EFFECTS OF SODIUM AND PROTEIN ON THE CALCIUM-MUREXIDE REACTION

by

Louis H. Peters

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APPROVED

[redacted]

C. Keith Claycomb, Ph.D.
Professor of Biochemistry
Chairman, Thesis Committee

[redacted]

Ellis B. Jump, D.M.D., Ph.D.
Chairman, Graduate Education Committee

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INTRODUCTION

Calcium ion concentration in biological fluids may be determined spectrophotometrically with the calcium-murexide color reaction. Preliminary investigation indicated that sodium ion concentration in some way affected this color reaction, and therefore varying concentrations of sodium ion in biological fluids could alter the results. A second substance likely to interfere with the determination is protein; hence, plasma calcium ion concentration is usually determined after the plasma proteins (with the protein-bound calcium) are removed. Direct determination without prior removal of the proteins would greatly simplify the procedure but would be contraindicated if a protein effect existed. This study was made in an attempt to verify the existence of sodium and protein effects and evaluate the extent to which they affect the calcium-murexide reaction.

Calcium of the plasma may be divided into nondiffusible (protein-bound) and diffusible (nonprotein-bound) fractions, with the calcium in the latter fraction existing both as free ions and in diffusible complexes with groups such as phosphate, citrate and other organic acid anions. The ionized calcium is in dynamic equilibrium with the protein-bound form in a relation which may be shown by the usual mass law equation, $\frac{(\text{CaProteinate})}{(\text{Ca})(\text{Protein})} = K_f$, with the charges omitted for convenience.

When written in this form, K_f is the formation constant of the calcium proteinate complex. The degree of association of calcium with protein depends on the pH, temperature and ionic strength. Were the above equation inverted, the new constant would be K_d, the dissociation constant of the complex.

Of the forms of calcium present in plasma, the ionic fraction is considered to be physiologically active and it is therefore of prime interest. Its level is regulated both directly and indirectly by parathyroid hormone, with secretion of the parathyroid hormone being regulated in turn by a feedback mechanism involving the level of ionic calcium in the blood supply to the parathyroid glands.

Until about a decade ago, there was no strictly chemical method for measuring ionic calcium and its concentration was usually estimated by one of two methods. The first was a determination of the diffusible or ultrafiltrable calcium as an approximation of the ionic calcium, the assumption being that the diffusible complexes make up only an insignificantly small and relatively constant part. The second method was a bioassay technique which involved measuring the amplitude of ventricular contraction of a frog heart as a function of the calcium ion concentration in the bathing solution. This latter method was developed by McLean and Hastings(1) in 1934 and was the basis of their subsequent nomogram(2) for estimating the ionic calcium concentration in human serum from the total calcium and protein concentrations.

Quantitative chemical determination of calcium ion concentration was made possible when Schwarzenbach and Gysling(3)

in 1949 reported the results of a thorough study of formation of murexide complexes by calcium and a number of other cations. Purple free murexide (Fig. 1) combines with calcium in a 1:1 ratio to form an orange-colored complex. Since these two forms have absorption maxima of 530 and 480 mu respectively,

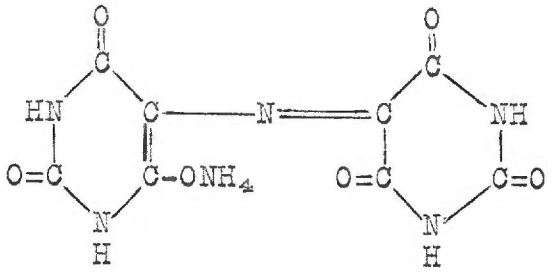

Fig. 1. Murexide

this indicator lends itself nicely to the spectrophotometric determination of ionic calcium. The pH and ionic strength affect the degree of dissociation both of murexide and the calcium murexide complex, and therefore must be held constant during the determination.

The ionic calcium concentration is usually determined after removal of the protein either by ultrafiltration through a collodion or cellophane membrane or by ultracentrifugation. Rose(4) prepared plasma ultrafiltrates using a collodion membrane and 650 mm Hg pressure and then measured the ionic calcium with murexide. He measured total ultrafiltrable calcium by the method of Kenny and Toverud(5) and total plasma calcium by the method of Kramer and Tisdall(6). A constant CO_2 tension was maintained throughout the ultrafiltration process by bathing the system in 5% CO_2 in O_2 and by bubbling the same

gas mixture through the ultrafiltrate. Initially this gas mixture was used for all samples and the determinations were made at room temperature. In subsequent work(7), the temperature was held at 37°C and the carbon dioxide tension was held at the alveolar carbon dioxide tension of the subject, as determined at the time of sampling. From Rose's data ((4) Table 3, p. 238), one may calculate the mean ionic calcium and mean ultrafiltrable calcium. These calculations yield values of 5.88 mg% (1.47 mM or 2.94 meq/L) for the mean plasma ionic calcium concentration and 6.19 mg% (1.55 mM or 3.10 meq/L) for the mean total ultrafiltrable calcium concentration. The concentration may also be expressed in terms of percentage of total plasma calcium. Calculation yields values of 56.4±2.3% and 59.4±2.4% for the mean ± standard deviation of ionic and total ultrafiltrable calcium concentrations respectively.

Toribara et al.(8) designed a special tube for ultrafiltration, with the plasma sample contained in a sock of cellophane dialysis tubing which rested on a sintered glass disc. The entire system was flushed with 5% CO_2 in O_2 after the dialysis bag was in place and the tube was then stoppered and centrifuged. The ultrafiltrate which collected below the supporting disc was subsequently removed through a side opening. These authors found a mean ultrafiltrable calcium of 65.9% of the total serum calcium when the ultrafiltration was carried out at 36°C.

To attain greater centrifugal force and hence faster filtration, Prasad and Flink(9) suspended a gauze bag in a 15 ml centrifuge tube and placed the dialysis bag in this.

They determined both total ultrafiltrable and total serum calcium by the method of Clark and Collip(10). Except for flushing the tube with 5% CO_2 in O_2 before ultrafiltration, the only precaution they recommended was the anaerobic handling of the serum samples, claiming that bubbling CO_2-O_2 mixtures through the samples caused an increase in the measured ultrafiltrable calcium which they termed an artifact of CO_2. The mean diffusible calcium reported by these authors was 1.31 mM or 53.3% of the total serum calcium, with respective standard deviations of ±0.054 mM and ±2.48%. This mean is significantly lower ($p<.01$) than that calculated from the data of Rose, 1.55 mM and 59.4%.

Loken et al.(11) measured "free" calcium ("free" designating calcium not bound by protein) with a flame photometer following ultracentrifugation of serum samples. Their mean value for free calcium was 53.1% of the total serum calcium with a standard deviation of 2.6%, which is in agreement with the findings of Prasad and Flink(9). With regard to carbon dioxide content, they concluded that their experiments "clearly show that the alteration in percentage of free calcium is related to changes in ionic concentration rather than to a specific effect of bicarbonate ion"((11) p. 3658).

Kara, Samachson and Spencer(12) added Ca^{45} to serum samples, ultrafiltered, and then determined the ratio of radioactive calcium in the ultrafiltrate to that in the serum. They found a normal mean ultrafiltrable calcium of 55.14% with a standard deviation of ±1.55%. Serