, Brett, Keith, Sabrina and Tiffany-Ann.

DEDICATION

For my wife, Kathryn

PREFACE

This thesis is presented in the manuscript format according to the rules of the Graduate School of the University of Rhode Island. Two manuscripts are included.

I. An Inexpensive Method for the Determination of Serum Ionized Calcium

II. Mechanisms of Antidepressant Action: Uptake Blockade or Serum Ionized Calcium

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURE PAGE

MECHANISMS OF ANTIDEPRESSANT ACTION: UPTAKE BLOCKADE OR SERUM IONIZED CALCIUM

AN INEXPENSIVE METHOD FOR THE DETERMINATION OF SERUM IONIZED CALCIUM

INTRODUCTION

Serum ionized calcium has long been viewed as the physiologically active form of calcium (20) but the laborious techniques required for its determination have limited both clinical and experimental studies. In 1967, Ross (21) introduced the calcium ion-specific electrode (ISE) and a short time thereafter Orion Research, Inc. made available a flow-through calcium ISE designed to measure ionized calcium in biological fluids anaerobically. This moderately priced electrode is no longer available and was replaced by a much more expensive microprocessor-controlled apparatus. Although other sophisticated instruments are commercially available, it appears that the paucity of literature concerning serum ionized calcium may be a direct result of the high cost of instrumentation.

Reducing the cost factor was approached by integrating an inexpensive static-type calcium ISE into an easily fabricated plastic electrode chamber to permit temperature-controlled, anaerobic sampling (0.6 ml) with the aid of commonly available laboratory equipment including a sensitive pH/mV meter and a circulating water bath. Since the static-type ISE is not recommended for use with serum samples, this report includes a characterization and application for such use. Also included are the construction details of the electrode chamber.

MATERIALS AND METHODS

Apparatus The potentiometric components of the experimental apparatus, obtained from Orion Research, Inc. (Cambridge, Mass.), include an electrode body (model 93-00) attached to a calcium ISE sensing module (model 93-20) and a single junction reference electrode (model 93-01) utilizing 4M KCl saturated with silver as the internal filling solution. The electrode potential was measured with an Orion digital pH/mV meter (model 701A) in the relative mV mode. Sample temperature was controlled by circulating water through the plastic sampling chamber using a constant temperature circulating pump (HETO, Denmark; model 7402).

Serum pH was measured using a blood gas analyzer (IL model 413).

Sampling Chamber - Construction Details The plastic chamber was constructed of Plexiglas(R) with critical dimensions denoted in the pictorial representations (figures 1 and 2). A photograph of the assembled apparatus is shown in figure 3.

The plastic was cut to the required sizes indicated in table 1. Sides of the upper and lower assembly were fitted with 1/8" male hose adapters (Cole-Palmer; Chicago, Il.). Holes for the electrode bodies were drilled using a spade

bit (5/8") while the top and bottom plates of the upper assembly were held together to insure proper hole alignment.

The bottom plate of the upper assembly forms a major part of the sample compartment. Sample entry and exit ports were made by drilling 1/16" channels obliquely from the center of the front and back edges to near the bottom of the electrode body holes. A 1/16" trough was created by filing the underside of the bottom plate between the electrode body holes. Threaded ends of female Instac(R) Luer-Boss adapters (Lee Co.; Westbrook, Ct.) were ground off, and the resultant flat surface sanded lightly. These adapters were components of the sample entry and exit ports. They were fitted over the channels, secured into position with a drop of methylene chloride and further reinforced with a bead of epoxy cement.

The electrode chamber was assembled and held in position with elastic bands. Methylene chloride was applied to all contacting surfaces with the aid of a glass syringe and stainless steel needle. Upon removal of the elastic bands, successive applications of plastic shavings dissolved in methylene chloride (5% and then 15% w/v) were made to provide leak-proof seals.

The electrodes were inserted into the holes and secured into position with 1/2" x 1/16" Buna-N O-rings (Arthur H.

Thomas Co.; Phila., Pa.). A sheet of teflon served as the remaining part of the sample compartment. The distance between the active surface of the electrodes and the teflon sheet, along with the position of the O-rings, determined the sample volume required for analysis. Generally, 0.6 ml was required to completely fill the sample compartment. The teflon was backed with cork sheeting and the lower assembly, cork, teflon sheet and upper assembly were all held together with steel rods and turnbuckles (figure 3).

Use of the Sampling Chamber The assembled electrode chamber was oriented at 45 degrees with respect to the sample entry port and a vertical plane. This orientation prevented air from becoming trapped within the sample compartment. The sample was introduced into the sample compartment with a syringe. The exit port was capped with a Leur-Lock end cap when the sample began to emerge from the port. The chamber was flushed 3 times with 3 ml of isotonic saline between the introduction of samples and standards.

Reagents All chemicals were of reagent grade (Fisher Scientific, Pa.) and solutions were prepared with distilled - deionized water. Standards were prepared from stock solutions (M) of NaCl (4.0), KCl (0.5), $MgCl_2$ (0.55), $CaCO_3$ (0.1) dissolved in HCl (0.24), $CaCl_2$ (0.1), HCl (1.0) and NaOH (1.0). Tris-(hydroxymethyl)-aminomethane (TRIS, 1.0) and disodium ethylenediamine tetraacetic acid (EDTA,

0.05) was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Calculations A) Ionic strength- In the preparation of standards at the ionic strengths given, the contribution of components except NaCl towards ionic strength (following adjustment of the solution to pH 7.4 with NaOH) was calculated using the classical formula

$$u = 1/2 \sum CiZi^2$$

where:

u = ionic strength

Ci = molar concentration of the ion, i

Zi = the charge associated with ion, i.

An appropriate amount of NaCl was then added to bring the solution to the desired ionic strength. All standards contained 5.0 mM KCl and 0.55 mM $MgCl_2$ along with varying amounts of $CaCl_2$ and NaCl.

B) Individual Ion Activities- A variety of conventions are available for estimating the activity coefficients of individual ions at various ionic strengths. The data of Kielland was used by applying polynomial regression analysis on the logarithm of the ionic strength as the independent variable versus the activity coefficient. Second degree polynomials fit the data well ($P<.001$) with maximum residuals of less than 5 percent. A comparison of the values obtained by various conventions along with those

estimated by the regression analysis (PR-K) are presented in table 2 along with the regression parameters.

In this report, molar concentrations will be represented by brackets and activity by parentheses.

C) Serum Ionized Calcium- These values were obtained by use of the equation [Ca++] = antilog ((intercept - mV reading)/ slope). The mV readings of two calcium standards (0.5 and 5.0 mM) were taken in duplicate both before and after a series of samples were run. The slope was calculated by taking the difference in mV readings of average values obtained for low calcium and high calcium standards. The average mV reading of the 1.0 mM calcium standards bracketing the sample was used in the equation to calculate the intercept for that sample. Early studies conducted with this system resulted in the observation that steady baseline drifts occured over the range of concentrations of standards employed. In addition, a paroxysmal shift in the mV reading for a sample or standard was observed. Errors associated with both types of shifts are attenuated by this calculation procedure.

Selectivity Ratios The equimolar concentration (0.1 M), separate solution method as described by Srinivasan and Rechnitz (25) was used to determine selectivity ratios while the electrodes were removed from the sample chamber.

Selectivity ratios are given by:

$$K_{ca-i} = \left(\text{antilog}\left(\frac{Eca - Ei}{S}\right)\right)\left(\frac{(Ca^{++})}{(i)^{2/Zi}}\right)$$

where:

K ca-i = selectivity ratio of the calcium ion specific electrode for the interfering ion, i.

E ca = observed potential of a 0.1M $CaCl_2$ solution

E i = observed potential of a 0.1M solution of the interfering ion, i.

S = 2.3 RT/2F = Nernstian slope with theoretical values of 29.58 at 25°C and 30.77 at 37°C.

(Ca++) = activity of calcium in moles/L.

(i) = activity of the interfering ion in moles/L.

Calcium Albuminate Kinetics Bovine serum albumin (BSA, Sigma Co.) at a concentration of 6g/dl was prepared in 50 mM acetate buffer (pH 4.4), dialyzed at 4°C for 24 hours with two forty-fold volume changes and subsequently dialyzed in a similar manner in 50 mM TRIS/HCl buffer (pH 7.4). Dialysis at the isoelectric point of albumin served to reduce initially elevated potentials observed following the preparation of proteins in TRIS buffer directly. The elevated potentials were due primarily to low levels of calcium liberated from the BSA; a finding confirmed qualitatively by atomic absorption spectroscopy. Following dialysis the resultant concentration of BSA was 4 g/dl, and to 10ml of this solution, 5, 10, and 20ul aliquots of

$CaCl_2$ (200mM) were added incrementally and ionized calcium determined. TRIS was selected as an appropriate buffer since the data of Mohan and Bates (14) showed calcium does not bind to TRIS over the range 0.01 to 0.1 M.

Statistical Analysis The method of least squares was used to calculate regression parameters of second and first degree polynomials. Tests of significance between regression coefficients were performed using the t-statistic as described by Steel and Torrie (26). Where appropriate, the independent sample or repeated measures t-statistic was applied.

Serum Samples Human blood samples were collected using 10 or 15 ml "Vacutainers" (red top, no additive; Becton-Dickson; Rutherford, N.J.) via the median cubital vein. The blood was allowed to clot at room temperature for 20 minutes, centrifuged at 1000 x g x 10′ and serum removed anaerobically using a disposable syringe and needle. The serum (1 ml) was transferred to a 2.0 ml Vacutainer with the aid of a syringe and either stored on ice until ready for assay or frozen at $-20^{o}C$.

Rat blood was collected using a disposable 3 ml syringe following light ether anesthetization by puncturing the inferior vena cava. The blood was transferred anaerobically into a 3 ml Vacutainer and processed as described for human

serum.

Test of Anaerobic Conditions Individual serum samples were collected anaerobically in 1 ml tuberculin syringes and put on ice. Prior to measuring ionized calcium, pH measurements were made and the remaining sample was processed through the sample chamber and the pH was again measured upon its removal.

Static Ise Electrode Versus the Orion SS-20 Sera of 10 normal volunteers (ages 22 to 42 for males and 21 to 32 for females) were run in triplicate on the Orion SS-20 according to the manufacturer's instructions and also on the apparatus described in this report. The remaining serum was pooled anaerobically and six 1 ml samples withdrawn and placed into 2 ml Vacutainers for each of the following treatment groups. A small volume (up to 20 ul) of $CaCl_2$ or EDTA was added to each Vacutainer.

Application Male COBS/CD rats (Charles River Co.; Wilmington, Mass.) weighing 350±50 grams were group housed and fed food and water ad libitum. Ten and thirty minutes prior to sacrifice, rats were injected subcutaneously (1ml/kg) with saline, $CaCl_2$ or EDTA. Total serum calcium was measured using the O-cresophthalein complexone reaction (Sigma Co.).

RESULTS

Electrode Response Time The resultant effects of incorporating the electrodes into the chamber on electrode response time were evaluated using standards and samples at both 25 and 37°C. The results illustrated in figure 4 were reported as deviations (in mV) from the 5 minute readings since the readings were shown to be stable from 5 to 30 minutes. Shorter response times were obtained with measurements determined at 25°C. Although the data converge on an equilibrium value at 5 minutes, it should be made clear that the mV readings for samples and standards were different at the two temperatures studied.

Based upon the data shown in figure 4, some quantitative differences associated with ionized calcium measured at the two temperatures would be expected. While mV measurements made at 37°C following a delay of 4 minutes deviate from the equilibrium value by approximately 0.11 and 0.17 mV for samples and standards, respectively, they do not significantly differ from each other ($P>0.05$). Since the standard deviations associated with measurement at this time point are small, the 4 minute reading would be expected to give results virtually identical to the equilibrium value. The 25°C measurements made at 1 minute however, while they average close to the equilibrium value, are themselves significantly different from each other ($P<0.05$) with the

standards reading approximately 0.25 mV higher than the samples. To correct for this effect, the difference in mV (0.25) is either added to the observed potential for the sample, or subtracted from the average observed potential for the standards bracketing the sample. This value is approximately 0.02 mM calcium.

Figure 5 illustrates the effect of temperature. The difference between the value obtained at 25°C corrected for equilibrium effects and those at 37°C is 0.04 mM Ca, or approximately 4%, a value consistent with those previously observed in the literature (2, 10).

Activity, Selectivity and Molar Concentrations Ion specific electrodes of the type employed here have been shown to respond in accordance with the extended Nicolsky-Eisenman equation (7).

$$E = E^{o} + S \log ((Ca^{++}) + K_{Ca-i} (i)^{2/Z_i})$$

where:

E = observed potential

E^{o} = sum of potentials originating from the reference electrode, the filling solution of the ion-specific electrode and the liquid junction potential.

Remaining terms as previously defined.

Conformance to this equation was used as a criterion for assessing the performance characteristics of the electrode

system.

Table 3 depicts the results of selectivity ratios evaluated for ions of primarily physiologic interest for each of two lot numbers of calcium sensing modules. The selectivity ratios obtained experimentally, when compared with calculated values presented in table 4, determine which ions significantly contribute to the observed potential through a selectivity effect.

Table 5 shows the regression parameters for calcium standards (0.5 to 1.7 incremented by 0.3 mM) prepared at the ionic strengths indicated. Regression parameters reveal identical slopes only when the activity and selectivity effects are accounted for in the analysis, as predicted by the Nicolsky-Eisenman equation.

Calcium-Albuminate Kinetics The results of this experiment depicted in figure 6A indicate apparently two saturable calcium binding components associated with BSA. The data, analyzed as described by Moore (16) and illustrated in figure 6B, reflects the two saturable sites, each of which may be fitted accurately using linear regression analysis. Replication of the experiment resulted in essentially identical findings concerning the number of saturable components. The average results of three independent experiments gave calculated dissociation constants (pK) for

the high and low concentration ranges (subscripted respectively H + L) and number of binding sites (n) of (mean ± S.D.) pK_H = 1.9±.4, pK_L = 2.9±.1, n_H = 12.7±2.2 and n_L = 1.9±.4. More importantly, after the electrodes had been exposed to solutions containing proteins, the change in the observed potential of the aqueous standards from the beginning to the end of the run was consistent with within-day variation characteristically observed when the electrodes had been used only with pure aqueous standards, suggesting that the electrodes can withstand the presence of proteins.

pH Effects A) Protein-Free Solution- Ionized calcium was determined at various levels of pH using 1.0 mM calcium prepared in 50 mM TRIS at pH 6.7, 7.4 and 7.9 with NaCl adjusted to a resultant ionic strength of 0.16 moles/L. The relationship between ionized calcium and pH was found to be Δ[Ca++] mM = -0.087 ΔpH, a value virtually identical to the relationship offered by Grima and Brand (9). These data suggest small but significant errors are associated with pH changes over the physiological range.

B) Protein Solutions- Studies similar to those reported in the calcium-albuminate kinetics section were performed at pH 5.5 where it was observed that 0.43 mM Ca was bound to albumin at 2.5 mM total calcium. Comparing the value obtained at pH 7.4 of 1.3 mM bound, described in the

preceding section, results in the pH dependent relationship Δ[Ca++] mM = -0.46 ΔpH and adjusting for the protein concentration of 4 g/dl in this study compared with 4.73 g/dl used by Moore, the correlation coefficient becomes -0.39 which is reasonably close to the value of -0.42 reported by Moore (16). The range of values varies from -0.49 to -0.35 (6, 18, 24). It may be concluded that the electrode system is responsive to the change in free calcium associated with varying pH in protein solutions.

C) Test of Anaerobic Conditions- The results (table 6) suggest that a significant increase in pH may result, but the magnitude of the change, i.e. (Δ[Ca++] = -0.39 x ΔpH = -0.39 x (.047) = -.018) is less than the error associated with the precision of the measurement.

Electrode Performance During a 23 day period both serum samples and standards were measured and the day to day and within day variations of the standards were calculated with the results presented in table 7. The data illustrates several important considerations. Electrode stability is fairly good as reflected by a maximum coefficient of variation of 1.5% associated with the 0.5 mM standard. Within day variation is appreciable and warrants the calculation protocol described in the methods section, whereby each sample is bracketed by the standards. The slope does not change appreciably from the beginning to the

end of the run, justifying the use of the average mV slope determined for the day in the calculation of ionized calcium. The relative mV readings for a given sensing module may differ significantly from any other module as previously shown in table 3. In addition, the data used for the preparation of tables 5 and 7 utilized two different sensing modules. The average mV reading for standards given in table 7 may be compared with calculated mV values for the same calibration standards obtained using the regression parameters of table 5, u = .16, [Ca++], 25°C. These values are 30.51, 38.67 and 57.63 mV for 0.5, 1.0 and 5.0 mM calcium respectively.

Static-type Electrode versus the Orion SS-20 The means of triplicate serum sample determinations run on the SS-20 and the static-type electrode system are compared in figure 7. The correlation was considered to be excellent with over 97% of the variation accounted for by regression.

Sample Storage Human serum samples frozen at -20°C for varying periods of time were assayed to determine the effects of storage. Figure 8 illustrates a significant increase in calcium following storage, a result at variance with most reported studies. This result suggests that the effect of storage conditions deserves more thorough characterization. Under the given conditions, unless measurements are made immediately, the duration of freezing

should be identical from one sample to the next and should not exceed two weeks.

Application Results The effects upon the observed serum total and ionized calcium, as a result of the administration of $CaCl_2$ or EDTA, are presented in table 8. Rats administered EDTA showed a profound decrease in both total and ionized serum calcium whereas a significant increase in serum ionized calcium was observed for $CaCl_2$ treated aminals despite a small but insignificant increase in their total serum calcium. The results confirm the in-vitro effects of $CaCl_2$ and EDTA and support the view that the system described here can easily resolve 10% changes in serum ionized calcium.

DISCUSSION

Several criteria must be met before calcium ion-specific electrodes may be used to determine serum ionized calcium. These criteria are associated with either the physical conditions under which measurements are made, the performance of the electrode or the composition of the sample. Since the static-type electrodes have not been recommended for the determination of ionized calcium in serum, this report focuses on the various criteria and demonstrates how each have been satisfied in terms of the experimental results.

Concerning the physical conditions surrounding the measurement itself, the determination should be made anaerobically on a sample of fixed volume at constant temperature. Any instrument designed to measure serum ionized calcium should be capable of retaining the original pH of the sample following the measurement, since alterations in pH can substantially alter the measurement. The ISE sampling chamber provides for quasi-anaerobic sample processing, as shown by the pH data provided.

Arnold et al. (2) demonstrated the dependence of the measurement upon sample volume, which is fixed in the apparatus described here at 0.6 ml. This value is not considerably different from the volume of sample required

for use of the SS-20.

The ideal conditions for determining serum ionized calcium are those which most closely reflect physiologic conditions. A variety of temperatures have been used including 20°C (2), 25°C (12), 26°C (19), 27°C (22) and 37°C (5, 6, 8, 13, 18). Although we have demonstrated the capability of operating the instrument at 37°C, 25°C was selected since the shorter equilibration time allows for a considerably greater number of samples to be processed per day. The difference of 4% lower values at the higher temperature does not justify its use especially in view of the slightly greater degree of variability associated with measurements made at the elevated temperature.

The static-type electrode is normally placed into a sample which is continuously stirred. The electrode chamber does not allow for continuous stirring and thereby may result in a prolongation of the time required to achieve equilibrium at the sample electrode interface. In addition, since the sample compartment volume is fixed, the flow rate of the reference electrode filling solution may be impeded and as a result contribute to unstable readings. Although response times were prolonged compared with the data provided by the manufacturer, the readings were stable.

Early models of the static-type electrode were

reportedly poisoned by proteins rendering the electrode unsuitable for use in the determination of serum ionized calcium (16). Later models were shown to exhibit a prolongation of their response times and prompted the practice of incorporating triethanolamine and trypsin into the standards in an attempt to remove the residual proteins from the membrane. The use of these organics has become a subject of controversy (20). The study of the kinetics of calcium binding to albumin was viewed as a viable experimental approach to assess the value of the static-type electrode considering the intended application. Temperature comparisons adjusted for response times and calcium albuminate kinetics reflect the refractiveness of the membrane to any potential poisoning effects of the proteins. Response times are not altered appreciably by the presence of proteins. Ca-albuminate studies did not result in an extraordinary effect in the mV readings when the observed potential of standards taken prior to sample assay were compared with values following exposure of the membrane to proteins. Pedersen (17) provided evidence to suggest that 12 of the 16 histidine residues serve as the binding sites for calcium to human albumin, a number not far from the experimentally determined value reported here for the high concentration range (2 - 5 mM) of total calcium. The arithmetic means of the pK's at 2.4 and n's at 7.3 compare reasonably well with those obtained by Moore (16), pK = 2.2 and n = 8.4, who reported only one saturable component.

An electrode should be capable of withstanding changes in pH over the range of physiological interest. The electrode employed here is not entirely independent of pH effects but the magnitude of the change in potential as a function of pH is not sufficiently large to warrant further consideration. The pH dependence data of calcium albuminate kinetics is in agreement with values reported by others.

ISEs measure ion activity and not the molar concentration of the ion itself. Their use in the measurement of serum ionized calcium is justified by the view that standards prepared at the same ionic strength as samples results in identical activity coefficients for the ions of interest. This is not entirely true, however, since it has been shown that the activity coefficient of sodium varies at constant ionic strength and varying mole fraction in a solution containing sodium and calcium. Similarly, the activity coefficient of calcium varies in proportion to its mole fraction at constant ionic strength (15). These effects have generally been ignored and to consider them here would require considerable deviations from the traditional use of the calcium ISE. However, it is noteworthy in terms of obtaining an appreciation for the limitations of this method.

The ionic strength of standards is usually near that of serum which is considered to be approximately 0.16 moles/L

with the principal contributors being Na and Cl. The composition of standards employed for the determination of serum ionized calcium varies from a sodium concentration of 140 to 160 mM (2, 23). Some investigators employ physiological concentrations of K and Mg while others include organics (TRIS or trypsin and triethanolamine).

The effects of changes in ionic strength on the observed potential is demonstrated in table 9. These calculations are in close agreement with those of Moore (16). Assuming the electrode is specific for calcium, i.e. selectivity ratios for potentially interfering ions are small, the magnitude of error both in mV and apparent ionized calcium is shown for a 10 and a 70 mM sodium change. The implications of the values reported in this table are (1) assuming ionized calcium concentrations are closely regulated, the values reflect the degree of variability that would be expected by a comparison of studies in the literature which employ standards of differing ionic strength; (2) assuming sodium and not calcium concentrations of serum samples vary considerably, the values reflect the errors associated with an apparent change in the concentration of ionized calcium. Similarly, the errors are calculated for the combined effect of changing ionic strength on the activity of the ion plus the selectivity component. Selectivity effects tend to offset the reduction in apparent ionized calcium brought about by

changing ionic strengths. The objective of the measurement is to determine the molar concentration of ionized calcium and not the activity, therefore, a significant selectivity component actually serves to minimize the error brought about by changes in the concentration(s) of interfering electrolyte(s).

SUMMARY

The method described here may be used for the determination of serum ionized calcium. The physical conditions of the measurement, including sample volume (0.6 ml) and temperature (25 or 37°C), are fixed. Stasis of the sample may contribute to a prolonged but not prohibitive response time. Other than the prolonged response time, the performance characteristics of the electrodes were not altered by incorporating them into the electrode chamber. Exemplifying the utility of this method were the results of protein binding studies, in-vitro and ex-vivo $CaCl_2$ and EDTA administration and a comparison with the Orion SS-20. In consideration of the data presented, the cost and simplicity of construction, the method described should serve to make the determination of serum ionized calcium accessible to a greater number of investigators and encourage research in pharmacological and physiological processes thought to be mediated by serum ionized calcium.

ACKNOWLEDGEMENT

Special thanks to Horace Martin, M.D., Ph.D., Stuart Wolstenholme and Joanne Holossitas for providing access to the Orion SS-20 for this study.

REFERENCES

1. Ammann, D., Gussi, M., Pretsch, E. and Simon, W.: Improved calcium ion-selective electrode based on a neutral carrier.
Anal. Letters 8: 709-720, 1975.

2. Arnold, D. E., Stansell, M. J. and Malvin, H. H.: Measurement of ionic calcium using a specific ion electrode.
Amer. J. Clin. Pathol. 49: 627-634, 1968.

3. Bates, R. G. and Alfenaar, M.: Activity standards for ion-selective electrodes. In Ion-Selective Electrodes, R. A. Durst (ed.)
Nat'l. Bur. Stds., Spec. Publ. 314, Ch 6: 191-214, 1969.

4. Brand, M. J. D. and Scott, W. J.: Electrolyte activities in human blood plasma In Proceedings of a Workshop on pH and Blood Gases
Nat'l. Bur. Stds., Spec Publ 450, 301-310, 1977.

5. Chan, G. M., Ash, K.O., Heutschel, W. and Wu, J.: Effects of bilirubin on ionized calcium.
Clin. Chem. 27: 204-205, 1981.

6. Drop, L. J., Fuchs, C.and Stulz, P. M.: Determination of blood ionized calcium in a large segment of the normal adult population.
Clin. Chim. Acta 89: 503-510, 1978.

7. Eisenman, G.: The electrochemistry of cation-sensitive

glass electrodes In Advances in Analytical Chemistry and Instrumentation, C. N. Reilley (ed.), John Wiley and Sons, Inc., N.Y., Ch 4: 213-233, 1965.

8. Fuchs, C., Dorn, D., McIntosh, C., Scheler, F. and Kraft, B.: Comparative calcium ion determinations in plasma and hole blood with a new calcium ion analyzer.
Clin. Chim. Acta 62: 99-102, 1976.

9. Grima, J. M. and Brand, M. J. D.: Activity and interference effects in measurement of ionized calcium with ion-selective electrodes.
Clin. Chem. 23: 2048-2054, 1977.

10. Hansen, S. O. and Theodorsen, L.: The usefulness of an improved calcium electrode in the measurement of ionized calcium in serum.
Clin. Chim. Acta 31: 119-122, 1971.

11. Kielland, J.: Individual activity coefficients of ions in aqueous solutions.
J. Amer. Chem. Soc. 59: 1675-1678, 1937.

12. Ladenson, J. H. and Bowers, Jr., S. N.: Free calcium in serum I. Determination with the ion-selective electrode and factors influencing the results.
Clin. Chem. 19: 565-574, 1973.

13. Madsen, S. and Olgard, K.: Evaluation of a new automatic calcium ion analyzer.
Clin. Chem. 23: 690-694, 1977.

14. Mohan, M. S. and Bates, R. G.: Calibration of ion-selective electrodes for use in biological fluids.
Clin. Chem. 21: 864-872, 1975.

15. Moore, E. W.: Hydrogen and cation analysis in biological fluids in-vitro. In Glass Electrodes for Hydrogen and Other Cations
G. Eisenman (ed.) Marcel Dekker, Inc., New York
Ch 15: 412-441, 1967.

16. Moore, E. W.: Studies with ion-exchange calcium electrodes in biological fluids: Some applications in biomedical research and clinical medicine. In Ion-Selective Electrodes,
R. A. Durst (ed.) Nat'l. Bur. Stds.,
Spec. Publ. 314, Ch 2: 215-285, 1969.

17. Pedersen, K. O.: Binding of calcium to serum albumin II. Effect of pH via competitive hydrogen and cation binding to the imidazole groups of albumin.
Scand. J. Clin. Lab. Invest. 29: 75-83, 1972.

18. Pedersen, K. O.: An analysis of measured and calculated calcium quantities in serum.
Scand. J. Clin. Lab. Invest. 38: 659-667, 1978.

19. Raman, A.: Determination of ionized calcium in serum with a calcium electrode.
Biochem. Med. 3: 369-375, 1970.

20. Robertson, W. G.: Measurement of ionized calcium in body fluids - A review.

Ann. Clin. Biochem. 13: 540-548, 1976.

21. Ross, J. W.: Calcium selective electrodes with liquid ion exchanger.
Science 156: 1378-1380, 1967.

22. Schwartz, H. D., McConville, B. C. and Christopherson, E. F.: Serum ionized calcium by specific ion electrode.
Clin. Chim. Acta 31: 97-107, 1971.

23. Schwartz, H. D.: Serum ionized calcium by electrodes: New technology and methodology.
Clin. Chim. Acta 64: 227-239, 1975.

24. Seamonds, B., Towfighi, J. and Arvan, D. A.: Determination of ionized calcium in serum by use of an ion-specific electrode.
Clin. Chem. 18: 155-160, 1972.

25. Srinivasan, K. and Rechnitz, G. A.: Selectivity studies on liquid membrane ion-selective electrodes.
Anal. Chem. 41: 1203-1208, 1969.

26. Steel, R.G.D. and Torrie, J. H.: Principles and Procedures of Statistics
McGraw-Hill, N.Y. Ch 9: 161-182, 1960.

Table 1. Critical Dimensions and Descriptions of Plastic (Plexiglas(R)) Serum Sampling Chamber

Qty.	Item	Size	Description
UPPER ASSEMBLY			
2	sides	2"x2.5"x.25"	with 1/8" MIPT male adapter fitted in one corner
1	top	2"x2.5"x.25"	with two 5/8" holes drilled
2	front & back	2"x2.5"x.25"	
1	bottom	2"x2.5"x.5"	with two 5/8" holes drilled
LOWER ASSEMBLY			
2	sides	2"x1"x.25"	with 1/8" MIPT male adapter fitted in one corner
2	front & back	2"x1"x.25"	
2	top & bottom	2"x2.5"x.25"	

Table 2. Comparison of Various Conventions Used for the Determination of Single Ion Activity Coefficients at 25°C

Ion	Ionic Strength	pH[a]	MacInnes[a]	Extended Debye-Huckel[b]	Davies[c]	Bates[d]	x±2S.D.	PR-K[e]
Ca	.001	.8721	---	.8697	.8655	.8684	.8689±.0055	.8688
	.01	.6677	---	.6758	.6525	.6689	.6662±.0196	.6773
	.09	---	---	.4129	.3185	.3983	.3766±.1016	.4123
	.10	.3895	.3959	.3798	.3025	.3864	.3686±.0885	.3976
	.125	---	---	.3761	.2696	.3623	.3360±.1158	.3658
	.16	---	---	.3501	.2346	.3375	.3074±.1267	.3296
Na	.001	.9661	---	.9651	.9640	.9651	.9650±.0018	.9663
	.01	.9036	---	.9022	.8927	.9025	.9003±.0101	.9018
	.09	---	---	.7803	.7170	.7862	.7612±.0767	.7828
	.10	.7798	.8709	.7731	.7042	.7797	.7815±.1185	.7757
	.125	---	---	.7575	.6754	.7660	.7330±.1001	.7602
	.16	---	---	.7400	.6406	.7510	.7105±.1216	.7424

a Bates & Alfenaar (3)
b. Grima & Brand (9)
c Brand & Scott (4)
d Ammann et al. (1)
e Polynomial Regression applied to the transformed data of Kielland (11)
where:

$$\text{activity coefficient} = \frac{b0}{} + \frac{b1}{} \log u + \frac{b2}{} (\log u)^2$$

	b0	b1	b2
Ca	.0296	-.4121	-.0441
Na	.5880	-.2185	-.0308
Mg	.1263	-.3512	-.0346
K	.5281	-.2602	-.0380

Table 3. Selectivity Ratios Determined for Ions of Physiologic Interest

i	[i][a]	u	Gi[b]	E(LS-1)[c]	Kca-i(LS-1)[d]	E(LQ-1)	Kca-i(LQ-1)
Ca	0.1	0.3	.2330	91.71	---	90.3	---
Na	0.1	0.1	.7757	0.51	2.71x10	-11.3	1.13x10
K	0.1	0.1	.7503	-0.32	2.7 x10	-8.2	1.55x10
Mg	0.1	0.3	.3007	-1.84	4.5 x10	-7.3	3.1 x10

a. [i] = molar concentration of ion, i.

b. Gi = activity coefficient of ion, i.

c. E = observed potential using the sensing module indicated in parentheses.

d. Kca-i = selectivity ratio associated with calcium ion-specific electrode sensing module for ion, i.

TABLE 4. Errors Associated with Calculated[a] Values of Selectivity Ratios

	Kca-i	
I	u=.09	u=.16
Na	7.32x10	2.09x10
K	2.38x10	2.19x10
Mg	7.88x10	7.88x10

a. Yielding errors in apparent calcium in excess of 0.1 mM using the Nicolsky-Eisenman equation.

TABLE 5. Linear Regression Parameters for Standards at Varing Ionic Strengths

Comparing Molar Concentration, Molar Activity and Molar Activity + Selectivity

Variable[a]	u (25°C)	Slope	Intercept	r^2	S.E. of estimate	HO: Su1 = Su2[b]	
		mV	mV		mV		
	.09	29.07	126.08	.9964	.1469	N.S.	
[Ca++]	.125	28.08	123.227	.9959	.1582		P<.001
	.16	27.12	120.034	.9989	.0736	N.S.	
	.09	29.075	137.903	.9964	.1481	N.S.	
(Ca++)	.125	28.08	135.498	.9959	.1530		P<.001
	.16	27.098	133.030	.9988	.0794	P<.05	
	.09	30.102	140.939	.9964	.1490	N.S.	
(Ca++ +	.125	30.023	141.267	.9960	.1504		N.S.
Kca-na Na^2)	.16	30.478	143.237	.9990	.0711	N.S.	
		30.201*	141.814*				
	u (37°C)						
[Ca++]	.09	35.025	151.858	.9911	.2816	P<.001	
	.16	29.967	136.144	.9811	.3234		
(Ca++ +	.09	36.26	168.990	.9911	.2813	N.S.	
Kca-na Na^2)	.16	34.71	165.37	.9805	.3674		

* arithmetic mean

a. brackets represent molar concentration of the ion, parentheses represent activity of the ion in moles/L.

b. the null hypothesis: slope at one ionic strength is equal to that at another ionic strength.

Table 6. Effect of Sample Processing on pH

	serum pH before	[Ca++] mM	serum pH after
	7.369	1.030	7.446
	7.362	1.112	7.415
	7.362	1.127	7.422
	7.365	1.127	7.399
	7.385	1.135	7.398
	7.372	1.135	7.415
mean	7.369	1.011	7.416
S.D.	±.009	±.041	±.018
C.V.	.12%	4.0%	.24%

Table 7. Electrode Performance Characteristics

	Variation (mean±S.D.) n = 23	
Ca standards	Within Day	Between Days
mM	mV	Rel. mV
0.5	1.40±0.65	35.61±1.41
1.0	1.14±0.59	44.09±1.44
5.0	1.14±0.50	63.80±1.21
slope	0.45±0.27	28.19±0.94

Table 8. The Effect of Subcutaneous Administration of $CaCl_2$ or EDTA upon Total and Ionized Serum Calcium in Rats

			Percent of Control ± S.D.			
Time(min)	Treatment[a]	n	[Ca++][b]	P	[Ca][c]	P
	Control[d]	8	100.0±2.2		100.0±4.6	
10	EDTA-100	8	78.5±3.5	<.001	81.5±2.2	<.001
		5	71.5±9.4	<.001	78.6±6.9	<.001
30	CaCl -250	4	111.4±11.5	<.020	102.1±5.2	N.S.
	350	6	108.1±3.6	<.001	104.2±4.1	N.S.
	500	4	113.6±8.4	<.010	111.6±7.5	N.S.

a. The numbers shown are doses of $CaCl_2$ and EDTA in mg/kg.

b. Serum ionized calcium.

c. Total serum calcium.

d. Saline injected controls for 10 and 30 minutes were not significantly different from each other. The pooled control values were [Ca++] = 1.149±.025, [Ca] = 2.418±.112 mM.

Table 9. Estimates of the Magnitude of Error Associated with Either Activity (a) Effect Alone or Combined Activity Plus Selectivity Effects (a+k)

Effect	Condition	ΔmV^a	$\Delta[Ca^{++}]^b$
(a+k)	135-145mM Na	-0.220	-0.019
(a)	135-145mM Na	-0.390	-0.033
(a+k)	90-160mM Na	-1.90	-0.149
(a)	90-160mM Na	-2.94	-0.221

a. for each condition $E = E^o + S \log(Ca^{++} + K_{ca-na} Na^{+2})$ is calculated and the difference tabulated as ΔmV
$E^o = 141.814$ $S = 30.201$ means of values taken from table 5, 25°C

b. for each effect $[Ca^{++}] = \text{anti-log}((E-E^o+\Delta mV)/S))$ and the deviation from 1.0mM tabulated as $\Delta[Ca^{++}]$
$E^o = 120.034$ $S = 27.12$ values taken from table 5.
E for 1.0mM $[Ca^{++}]$ calculated to be 38.674

EIGURE 1

TITLE: Pictorial representation of the calcium ion-specific electrode chamber

EIGURE 2

TITLE: Pictorial representation of the bottom plate of the electrode chamber

EIGURE 3 Photograph of the experimental apparatus assembled for the determination of serum ionized calcium

EIGURE 4

TITLE: Response times of the electrode system incorporated into the electrode chamber

LEGEND

Each point illustrated is the mean of 6-8 determinations with the standard deviation depicted.

EIGURE 5

TITLE: The effect of sample processing temperature on serum ionized calcium.

LEGEND

Each point illustrated is the mean of 10 individual determinations with the standard deviation depicted.

FIGURE 6

TITLE: Calcium binding to bovine serum albumin

LEGEND

A) Each point reflects the calculated result of sequential additions of aliquots of a $CaCl_2$ stock solution with readings taken at 25°C, 1 minute following each addition.

B) The data of figure A replotted to give an estimate of the parameters of calcium binding to bovine serum albumin.

FIGURE 7

TITLE: Correlation between ionized calcium measured on the Orion SS-20 with that determined using the static-type electrode

LEGEND

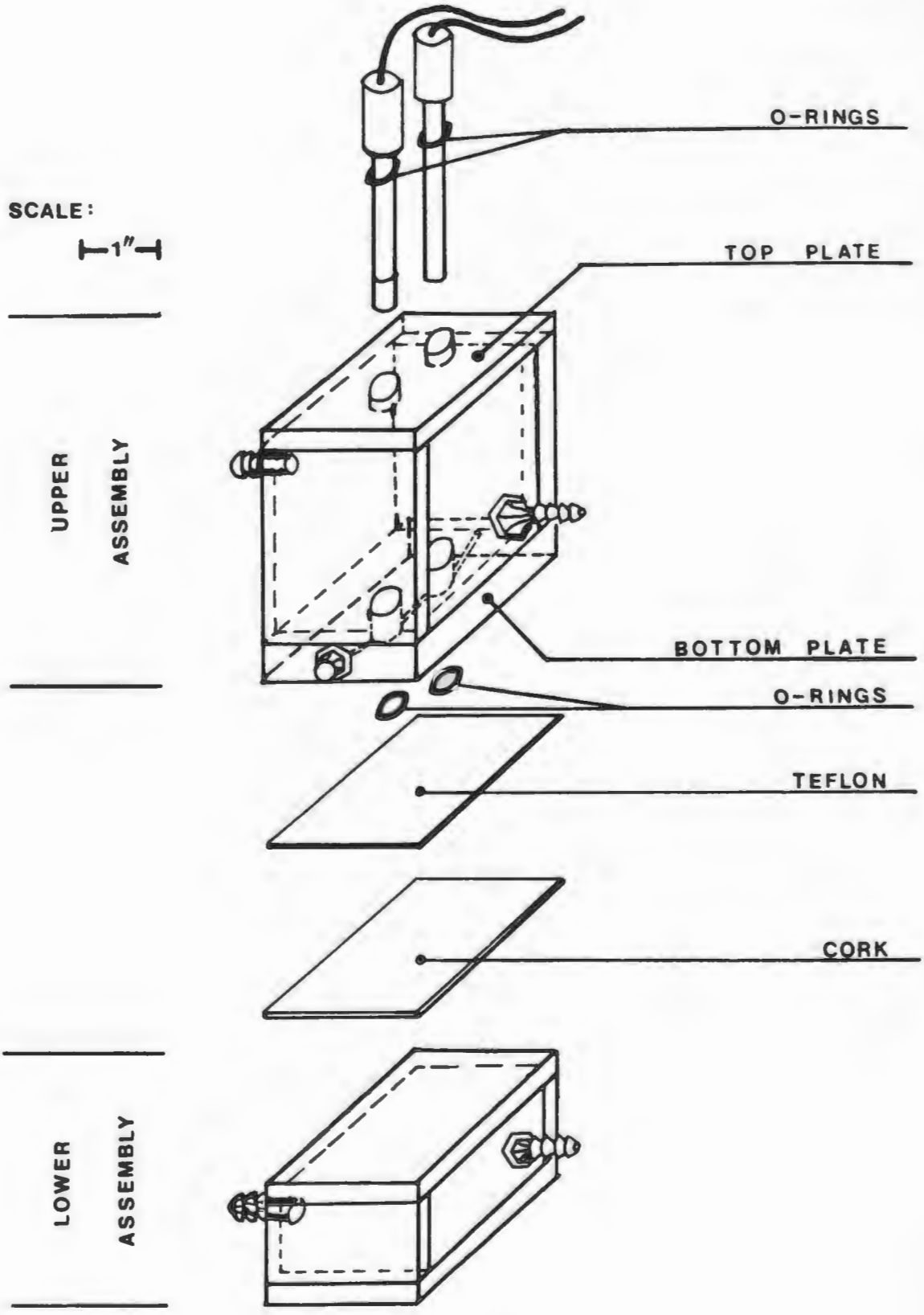
Open circles denotes serum samples to which either EDTA or $CaCl_2$ have been added. Closed circles depict values obtained using normal serum samples. Each point illustrated is the mean of triplicate determinations.

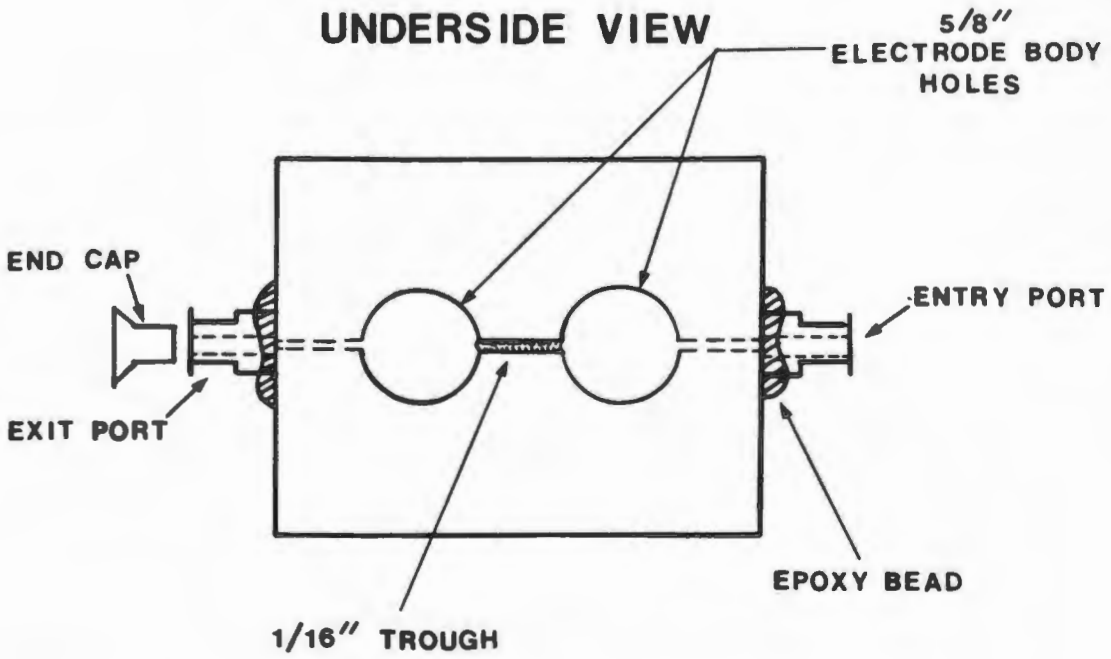
FIGURE 8

TITLE: Effect of the duration of serum sample storage at -20°C

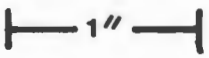
LEGEND

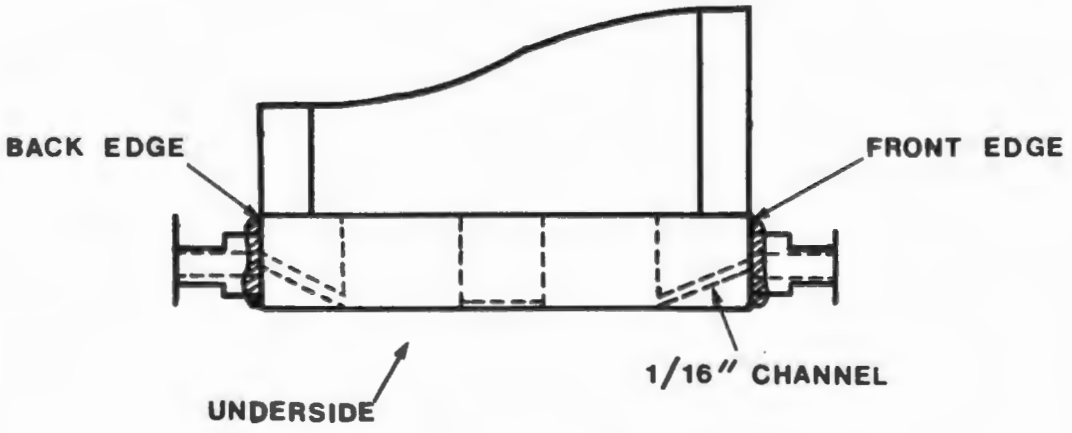
Each value illustrated is the mean of triplicate determinations with the standard deviation depicted.


SCALE:
1"
O-RINGS
TOP PLATE
UPPER ASSEMBLY
BOTTOM PLATE
O-RINGS
TEFLON
CORK
LOWER ASSEMBLY

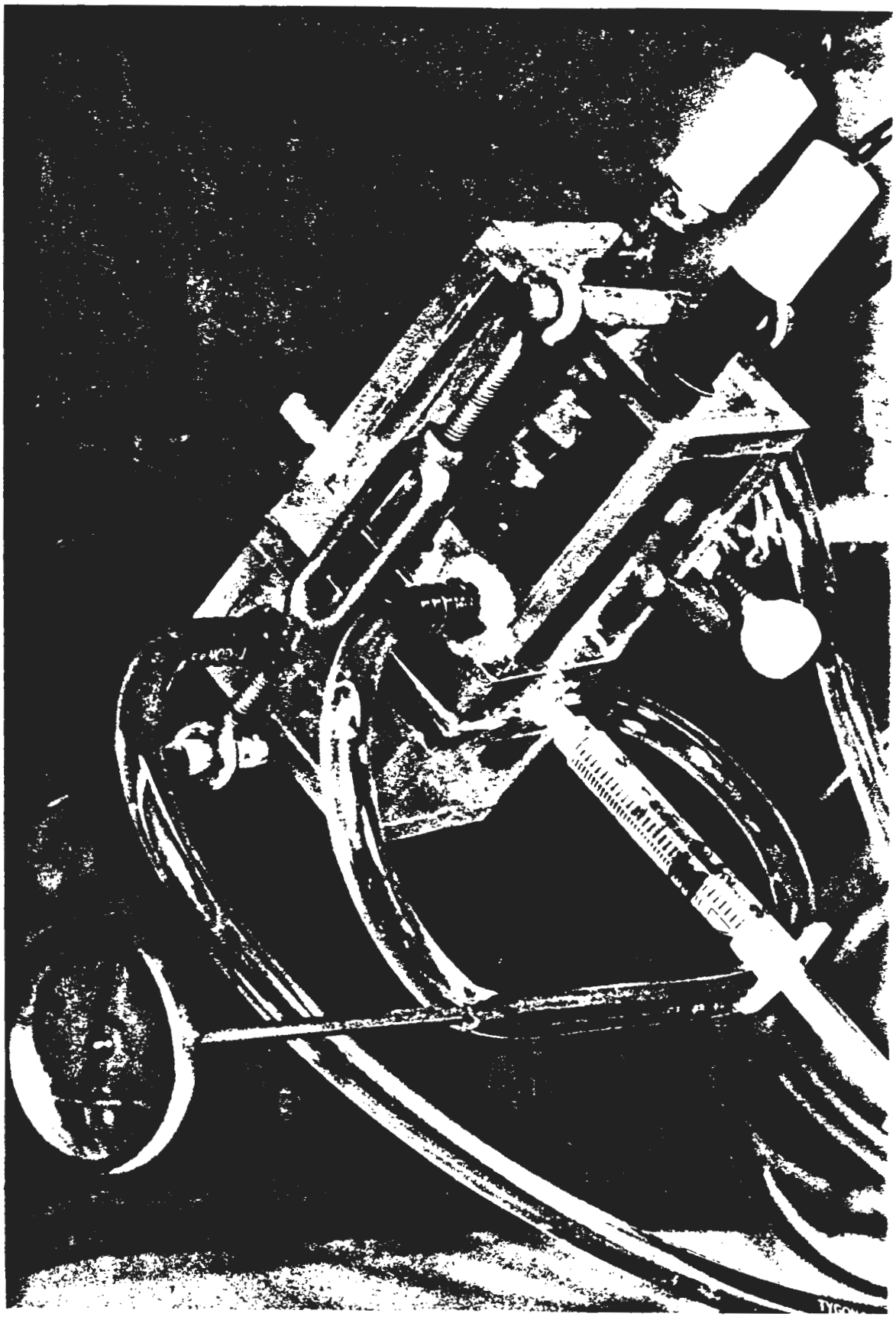


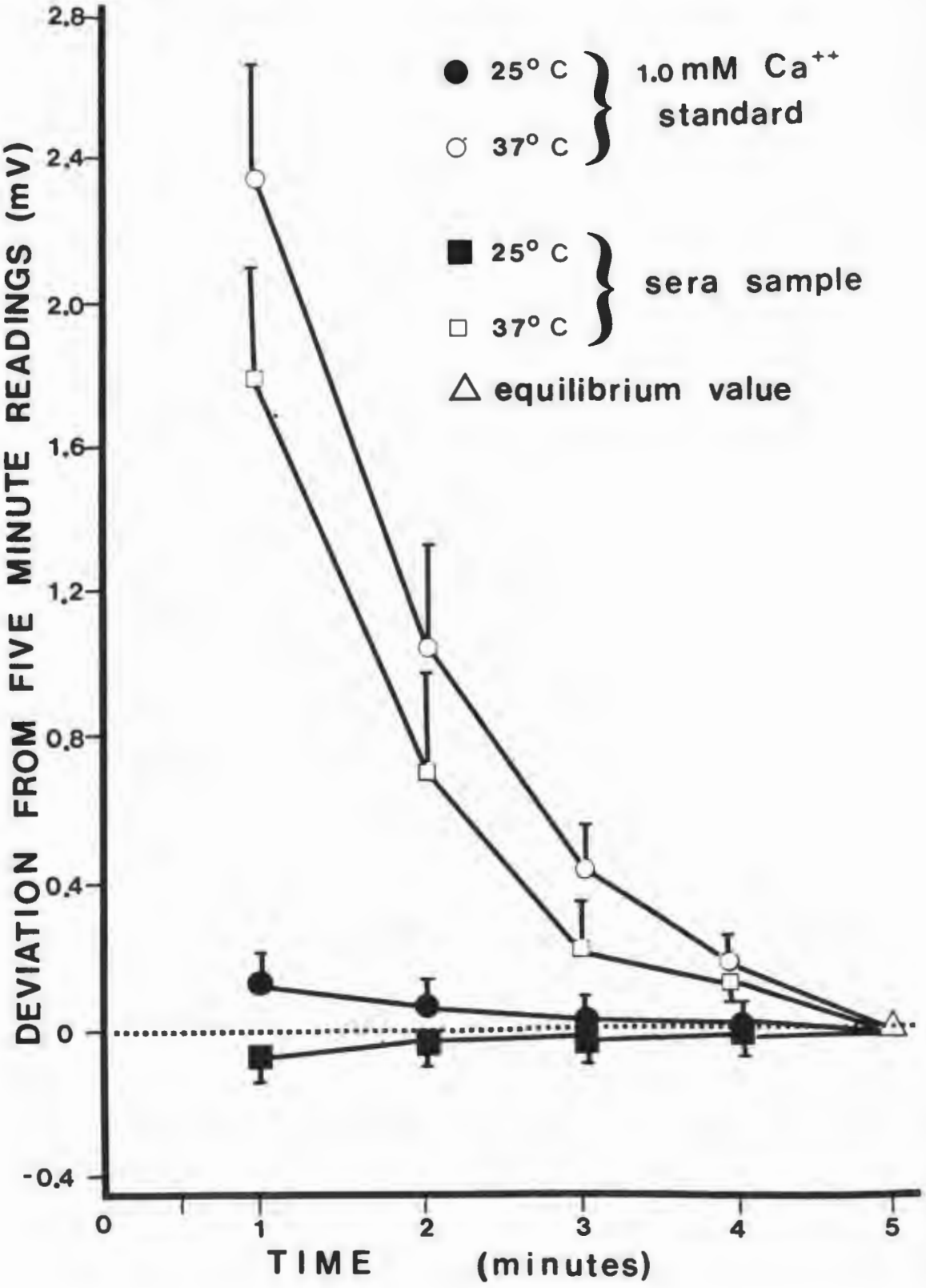
SCALE:

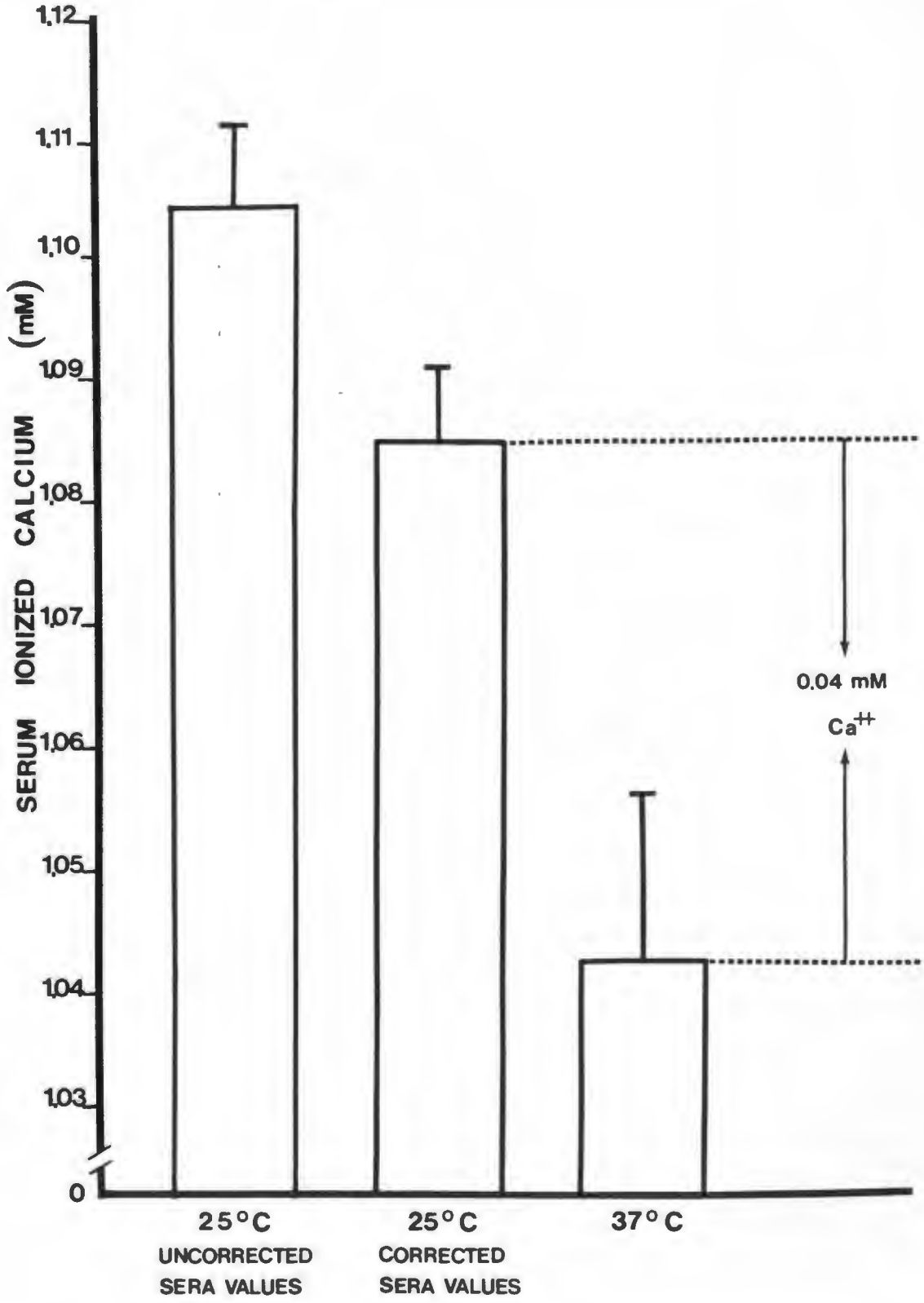




SIDE VIEW

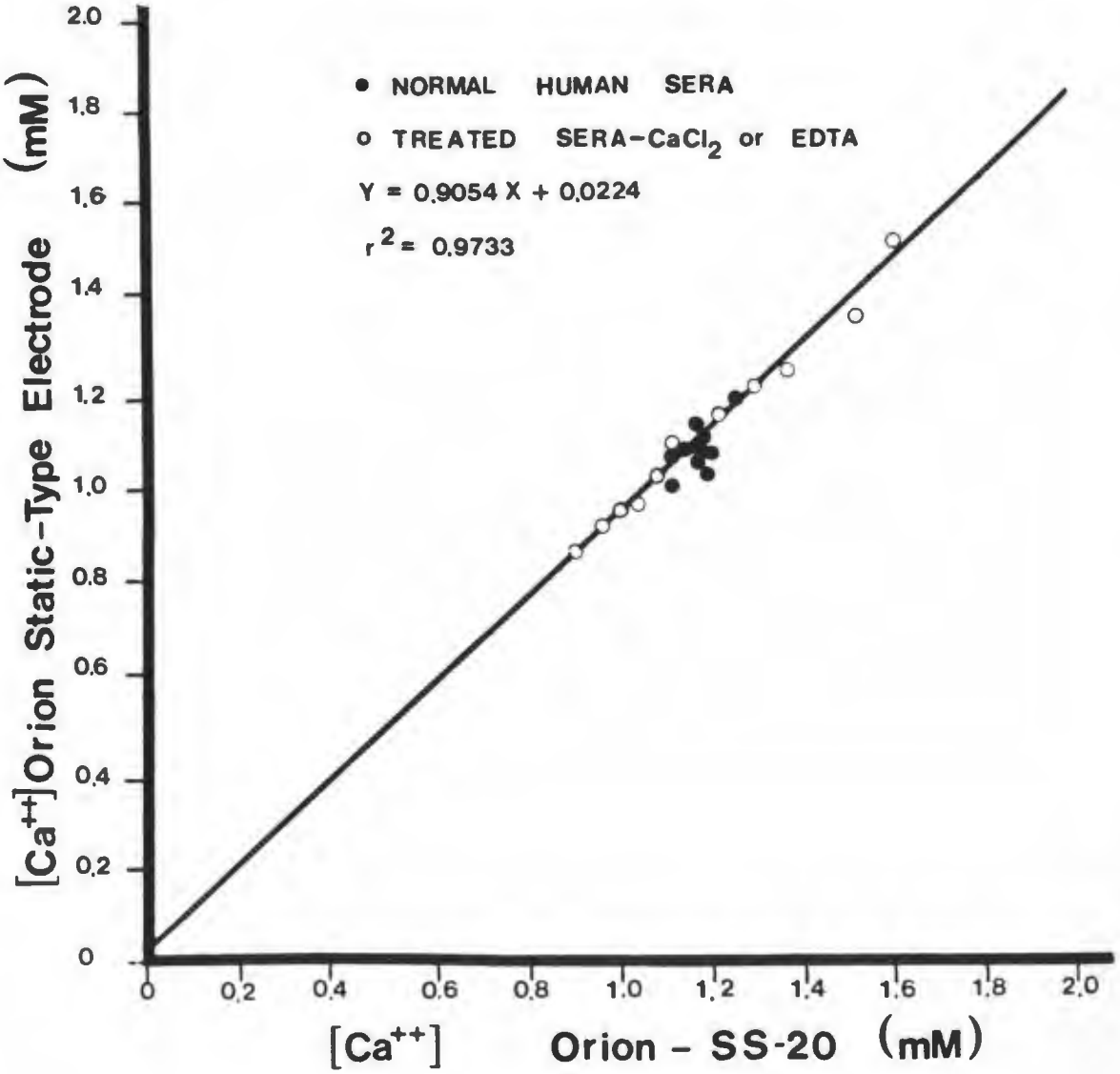


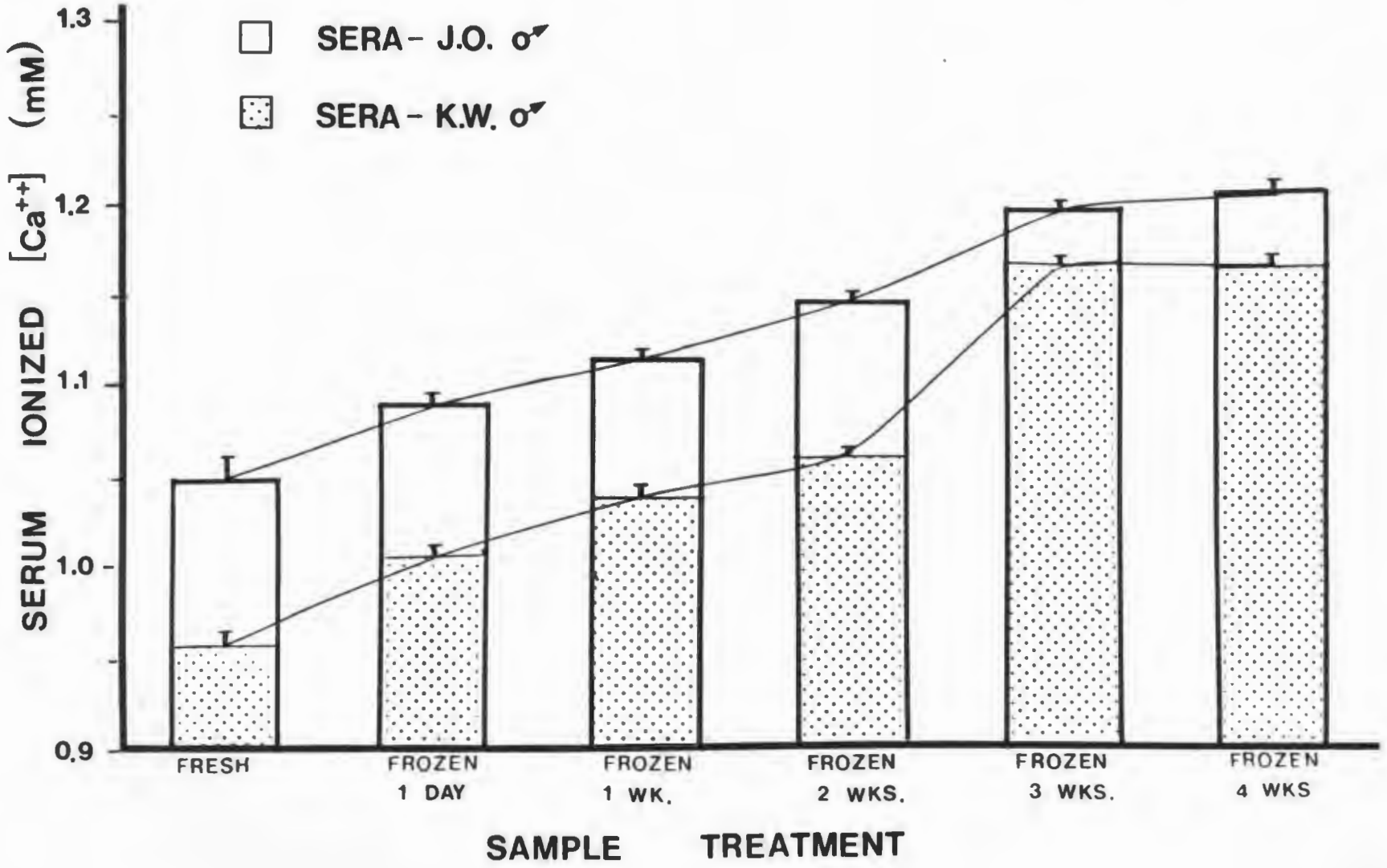

2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
-0.4
DEVIATION FROM FIVE MINUTE READINGS (mV)
25° C
37° C
1.0 mM Ca++ standard
25° C
37° C
sera sample
equilibrium value
0
1
2
3
4
5
TIME (minutes)


1.12
1.11
1.10
1.09
1.08
1.07
1.06
1.05
1.04
1.03
0
SERUM IONIZED CALCIUM (mM)
0.04 mM
Ca++
25°C
UNCORRECTED
SERA VALUES
25°C
CORRECTED
SERA VALUES
37°C

Calcium Bound (moles)/ Albumin (moles)
3.0
2.0
1.0
0
1.0
2.0
3.0
4.0
5.0
Total Ca++ M x 10−3

[Alb]/[Ca−Alb] M
2.5
2.0
1.0
pkl = 3.07
nl = 2.2
r2 = .9842
pkh = 1.93
nh = 15.1
r2 = .9875
0
6
12
18
24
30
36
42
48
1/[Ca++] M x10−2


• NORMAL HUMAN SERA
o TREATED SERA-CaCl2 or EDTA
Y = 0.9054 X + 0.0224
r2 = 0.9733
[Ca++]Orion Static-Type Electrode (mM)
[Ca++] Orion - SS-20 (mM)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0


SERA - J.O. ♂
SERA - K.W. ♂
SERUM IONIZED [Ca++] (mM)
1.3
1.2
1.1
1.0
0.9
FRESH
FROZEN 1 DAY
FROZEN 1 WK.
FROZEN 2 WKS.
FROZEN 3 WKS.
FROZEN 4 WKS
SAMPLE TREATMENT

MECHANISMS OF ANTIDEPRESSANT ACTION:
UPTAKE BLOCKADE OR SERUM IONIZED CALCIUM

INTRODUCTION

The most popular view concerning the mechanism of tricyclic antidepressant action mediating the clinical response remains equivocal in view of accumulating data. The biogenic amine hypothesis of affective disorders is based upon clinical and experimental findings and has been extensively reviewed (1,14,40,45). Briefly, the hypothesis states that depression may come about as a result of a "functional depletion" of one or more neurotransmitters in the brain. Functional depletion means there is too little neurotransmitter present or the neurotransmitter is incapable of being released. Alternatively, there may be a post-synaptic receptor deficit resulting in a loss of efficacy of released amines. Serotonin (44), dopamine (27) and acetylcholine (21) have all been implicated but norepinephrine has been, by far, the most extensively studied neurotransmitter (39).

The neurotransmitters mentioned, with the exception of acetylcholine, are believed to be inactivated within the synapse partly by a pre-synaptic, high-affinity, transport mechanism which serves to conserve the neurotransmitter by accumulating it back into the nerve ending from which it was released (19). Tricyclic antidepressants have been reported to be effective in blocking the neurotransmitter re-uptake mechanism resulting in an accumulation of these amines

within the synapse. Uptake blockade has been viewed as a mechanism whereby tricyclics may reverse the functional depletion of neurotransmitters and consequently elicit a therapeutic response.

One of the problems associated with the uptake blockade mechanism of therapeutic action proposed for the tricyclic antidepressants is the apparent lack of temporal correlation between the onset of the therapeutic effect and that of the experimentally observable biochemical effect. Tricyclics must be administered for two to three weeks before any therapeutic effect becomes evident (32). Uptake blockade of serotonin and norepinephrine into brain slices or a crude preparation of pinched-off nerve endings (synaptosomes) may be demonstrated in-vitro (7,35,38) and ex-vivo following acute (36,42) or chronic (2) administration to rats.

The most serious criticism of the uptake blockade mechanism of action of tricyclics has not been thoroughly challenged. Whereas uptake blockade may be demonstrated following acute or chronic dosing of antidepressants, a comparison of the magnitude and duration of this effect has not been made. Criticisms have therefor been based upon qualitative evidence. Cummulative dosing may be expected to result in effects which are either more prolonged and/or greater in magnitude than that elicited following acute drug administration in which steady-state blood levels have not

yet been achieved. The first hypothesis addressed in this study is that chronic protriptyline (PRO) administration results in a greater magnitude and/or duration of uptake blockade activity compared with that elicited by acute administration of the drug. Experimental support for this hypothesis would reaffirm uptake blockade as the explanation for the biochemical mechanism by which tricyclics elicit their therapeutic effects.

Several lines of investigation converge to suggest an alternative view of antidepressant activity to that of uptake blockade and features a role for calcium ions. Calcium metabolism in affective disorders has been studied with equivocal results. Courault et al. (6) reported elevations in both serum total and ionized calcium correlated with clinical recovery among depressives treated with either a tricyclic antidepressant or a monoamine oxidase inhibitor. Frizel and co-workers (13) failed to establish significant increases in ionized calcium concomitant with pharmacotherapy but their data reflect a trend towards decreases in this response measure during depression and increases following recovery. Other studies of calcium metabolism in depression did not employ serum ionized calcium as the response measure but rather total serum calcium, cerebrospinal fluid calcium or mineral balance methodologies were utilized. Here too, the results are less than clear with some reporting trends towards

increases in calcium following recovery from depression (9-12,17,18,31) and others reporting the reverse effect (4,20) or no effect (28). Clark et al. (5) reviewed the evidence for linking affective disorders with hypo-parathyroidism.

Thyroid hormone status has been associated with affective state as well (15). Stimulation of the hypothalamic-pituitary-thyroid axis is acutely but transiently effective in depression (8). Prange et al. (34) reported a reduction in the latency of the therapeutic response of tricyclics with concomitant thyroid hormone administration. The antidepressant activity of thyroid hormone and its relationship to calcium may be assumed by the observation that 47% of hyperthyroid individuals were shown to possess elevated levels of serum ionized calcium (3). In addition, there is evidence suggestive of a functional relationship between cells of the thyroid gland elaborating thyrocalcitonin and those which secrete thyroid hormone (28).

Biochemical observations reflecting the importance of calcium in neuronal functioning suggest that a calcium hypothesis of antidepressant activity may be complementary to and thereby extend the biogenic amine hypothesis of affective disorders. Calcium is required for the depolarization-induced release of neurotransmitters (33,37)

and perhaps the synthesis of norepinephrine as well (30). There is some evidence that the spontaneous release of neurotransmitter, usually viewed as calcium-independent, may be dependent to some extent on calcium (22).

The clinical and biochemical observations presented appear to justify the formulation of a calcium hypothesis of antidepressant activity. This report concerns the tests of three such hypotheses. The first is that chronic but not acute administration of the tricyclic antidepressant, protriptyline (PRO), results in an increase in serum ionized calcium. The second is that the thyroid hormone, triiodothyronine, results in an acute but transient elevation of serum ionized calcium. Lastly, combined thyroid hormone and tricyclic antidepressant administration results in an acute and sustained increase in serum ionized calcium. Evidence supporting any or all of these hypotheses would suggest that calcium may play an important role in mediating the observed clinical response.

MATERIALS AND METHODS

Assays Serum sodium and potassium determinations were performed by flame photometry (IL model 143-02). Glucose and blood urea nitrogen (BUN) were assayed with the Ministat-S Chemistry Analyzer (Biokinetix Corp., Mass.). Creatinine was measured by the Jaffe rate method using a Beckman Creatinine Analyzer 2. A Beckman Chloride/CO_2 Analyzer was used for their determinations. Inorganic phosphate, creatine phosphokinase (CPK), alkaline phosphatase and total serum calcium were all measured colorimetrically using kits provided by the Sigma Co. (St. Louis, Mo.). Serum pH, pCO_2 and pO_2 were analyzed using the IL model 413 Blood Gas Analyzer. Hydroxyproline was determined by the method of Kivirikko et al. (23). Magnesium was determined by the Titan Yellow colorimetric method (43). Protein was assayed using the method of Lowry and co-workers (26). Serum ionized calcium was measured potentiometrically by the method described in the preceding manuscript. Serum conductivity was determined using a Radiometer model CD3m conductivity meter and the data expressed as the molar equivalent of KCl standards.

Statistical Methods One way analysis of variance was employed with subsequent Newman-Kuels multiple comparisons applied as described by Winer (46).

^{3}H-Norepinephrine Uptake A modification of the method of Snyder and Coyle (41) was used to determine ^{3}H-norepinephrine uptake. Rats were sacrificed between the hours of 11:00 A.M. and 12:30 P.M. to minimize the contribution due to diurnal variation associated with norepinephrine uptake (25). Animals were lightly anesthetized with ether (2.5 minutes) and decapitated following the withdrawal of 3 ml of blood via syringe by puncturing the inferior vena cava. The brains were removed from the animals and the hypothalami dissected out as described by Glowinski and Iversen (16). The hypothalami were placed into ice-cold 0.32M sucrose until all dissections were completed.

After weighing the tissue, the volume of sucrose was adjusted to result in a 10% w/v concentration of tissue. The tissue was homogenized in a teflon-glass tissue homogenizer with a clearance of 0.03 cm (Arthur H. Thomas Co.; Phila., Pa.) using 10 up and down strokes at about 1,000 rpm. The supernatant fraction of a 1,000 x g x 10 min. centrifugation carried out at 4°C in a Sorvall RC-2B centrifuge was used as the crude synaptosomal homogenate (CSH).

The incubation medium was similar to that reported by Snyder and Coyle (41) and modified by deleting the calcium since this ion is not required for uptake and may in fact

contribute to a reduction in apparent uptake (44, personal observation). The incubation medium contained (mM): NaCl (118.5), KCl (4.8), KH_2PO_4(1.2). $MgSO_4$(1.2), $NaHCO_3$(24.8), disodium ethylene diamine tetra-acetic acid (0.13), the monoamine oxidase inhibitor, nialamide, (0.01) and ^{3}H-norepinephrine (0.0001; 2uCi/ml) and was oxygenated for 20 min. prior to use with O_2:CO_2(95:5) while the buffer was kept cold. Reagents were obtained from Sigma Co., the isotope from New England Nuclear (Mass.) and the nialamide was donated by Pfizer Pharmaceutical Co. (Groton, Conn).

Six aliquots (0.2 ml) of the CSH were equally divided among 0°C and 37°C incubation conditions using glass tubes (10 mm x 75 mm) which contained 0.8 ml of incubation medium. The resultant protein concentration was between 1.0 and 1.5 mg/ml with bovine serum albumin used as the standard. Following a ten minute incubation the reaction was stopped by submerging the 37°C tubes into an ice-bath. All tubes were then centrifuged at 1,000 x g x 60 min. The supernatant was aspirated and 1.0 ml of absolute ethanol was added to the pellet, dispersed by vortexing and transferred into a scintillation vial. All tubes were rinsed with an additional ml of ethanol and the rinse transferred into their respective scintillation vials to which was added 10 ml of toluene phosphor (5.5 g/l 2,5-diphenyl oxazole and 125 mg/l B-bis-(2-phenyloxazole)-benzene in toluene). Scintillation reagents were obtained from New England

Nuclear.

Uptake was calculated by subtracting the mean value of the 0°C incubations from those obtained at 37°C. The radioactivity was measured using a Packard Tri-Carb model 3335 liquid scintillation spectrometer. Uptake was expressed as ng norepinephrine/mg protein/10 min.

Bone Calcium The right femur was removed from the animal and connective tissue dissected free using a scalpel and forceps. The femur was dissolved in 10 ml of concentrated nitric acid and the acid was boiled off. The residue was reconstituted with 100 ml of distilled and deionized water and calcium determined colorimetrically.

Animals-Protriptyline Study Male COBS/CD rats (Charles River Co.; Wilmington, Mass.) weighing 250-275 grams at the time of arrival were group housed (5/case), fed food and water ad libitum and left to acclimate for 1 week prior to initiating the experiment. Each animal received 18 daily injections (1 ml/kg, i.p.) with controls receiving physiological saline, the acute group given 17 days of saline and on the last day 1 dose of PRO (10 mg/kg). The subchronic group received 12 days of saline injections and PRO for the last 6 days. The chronic group received 18 days of PRO.

Five rats from each treatment group were sacrificed each day in random order. The hypothalami of rats from a given treatment group were pooled to result in N=1 for the day. Each week a given time point following the last injection was under study to result in N=5 for each treatment condition and time point.

Animals=Chronic Protriptyline and Tri-iodothyronine Administration Animals were group housed (3/cage) and treated for 14 consecutive days with either saline or PRO (5 mg/kg) in combination with varying doses of tri-iodothyronine (1, 10 or 100 ug/kg). Three hours following the last injection animals were anesthetized with ether and blood collected via syringe from the inferior vena cava.

High Dose Protriptyline = Balance Study Rats were individually housed in metabolic cages with food and water consumption manipulated in the weight adjusted control (C20) group to equal that of the drug treated group (P20). Urine assays were performed daily with blood and bone determinations conducted following the sixth treatment day.

RESULTS

Effects of Ether Anesthesia on ^{3}H-Norepinephrine Uptake and Serum Ionized Calcium Since sera must be obtained from anesthetized rats, the effect of ether upon norepinephrine uptake and serum ionized calcium was determined. The results presented in figure 1 demonstrates that ether administration did not result in any significant change in norepinephrine uptake either in saline injected controls or in animals given an acute dose of PRO (10 mg/kg). Similarly, the serum ionized calcium of animals provided with an indwelling catheter in the vena cava 1 week prior to sampling was not different from anesthetized, uncatheterized controls (data not shown).

Protriptyline Study The effect of PRO upon the uptake of norepinephrine (figure 2) was clearly evident at 2 hours after the last injection with all treatment groups different from controls ($P<0.01$) but none different from each other. Uptake blockade was most pronounced at 6 hours following the last dose of PRO and returned to control values at 24 hours. Among the various treatment groups, the values for norepinephrine uptake were not significantly different from each other at any given time point. In all treatment groups, the 2 and 6 hour time points were significantly different from controls ($P<0.01$). Within the 12 hour time point, the acute group did not differ significantly from

controls, whereas the subchronic and chronic groups did ($P<0.01$).

Serum total and ionized calcium remained unaltered throughout the various times and durations of treatment with PRO (table 1).

Tri-iodothyronine (I_3) Study Tri-iodothyronine administration failed to alter serum total or ionized calcium irrespective of the amount and duration of administration as well as the time of sacrifice following the last injected dose (table 2).

Chronic Protriptyline and Tri-iodothyronine A variety of response measures known or suspected to be directly or indirectly reflective of the status of ionized calcium was determined in rats treated with PRO (5 mg/kg) in combination with tri-iodothyronine at varying doses. The results, presented in table 3, fail to reveal any physiologically relevant changes among the response measures.

There were no significant differences associated with serum total or ionized calcium or glucose. Although BUN and creatinine values differ from controls, it is the ratio of these values which are diagnostic of kidney dysfunction and the ratios do not vary appreciably (37.4, 30.3, 44.9 and 44.4 for the control and PRO from low to high dose of

thyroid hormone, respectively). Serum electrolytes are unaltered as a result of the various treatments. Blood gases are also not significantly different across treatment groups. Serum conductivity and proteins remain unchanged as well.

High Dose Protriptyline Although total serum calcium, inorganic phosphate, alkaline phosphatase, hydroxyproline, femure weight and femur volume did not differ significantly, there were significant differences in body weight, serum proteins, bone density and the density of calcium associated with bone among animals treated with PRO (20 mg/kg) for 14 days (table 4).

High Dose Protriptyline = Balance Study Table 5 illustrates the results of daily urine analyses and reflects the adequacy of the attempt to match consumption of food and water within the control group with those animals treated with PRO. The data suggest, by failure to uncover significant differences, that the attempt to control dietary consumption was adequate. The urine data reflects the constancy of homeostasis throughout controls and drug treated groups.

Table 6 depicts the results of blood assays and bone studies. All response measures failed to show significant differences with the exception of alkaline phosphatase found

to be significantly lower in the P20 group.

Low Dose Tricyclics PRO (0.5 mg/kg) and imipramine (5 mg/kg) administered to rats in an attempt to reflect doses used in humans was studied to cover the possibility of uncovering any biphasic dose-dependent effects on the response measures indicated in table 7. There were no significant differences among the various treatments.

DISCUSSION

Four hypotheses concerning the mechanisms of action of antidepressant substances have been addressed. All utilize the same criterion for establishing a probable causal relationship between the biochemical and therapeutic effect, namely, a temporal relationship. Since tricyclics are only effective following chronic administration and the converse is true for thyroid hormone, alterations in the response measures studied which reflect the temporal considerations of the therapeutic agents may offer strong support for each hypothesis in question.

The results of chronic protriptyline administration upon the uptake of ^{3}H-norepinephrine suggest that multiple dosing may result in a longer duration of action but fail to confirm that the magnitude of the effect is more pronounced. It may be reasonable to argue that the significantly more prolonged duration of action following multiple dosing supports the view that uptake blockade may be the primary effect resulting in the therapeutic response, however, two important considerations make this argument less tenable. The first is concerned with the data presented here. Although at the 12 hour time point norepinephrine uptake following acute administration of protriptyline was not significantly different from control values and both multiple dosing regimes were, the acute value was not found

to be significantly different from the data obtained for subchronic and chronic schedules of drug administration. It may be offered that although subchronic and chronic drug administration resulted in differences from controls during this experiment, significance may not necessarily be reflected following a duplication of the experiment. The rational for this interpretation considers that the values obtained from all three treatment groups are a result of random sampling since they did not significantly differ from one another. Another random sampling may result in approximately the same means but for different treatment groups. It would have been more convincing if the time point in question was not only insignificant when compared with controls but significantly different from the values obtained following multiple dosing.

Another consideration deals with the actual use of tricyclics in the clinical setting. Seldom, if ever, are the tricyclics prescribed for use in depression by single daily dosing. Generally, they are administered three or four times a day in divided doses. This would make the response at 12 hours a less significant finding than would otherwise be the case. Although compliance to dose schedules is a well known problem among patients with affective disorders and may argue for the relevance of the 12 hour time point, this may be dismissed since most published studies with which we draw upon for establishing

the latency to the therapeutic effect have been performed using hospitalized patients in which compliance related problems are not significant.

Failure to demonstrate a greater magnitude of effect following multiple dosing schedules along with the considerations discussed concerning the duration of the biochemical effect suggest that uptake blockade is not temporally correlated with the clinical response.

The alternative hypotheses regarding elevations in serum ionized calcium as a function of tricyclic, thyroid hormone or their combined administration also failed to receive experimental support using rats. Of the doses and durations of administration tested using protriptyline and/or tri-iodothyronine, significant differences were not demonstrable considering either the primary response measure or any of the secondary variables tested which are known or suspected to be reflective of alterations in calcium homeostasis. The significant findings of the high dose protriptyline experiment (table 4) failed to be confirmed when the control group was adjusted for the effects due to inanition (table 6). Under conditions in which dietary considerations were not a problem, as in the therapeutically relevant dosage range studied (table 7), significant differences were not discernable.

Although the data fail to confirm any of the three calcium related hypotheses, two arguments may be made in defense of the value of the hypotheses and underscore the need for continuing investigation. The clinical data demonstrating an effect of tricyclics temporally correlated with the therapeutic effect were obtained from depressives. There are no generally well accepted animal models of depression especially in rats. Tricyclics, unlike psychic stimulants, are without such effects in non-depressed individuals. Perhaps in order to observe a stimulatory effect of tricyclics upon serum ionized calcium, the organism must be depressed.

The observation of elevated serum ionized calcium among hyperthyroid individuals, who are not depressed, does not agree with the findings presented here using rats. However, a recent report concerning calcium metabolism in rats concludes that bone calcium is not exchangeable with the calcium of serum in this species as occurs in dogs or chicks (24). For this reason the rat may be considered a poor animal model in which to study the effects of tricyclics upon serum ionized calcium. As a result of these considerations it is suggested that the calcium hypotheses of antidepressant action should receive thorough investigation within clinically depressed patients.

SUMMARY

Uptake blockade of norepinephrine into rat hypothalamic synaptosomes was studied ex-vivo following acute, subchronic and chronic protriptyline administration. This experiment was performed to determine if multiple dosing results in a response which is greater in magnitude and/or duration than observed following acute drug administration in order to gather support for uptake blockade as the primary mechanism of action of tricyclics. The results suggest that multiple dosing does not alter this response measure in such a way as to convincingly demonstrate that uptake blockade is the primary mechanism of antidepressant action of tricyclics.

An alternative hypothesis concerning the antidepressant action of tricyclics and thyroid hormone invoking a role for serum ionized calcium was similarly untenable based upon the experimental results obtained. Protriptyline and/or tri-iodothyronine failed to alter serum ionized calcium or any of the secondary response measures which are known or suspected to be reflective of alterations in calcium homeostasis. Support for the calcium hypothesis was offered by emphasizing recently published data suggesting that rats may be a poor model for studying calcium homeostasis.

REFERENCES

1. Baldessarini, R.J.: Biogenic amine hypothesis in affective disorders. In The Nature and Treatment of Depression, Flach, F.F. and Draghi, S.C. (eds.), Wiley and Sons, Inc., N.Y. 347-385,1975.

2. Bergstrom, D.A. and Keller, K.J.: Adrenergic and serotonergic receptor binding in rat brain after chronic desmethylimipramine treatment. J. Pharmacol. Exp. Ther. 209:256-261,1979.

3. Burman, K.D., Monchik, J.M., Earll, J.M. and Wartofsky, L.: Ionized and total serum calcium and parathyroid hormone in hyperthyroidism. Ann. Int. Med. 84:668-671,1976.

4. Carman, J.S. and Wyatt, R.J.: Calcium: Bivalent cation in the bivalent psychoses. Biol. Psychiatr. 14:295-336,1979.

5. Clark, J.A., Davidson, L.J. and Ferguson, H.G.: Psychosis in hypoparathyroidism. J. Ment. Sci. 108:811-815,1962.

6. Coirailt, P.R., Desclos-De-La-Fonchais, R., Ramel, P. and Neiger, R.: Les variations du calcium sanguin total, du calcium sanguin ionise et du calcium urinaire/24 heures au cours de la sismotherapie et des traitements chimiotherapiques a action anxiolytique. Med. Exp. 1:176-186,1959.

7. Densler, H., Spiegel, H.E. and Titus, E.O.: Effects of

drugs on uptake of isotopic norepinephrine by cat tissues. Nature 177:44-45,1961.

8. Ehrensing, R.H. and Kastin, A.J.: TRH: Clinical investigations for nonendocrine actions in man. In Clinical Neuroendocrinology, Martini, L. and Besser, G.M. (eds.), Academic Press, N.Y. 133-142,1977.

9. Eiduson, S., Brill, N.Q. and Crumpton, E.: The effect of electroconvulsive therapy on spinal fluid constituents. J. Ment. Sci. 106:692-698,1960.

10. Farasalla, F.F. and Flach, F.F.: Studies of mineral metabolism in mental depression. J. Nerv. Ment. Dis. 151:120-129,1970.

11. Flach, F.F.: Calcium metabolism in states of depression. Brit. J. Psychiatr. 110:588-593,1964.

12. Flach, F.F., Liang, E. and Stokes, P.E.: The effects of electric convulsive treatments on nitrogen, calcium and phosphorous metabolism in psychiatric patients. J. Ment. Sci. 106:638-647,1960.

13. Frizel, D., Coppen, A. and Marks, V.: Plasma magnesium and calcium in depression. Brit. J. Psychiatr. 110,588-593,1964.

14. Garver, D.L. and Davis, J.M.: MINIREVIEW: Biogenic amine hypothesis of affective disorders. Life Sci. 24:383-394,1979.

15. Gibson, J.G.: Emotions and the thyroid gland:

A critical appraisal.
J. Psychosom. Res. 6:93-116,1962.

16. Glowinsky, J. and Iversen, L.L.: Regional studies of catecholamines in the rat brain.
J. Neurochem. 13:655-669,1966.

17. Gour, K.N. and Chaudhry, H.M.: Study of calcium metabolism in electric convulsive therapy (E.C.T.) in certain mental diseases.
J. Ment. Sci. 103:275-285,1957.

18. Harris, W.H. and Beauchemin, J.A.: Cerebrospinal fluid calcium, magnesium and their ratio in psychoses of organic and functional origin.
Yale J. Biol. Med. 29:117-124,1959.

19. Iversen, L.L.: Uptake process for biogenic amines. In Handbook of Psychopharmacology, Iversen, L.L., Iversen, S.D. and Snyder, S.H. (eds.), Plenum Press, N.Y., 3:381-442,1975.

20. Jimerson, D.C., Post, R.M., Carman, J.S., van Kammen, D.P., Wood, J.H., Goodwin, F.K. and Bunney, W.E. Jr.: CSF calcium: Clinical correlates in affective illness and schizophrenia.
Biol. Psychiatr. 14:37-51,1979.

21. Janowsky, D.S., El-Yousef, M.K., Davis, J.M. and Sekerke, H.G.: A cholinergic-adrenergic hypothesis of mania and depression.
Lancet ii:632-635,1972.

22. Kelly, R.B., Deutsch, J.W., Carlson, S.S. and Wagner,

J.A.: Biochemistry of neurotransmitter release. Ann. Rev. Neurosci. 2:399-446,1979.

23. Kivirikko, K.I., Laitinen, O. and Prockop, D.J.: Modifications of a specific assay for hydroxyproline in urine. Anal. Biochem. 19:249-255,1967.

24. Klein, L.: Steady-state relationship of calcium-45 between bone and blood: Differences in growing dogs, chicks and rats. Science 214:190-193,1981.

25. Lew, G,M. and Quay, W.B.: Twenty four hour rhythmic uptake of H^3-norepinephrine in-vitro by hypothalamus and medial lower brainstem. Int. J. Chronobiol. 2:209-213,1974.

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

27. McClure, D.J.: The role of dopamine in depression. Can. Psychiatr. Assoc. J. 18:309-312,1973.

28. Melander, E.T., Ericson, L.E., Sundler, F. and Westgren, U.: Intra-thyroidal amines in the regulation of thyroid activity. Rev. Physiol. Biochem. Pharmacol. 73:39-71,1975.

29. Mellerup, E.T., Bech, P., Sorensen, T., Fuglsang-Frederiksen, A. and Rafaelsen, O.J.: Correspondence: Calcium and electroconvulsive therapy of depressed patients.

Biol. Psychiatr. 14:711-714,1979.

30. Morgenroth, V.H.,III, Boadle-Biber, M.C. and Roth, R.H.: Activation of tyrosine hydroxylase from central noradrenergic neurons by calcium.
Mol. Pharmacol. 11:427-435,1975.

31. Naylor, G.J., Fleming, L.W., Stewart, W.K., McNamee, H.B. and LePoidevin, D.: Plasma magnesium and calcium levels in depressive psychosis.
Brit. J. Psychiatr. 120:683-684, 1972.

32. Oswald, I., Brezinova, V. and Dunleavy, D.L.F.: On the slowness of action of tricyclic antidepressant drugs.
Brit. J. Psychiatr. 120:673-677,1972.

33. Phillis, J.W.: MINIREVIEW: The role of calcium in the central effects of biogenic amines.
Life Sci. 14:1189-1201,1974.

34. Prange, A.J. Jr., Wilson, I.C., Rabon, A.M. and Lipton, M.A.: Enhancement of imipramine antidepressant activity by thyroid hormone.
Amer. J. Psychiatr. 126:457-469,1969.

35. Ross, S.B. and Renyi, A.L.: Inhibition of the uptake of tritiated catecholamines by antidepressant and related agents.
Europ. J. Pharmacol. 2:181-186,1967.

36. Ross, S.B. and Renyi, A.L.: Tricyclic antidepressant agents. II. Effect of oral administration on the uptake of ^{3}H-noradrenaline and

^{14}C-5-hydroxytryptamine in slices of the midbrain-hypothalamus region of the rat. Acta Pharmacol et Toxicol. 36:395-408,1975.

37. Rubin, R.P.: The role of calcium in the release of neurotransmitter substances and hormones. Pharmacol. Rev. 22:389-428,1970.

38. Salama, A.I., Insalaco,J.R. and Maxwell, R.A.: Concerning the molecular requirements for the inhibition of the uptake of racemic ^{3}H-norepinephrine into rat cerebral cortex slices by tricyclic antidepressant compounds. J. Pharmacol. Exp. Ther. 178:474-481,1971.

39. Schildkraut, J.J.: The catecholamine hypothesis of affective disorders: A review of supporting evidence. Amer. J. Psychiatr. 112:509-522,1965.

40. Schildkraut, J.J. and Kety, S.S.: Biogenic amines and emotion. Science 156:21-30,1967.

41. Snyder, S.H. and Coyle, J.T.: Regional differences in ^{3}H-norepinephrine and ^{3}H-dopamine uptake into brain homogenates. J. Pharmacol. Exp. Ther. 165:78-86,1969.

42. Squires, R.: Effects of noradrenaline pump blockers on its uptake by synaptosomes from several different brain regions: Additional evidence for dopamine terminals in the frontal cortex. J. Pharm. Pharmacol. 26:364-366,1974.

43. Teitz, N.W.: Fundamentals of Clinical Chemistry, W.B. Saunders Co., Phila., Pa. 919-920,1976.

44. van Praag, H.M.: Central monoamine metabolism in depressions: I. Serotonin and related compounds. Comprehen. Psychiatr. 21:30-43,1980.

45. van Praag, H.M.: Central monoamine metabolism in depressions. II. Catecholamines and related compounds. Comprehen. Psychiatr. 21:44-54,1980.

46. Winer, B.J.: Statistical Principles in Experimental Design, McGraw-Hill Book Co., N.Y., 191-195,1971.

Table 1. Effect of Protriptyline (10 mg/kg) Upon Serum Ionized and Total Calcium

Treatment[a]	ToS[b]	N[c]	[Ca++][d] Mean±S.D.	[Ca][e] Mean±S.D.
Saline	2	13	.947±.046	2.188±.179
Acute	2	15	.973±.039	2.246±.198
Subchronic	2	15	.965±.044	2.288±.181
Chronic	2	14	.969±.038	2.233±.234
Saline	6	14	1.005±.063	2.073±.110
Acute	6	15	1.029±.067	2.080±.119
Subchronic	6	15	1.019±.052	2.078±.089
Chronic	6	15	1.022±.052	2.019±.125
Saline	12	8	1.003±.026	2.206±.082
Acute	12	14	.977±.054	2.251±.128
Subchronic	12	15	.984±.041	2.253±.140
Chronic	12	13	.993±.039	2.176±.148
Saline	24	15	1.010±.026	2.156±.123
Acute	24	15	1.012±.035	2.220±.127
Subchronic	24	15	1.016±.027	2.181±.135
Chronic	24	13	1.010±.041	2.108±.097

a. Saline: 18 daily injections (i.p.) of saline
   Acute: 17 daily injections of saline followed by one injection of protriptyline
   Subchronic: 12 daily injections of saline followed by 6 of protriptyline
   Chronic: 18 daily injections of protriptyline
b. Time of Sacrifice after the last injection, in hours
c. Number of animals
d. Serum ionized calcium, mM
e. Total serum calcium, mM

Table 2. Effect of Tri-iodothyronine Administration on Serum Ionized and Total Calcium

Treatment[a]	DoA[b]	ToS[c]	N	[Ca++][d]	[Ca][e]
Saline	1	1	10	1.021±.048	2.298±.138
T_3 1,000	1	1	10	1.017±.064	2.345±.075
Saline	1	4	10	1.087±.054	2.363±.158
T_3 1,000	1	4	10	1.074±.056	2.425±.049
Saline	1	24	5	1.037±.046	2.063±.033
T_3 1,000	1	24	6	1.099±.063	2.128±.074
Saline	5	4	6	1.119±.011	2.211±.088
T_3 1,000	5	4	6	1.037±.037	2.101±.090
Saline	10	4	6	1.069±.051	2.246±.115
T_3 1	10	4	6	1.078±.029	2.156±.101
T_3 10	10	4	5	1.061±.035	2.188±.123
T_3 100	10	4	6	1.057±.031	2.118±.114
T_3 250	10	4	6	1.006±.088	2.006±.140
T_3 500	10	4	6	1.047±.041	2.038±.086
T_3 1,000	10	4	3	.953±.045	1.978±.091

a. Saline or tri-iodothyronine (T_3) in ug/kg
b. Days of Administration
c. Time of Sacrifice after the last dose, in hours
d. Serum ionized calcium, mM (Mean±S.D.)
e. Total serum calcium, mM (Mean±S.D.)

Table 3. The Effect of Concomitant Tri-iodothyronine (T_3) and Protriptyline (5mg/kg) Administration[a] for 14 Days Upon Response Measures Suspected to be Reflective of Calcium Homeostasis (Mean±SD, N=6)

Serum Response Measure	Saline Control	PRO+T (1ug/kg)	PRO+T (10ug/kg)	PRO+T (100ug/kg)
[Ca++], mM	1.26±.12	1.22±.10	1.21±.04	1.20±.04
[Ca], mM	2.53±.14	2.53±.10	2.49±.06	2.35±.30
[Na], mM	144.8±1.8	143.9±1.9	144.9±1.4	143.5±1.4
[K], mM	5.20±.78	5.76±.90	5.10±.50	5.20±.43
[Cl], mM	101.8±3.1	99.9±3.4	100.9±2.7	101.8±3.2
[PO_4---] mEq/L	10.3±1.3	13.0±.70	10.7±1.0	10.2±1.3
pH	7.46±.10	7.37±.08	7.45±.06	7.42±.09
pCO_2, mm Hg	63.0±15.6	72.6±18.4	58.8±7.5	68.8±11.9
Glucose mg/dL	173.3±47.9	179.2±31.2	140.0±14.8	157.5±31.9
BUN, mg/dL	21.7±3.3	16.4±2.9	17.5±2.7	16.0±2.1
Creatinine mg/dL	.58±.11	.54±.07	.39±.14	.36±.20
Protein g/dL	4.84±.46	4.31±.40	5.92±1.36	6.30±1.40
Conductivity (M KCl)	.090±.002	.090±.002	.091±.002	.091±.004

a. Protriptyline was administered i.p., whereas T_3 was given subcutaneously

Table 4. The Effect of a High Dose (20mg/kg) of Protryptyline Chronically Administered (14 days) on Body Weight, Blood Constituents and Bone (mean±S.D., N=10)

	Control	PRO	P
Body weight, gms.	486.2±57.8	376.0±41.9	<.01
BLOOD			
[Ca], mM	2.22±.19	2.02±.37	N.S.
[PO_4--], mEq/L	7.71±.94	7.62±.91	N.S.
Protein, g/dL	6.05±.10	5.64±.32	<.01
Alk. Phos. Sigma U/ml	4.75±.84	4.33±.77	N.S.
Hydroxy-proline ug/ml	3.34±.4	3.08±1.59	N.S.
BONE			
Femur weight, gms.	1.1651±.1274	1.0750±.0760	N.S.
Femur volume, ml	.746±.076	.730±.068	N.S.
Femur density, g/ml	1.562±.051	1.478±.099	<.01
Femur calcium density, mg/ml	234.3±14.1	211.4±10.2	<.01

Table 5A. The Effects of Subchronic (6 days) Protriptyline (20mg.kg) Administration Compared with Weight Adjusted Controls on Gross Indices of Metabolism (means±S.D., N=10)

Index	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Trt
Body Weight (gms)	289.0±28.7	289.0±35.2	281.3±29.3	275.0±21.6	275.4±21.6	268.7±26.2	Con
	309.2±34.5	300.0±30.2	284.5±12.6	287.0±19.2	286.8±20.0	280.7±20.9	PRO
Food Intake (gms)	17.5±2.6	5.7±2.2.	5.8±2.8	12.8±3.5	14.3±3.9	13.3±3.7	Con
	10.9±3.3	6.4±1.6	9.6±2.6	11.2±3.0	9.3±2.1	7.9±1.7	PRO
Water Intake (ml)	33.3±8.6	25.1±7.2	36.0±4.5	32.3±2.1	36.2±3.2	20.8±7.0	Con
	20.1±5.5	24.7±4.4	29.5±5.4	26.0±5.8	30.4±3.2	26.2±6.3	PRO
Urine output (ml)	8.4±2.2	11.2±3.2	13.6±2.6	12.1±1.9	17.4±2.3	12.2±2.7	Con
	5.7±0.8	4.4±0.7	3.7±1.5	7.1±1.7	13.8±3.2	9.3±1.6	PRO
Fecal output (gms)	3.3±0.6	1.4±0.4	1.5±0.2	1.4±0.4	2.4±0.5	1.8±0.4	Con
	2.1±0.5	1.6±0.5	2.20.6	1.6±0.5	1.7±0.4	2.2±0.5	PRO

Table 5B. The Effects of Subchronic (6 days) Protriptyline (20mg/kg) Administration Compared with Weight Adjusted Controls on Urinary Indices of Calcium Metabolism (mean±S.D., N=10)

Index	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Trt
Calcium output (mg)	.383±.225	.235±.109	.243±.132	.241±.104	.255±.184	.241±.135	Con
	.251±.192	.123±.085	.155±.156	.203±.123	.225±.111	.204±.046	PRO
Hydroxy-proline (umol)	8.9±2.4	9.2±3.6	9.0±4.4	7.6±4.3	10.4±3.8	8.2±2.7	Con
	8.6±1.6	9.0±3.1	10.2±6.5	9.4±3.6	10.9±3.8	9.6±2.5	PRO
Magnesium output (mmol)	.074±.065	.060±.048	.032±.028	.037±.016	.047±.036	.039±.027	Con
	.057±.038	.035±.021	.033±.036	.041±.030	.089±.143	.043±.030	PRO
Phosphate output (mmol)	.527±.174	.525±.155	.499±.199	.551±.266	.587±.182	.509±.208	Con
	.581±.163	.475±.172	.414±.306	.586±.435	.932±.314	.586±.166	PRO
Creatinine output (mmol)	.057±.025	.055±.023	.049±.030	.065±.030	.059±.021	.055±.019	Con
	.055±.015	.059±.018	.056±.035	.070±.034	.120±.057	.053±.016	PRO

Table 6. The Effect of a High Dose (20mg/kg) of Protriptyline Subchronically Administered (6 days) on Blood Constituents and Bone (mean±S.D., N=10).

	Weight Adjusted Controls	PRO
BLOOD		
[Ca++], mM	1.17±.13	1.13±.11
[Ca], mM	1.73±.31	1.65±.11
[PO_4--], mEq/L	8.31±.66	7.99±1.71
[Mg], mM	1.18±.03	1.19±.06
Alk. Phos. Sigma U/ml	5.33±1.30	3.38±1.33
CPK Sigma U/ml	26.11±8.27	25.70±11.96
Protein, g/dL	5.69±.43	4.98±.76
BONE		
Femur weight, gms.	.7536±.0533	.7605±.0450
Femur volume, ml	.533±.044	.544±.039
Femur density, g/ml	1.416±.0334	1.3998±.0461

Table 7. The Effect of Therapeutically Relevant Doses of Protriptyline (0.5mg/kg) and Imipramine (5.0mg/kg) Administered Subchronically (6 days) on Blood Constituents and Bone (mean±S.D., N=10)

	Control	PRO	IMI
BLOOD			
[Ca++], mM	1.21±.09	1.20±.11	1.19±.10
[Ca], mM	1.84±.29	1.85±.34	1.84±.25
[PO_4--], mEq/L	8.95±1.08	8.81±1.16	8.44±.87
[Mg], mM	1.15±.04	1.16±.02	1.16±.02
Alk. Phos. Sigma U/ml	6.73±.94	6.81±2.0	5.76±1.6
CPK Sigma U/ml	30.55±12.5	26.72±.82	32.83±18.1
Protein, g/dl	5.65±.55	5.82±.85	5.80±.81
BONE			
Femur weight, gms.	.7883±.0406	.8003±.0392	.7660±.0484
Femur volume, ml	.561±.041	.567±.029	.547±.033
Femur density, g/ml	1.4133±.0595	1.4120±.0378	1.4190±.0344

FIGURE 1

TITLE: The effect of ether administration on ^{3}H-norepinephrine uptake into rat hypothalamic synaptosomes

LEGEND

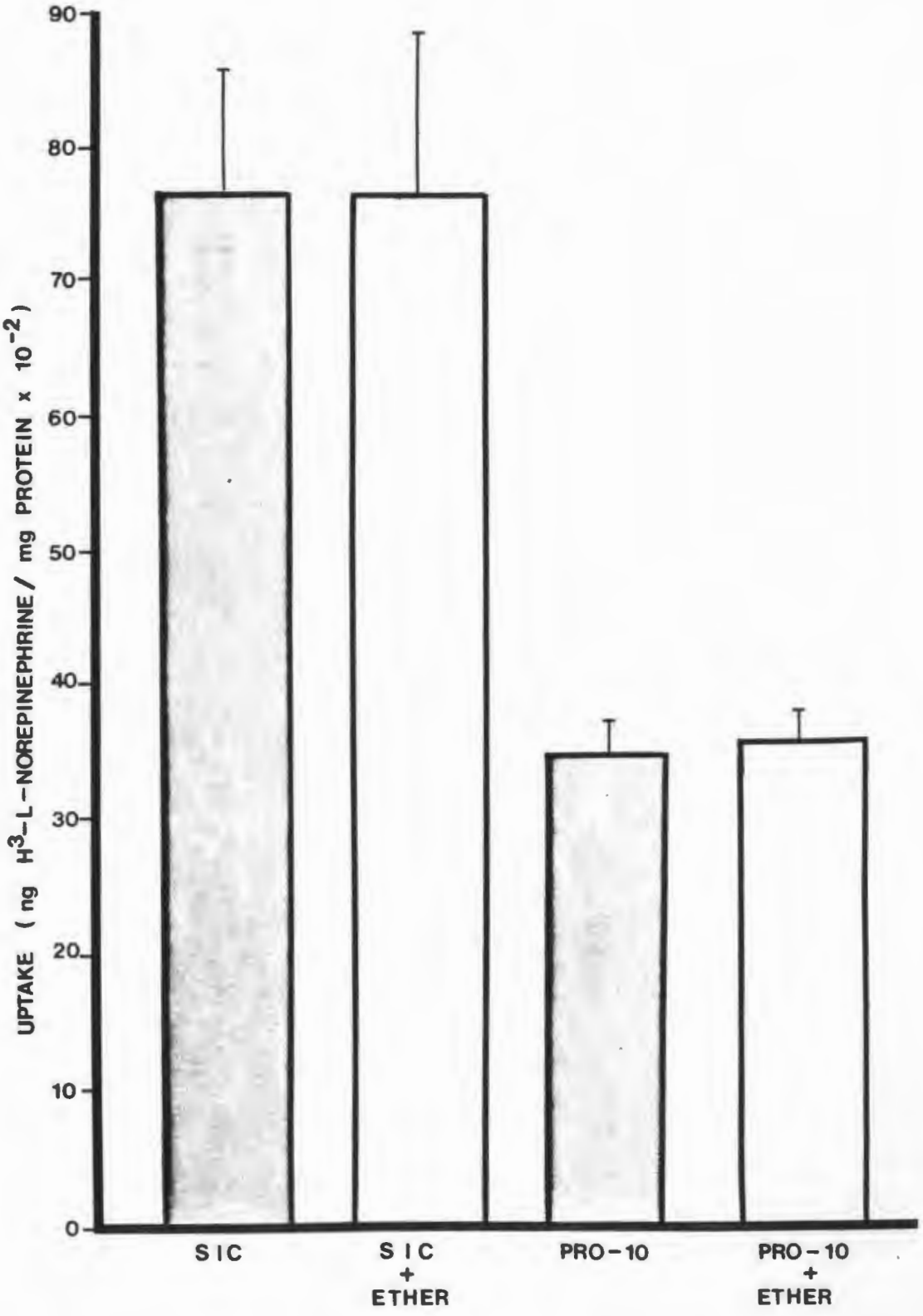
Depicted are the means and standard deviations of five determinations. SIC = saline injected controls; PRO-10 = protriptyline (10mg/kg) given 6 hours prior to sacrifice.

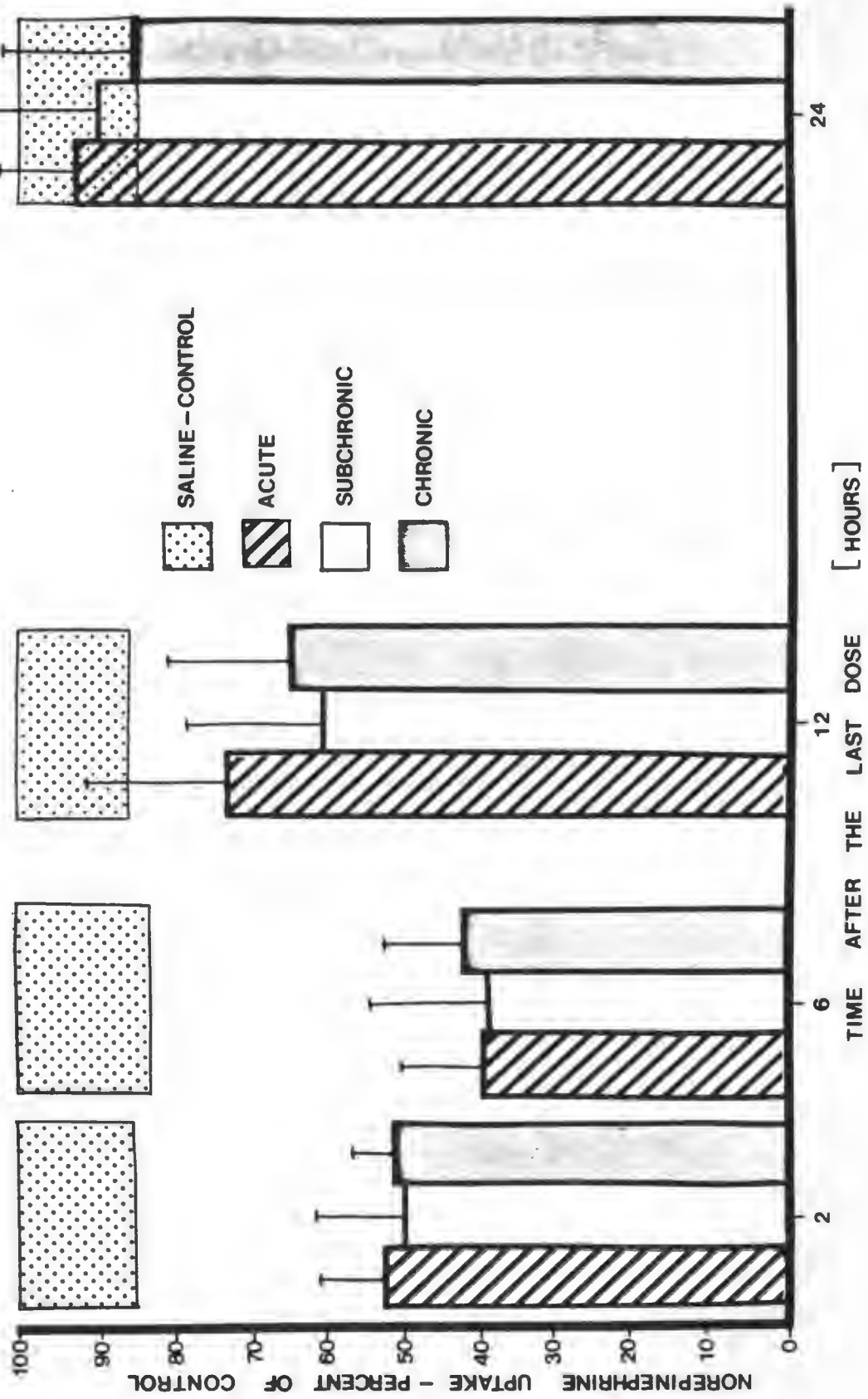
FIGURE 2

TITLE: The effects of acute, subchronic and chronic protriptyline administration upon the uptake of ^{3}H-norepinephrine into rat hypothalamic synaptosomes

LEGEND

Depicted are the means and standard deviations of five separate determinations.


90
80
70
60
50
40
30
20
10
0
UPTAKE (ng H^3-L-NOREPINEPHRINE / mg PROTEIN x 10^{-2})
SIC
SIC + ETHER
PRO-10
PRO-10 + ETHER


NOREPINEPHRINE UPTAKE - PERCENT OF CONTROL
100
90
80
70
60
50
40
30
20
10
0
2
6
12
24
TIME AFTER THE LAST DOSE [HOURS]
SALINE-CONTROL
ACUTE
SUBCHRONIC
CHRONIC

BIBLIOGRAPHY

Ammann, D., Gussi, M., Pretsch, E. and Simon, W.: Improved calcium ion-selective electrode based on a neutral carrier.
Anal. Letters 8: 709-720, 1975.

Arnold, D. E., Stansell, M. J. and Malvin, H. H.: Measurement of ionic calcium using a specific ion electrode.
Amer. J. Clin. Pathol. 42: 627-634, 1968.

Baldessarini, R.J.: Biogenic amine hypothesis in affective disorders. In The Nature and Treatment of Depression, Flach, F.F. and Draghi, S.C. (eds.), Wiley and Sons, Inc., N.Y. 347-385,1975.

Bates, R. G. and Alfenaar, M.: Activity standards for ion-selective electrodes. In Ion-Selective Electrodes, R. A. Durst (ed.)
Nat'l. Bur. Stds., Spec. Publ. 314, Ch 6: 191-214, 1969.

Bergstrom, D.A. and Keller, K.J.: Adrenergic and serotonergic receptor binding in rat brain after chronic desmethylimipramine treatment.
J. Pharmacol. Exp. Ther. 209:256-261,1979.

Brand, M. J. D. and Scott, W. J.: Electrolyte activities in human blood plasma In Proceedings of a Workshop on pH and Blood Gases
Nat'l. Bur. Stds., Spec Publ 450, 301-310, 1977.

Burman, K.D., Monchik, J.M., Earll, J.M. and Wartofsky, L.: Ionized and total serum calcium and parathyroid hormone in hyperthyroidism. Ann. Int. Med. 84:668-671,1976.

Carman, J.S. and Wyatt, R.J.: Calcium: Bivalent cation in the bivalent psychoses. Biol. Psychiatr. 14:295-336,1979.

Chan, G. M., Ash, K.O., Heutschel, W. and Wu, J.: Effects of bilirubin on ionized calcium. Clin. Chem. 27: 204-205, 1981.

Clark, J.A., Davidson, L.J. and Ferguson, H.G.: Psychosis in hypoparathyroidism. J. Ment. Sci. 108:811-815,1962.

Coirailt, P.R., Desclos-De-La-Fonchais, R., Ramel, P. and Neiger, R.: Les variations du calcium sanguin total, du calcium sanguin ionise et du calcium urinaire/24 heures au cours de la sismotherapie et des traitements chimiotherapiques a action anxiolytique. Med. Exp. 1:176-186,1959.

Dengler, H., Spiegel, H.E. and Titus, E.O.: Effects of drugs on uptake of isotopic norepinephrine by cat tissues. Nature 177:44-45,1961.

Drop, L. J., Fuchs, C.and Stulz, P. M.: Determination of blood ionized calcium in a large segment of the normal adult population. Clin. Chim. Acta 82: 503-510, 1978.

Ehrensing, R.H. and Kastin, A.J.: TRH: Clinical

investigations for nonendocrine actions in man. In Clinical Neuroendocrinology, Martini, L. and Besser, G.M. (eds.), Academic Press, N.Y. 133-142,1977.

Eidson, S., Brill, N.Q. and Crumpton, E.: The effect of electroconvulsive therapy on spinal fluid constituents. J. Ment. Sci. 106:692-698,1960.

Eisenman, G.: The electrochemistry of cation-sensitive glass electrodes In Advances in Analytical Chemistry and Instrumentation, C. N. Reilley (ed.), John Wiley and Sons, Inc., N.Y., Ch 4: 213-233, 1965.

Farasalla, F.F. and Flach, F.F.: Studies of mineral metabolism in mental depression. J. Nerv. Ment. Dis. 151:120-129,1970.

Flach, F.F.: Calcium metabolism in states of depression. Brit. J. Psychiatr. 110:588-593,1964.

Flach, F.F., Liang, E. and Stokes, P.E.: The effects of electric convulsive treatments on nitrogen, calcium and phosphorous metabolism in psychiatric patients. J. Ment. Sci. 106:638-647,1960.

Frizel, D., Coppen, A. and Marks, V.: Plasma magnesium and calcium in depression. Brit. J. Psychiatr. 110,588-593,1964.

Fuchs, C., Dorn, D., McIntosh, C., Scheler, F. and Kraft, B.: Comparative calcium ion determinations

in plasma and hole blood with a new calcium ion analyzer.
Clin. Chim. Acta 62: 99-102, 1976.

Garver, D.L. and Davis, J.M.: MINIREVIEW: Biogenic amine hypothesis of affective disorders.
Life Sci. 24:383-394,1979.

Gibson, J.G.: Emotions and the thyroid gland: A critical appraisal.
J. Psychosom. Res. 6:93-116,1962.

Glowinsky, J. and Iversen, L.L.: Regional studies of catecholamines in the rat brain.
J. Neurochem. 13:655-669,1966.

Gour, K.N. and Chaudhry, H.M.: Study of calcium metabolism in electric convulsive therapy (E.C.T.) in certain mental diseases.
J. Ment. Sci. 103:275-285,1957.

Grima, J. M. and Brand, M. J. D.: Activity and interference effects in measurement of ionized calcium with ion-selective electrodes.
Clin. Chem. 23: 2048-2054, 1977.

Hansen, S. O. and Theodorsen, L.: The usefulness of an improved calcium electrode in the measurement of ionized calcium in serum.
Clin. Chim. Acta 31: 119-122, 1971.

Harris, W.H. and Beauchemin, J.A.: Cerebrospinal fluid calcium, magnesium and their ratio in psychoses of organic and functional origin.

Yale J. Biol. Med. 29:117-124,1959.

Iversen, L.L.: Uptake process for biogenic amines. In Handbook of Psychopharmacology, Iversen, L.L., Iversen, S.D. and Snyder, S.H. (eds.), Plenum Press, N.Y., 3:381-442,1975.

Jimerson, D.C., Post, R.M., Carman, J.S., van Kammen, D.P., Wood, J.H., Goodwin, F.K. and Bunney, W.E. Jr.: CSF calcium: Clinical correlates in affective illness and schizophrenia. Biol. Psychiatr. 14:37-51,1979.

Janowsky, D.S., El-Yousef, M.K., Davis, J.M. and Sekerke, H.G.: A cholinergic-adrenergic hypothesis of mania and depression. Lancet ii:632-635,1972.

Kelly, R.B., Deutsch, J.W., Carlson, S.S. and Wagner, J.A.: Biochemistry of neurotransmitter release. Ann. Rev. Neurosci. 2:399-446,1979.

Kielland, J.: Individual activity coefficients of ions in aqueous solutions. J. Amer. Chem. Soc. 59: 1675-1678, 1937.

Kivirikko, K.I., Laitinen, O. and Prockop, D.J.: Modifications of a specific assay for hydroxyproline in urine. Anal. Biochem. 19:249-255,1967.

Klein, L.: Steady-state relationship of calcium-45 between bone and blood: Differences in growing dogs, chicks and rats.

Science 214:190-193,1981.

Ladenson, J. H. and Bowers, Jr., S. N.: Free calcium in serum I. Determination with the ion-selective electrode and factors influencing the results. Clin. Chem. 19: 565-574, 1973.

Lew, G,M. and Quay, W.B.: Twenty four hour rhythmic uptake of H^3-norepinephrine in-vitro by hypothalamus and medial lower brainstem. Int. J. Chronobiol. 2:209-213,1974.

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

Madsen, S. and Olsard, K.: Evaluation of a new automatic calcium ion analyzer. Clin. Chem. 23: 690-694, 1977.

McClure, D.J.: The role of dopamine in depression. Can. Psychiatr. Assoc. J. 18:309-312,1973.

Melander, E.T., Ericson, L.E., Sundler, F. and Westgren, U.: Intra-thyroidal amines in the regulation of thyroid activity. Rev. Physiol. Biochem. Pharmacol. 73:39-71,1975.

Mellerup, E.T., Bech, P., Sorensen, T., Fuglsang-Frederiksen, A. and Rafaelsen, O.J.: Correspondence: Calcium and electroconvulsive therapy of depressed patients. Biol. Psychiatr. 14:711-714,1979.

Mohan, M. S. and Bates, R. G.: Calibration of ion-

selective electrodes for use in biological fluids.
Clin. Chem. 21: 864-872, 1975.

Moore, E. W.: Hydrogen and cation analysis in biological fluids in-vitro. In Glass Electrodes for Hydrogen and Other Cations.
G. Eisenman (ed.) Marcel Dekker, Inc., New York
Ch 15: 412-441, 1967.

Moore, E. W.: Studies with ion-exchange calcium electrodes in biological fluids: Some applications in biomedical research and clinical medicine. In Ion-Selective Electrodes,
R. A. Durst (ed.) Nat'l. Bur. Stds.,
Spec. Publ. 314, Ch 7: 215-285, 1969.

Morgenroth, V.H.,III, Boadle-Biber, M.C. and Roth, R.H.: Activation of tyrosine hydroxylase from central noradrenergic neurons by calcium.
Mol. Pharmacol. 11:427-435,1975.

Naylor, G.J., Fleming, L.W., Stewart, W.K., McNamee, H.B. and LePoidevin, D.: Plasma magnesium and calcium levels in depressive psychosis.
Brit. J. Psychiatr. 120:683-684, 1972.

Oswald, I., Brezinova, V. and Dunleavy, D.L.F.: On the slowness of action of tricyclic antidepressant drugs.
Brit. J. Psychiatr. 120:673-677,1972.

Pedersen, K. O.: Binding of calcium to serum albumin II.

Effect of pH via competitive hydrogen and cation binding to the imidazole groups of albumin. Scand. J. Clin. Lab. Invest. 29: 75-83, 1972.

Pedersen, K. O.: An analysis of measured and calculated calcium quantities in serum. Scand. J. Clin. Lab. Invest. 38: 659-667, 1978.

Phillis, J.W.: MINIREVIEW: The role of calcium in the central effects of biogenic amines. Life Sci. 14:1189-1201,1974.

Prange, A.J. Jr., Wilson, I.C., Rabon, A.M. and Lipton, M.A.: Enhancement of imipramine antidepressant activity by thyroid hormone. Amer. J. Psychiatr. 126:457-469,1969.

Raman, A.: Determination of ionized calcium in serum with a calcium electrode. Biochem. Med. 3: 369-375, 1970.

Robertson, W. G.: Measurement of ionized calcium in body fluids - A review. Ann. Clin. Biochem. 13: 540-548, 1976.

Ross, J. W.: Calcium selective electrodes with liquid ion exchanger. Science 156: 1378-1380, 1967.

Ross, S.B. and Renyi, A.L.: Inhibition of the uptake of tritiated catecholamines by antidepressant and related agents. Europ. J. Pharmacol. 2:181-186,1967.

Ross, S.B. and Renyi, A.L.: Tricyclic antidepressant

agents. II. Effect of oral administration on the uptake of ^{3}H-noradrenaline and ^{14}C-5-hydroxytryptamine in slices of the midbrain-hypothalamus region of the rat. Acta Pharmacol et Toxicol. 36:395-408,1975.

Rubin, R.P.: The role of calcium in the release of neurotransmitter substances and hormones. Pharmacol. Rev. 22:389-428,1970.

Salama, A.I., Insalaco,J.R. and Maxwell, R.A.: Concerning the molecular requirements for the inhibition of the uptake of racemic ^{3}H-norepinephrine into rat cerebral cortex slices by tricyclic antidepressant compounds. J. Pharmacol. Exp. Ther. 178:474-481,1971.

Schildkraut, J.J.: The catecholamine hypothesis of affective disorders: A review of supporting evidence. Amer. J. Psychiatr. 112:509-522,1965.

Schildkraut, J.J. and Kety, S.S.: Biogenic amines and emotion. Science 156:21-30,1967.

Schwartz, H. D., McConville, B. C. and Christopherson, E. F.: Serum ionized calcium by specific ion electrode. Clin. Chim. Acta 31: 97-107, 1971.

Schwartz, H. D.: Serum ionized calcium by electrodes: New technology and methodology. Clin. Chim. Acta 64: 227-239, 1975.

Seamonds, B., Towfighi, J. and Arvan, D. A.: Determination of ionized calcium in serum by use of an ion-specific electrode. Clin. Chem. 18: 155-160, 1972.

Snyder, S.H. and Coyle, J.T.: Regional differences in ^{3}H-norepinephrine and ^{3}H-dopamine uptake into brain homogenates. J. Pharmacol. Exp. Ther. 165:78-86,1969.

Squires, R.: Effects of noradrenaline pump blockers on its uptake by synaptosomes from several different brain regions: Additional evidence for dopamine terminals in the frontal cortex. J. Pharm. Pharmacol. 26:364-366,1974.

Srinivasan, K. and Rechnitz, G. A.: Selectivity studies on liquid membrane ion-selective electrodes. Anal. Chem. 41: 1203-1208, 1969.

Steel, R.G.D. and Torrie, J. H.: Principles and Procedures of Statistics McGraw-Hill, N.Y. Ch 9: 161-182, 1960.

Teitz, N.W.: Fundamentals of Clinical Chemistry, W.B. Saunders Co., Phila., Pa. 919-920,1976.

van Praag, H.M.: Central monoamine metabolism in depressions: I. Serotonin and related compounds. Comprehen. Psychiatr. 21:30-43,1980.

van Praag, H.M.: Central monoamine metabolism in depressions. II. Catecholamines and related compounds.

Comprehen. Psychiatr. 21:44-54,1980.

Winer, B.J.: Statistical Principles in Experimental Design, McGraw-Hill Book Co., N.Y., 191-195,1971.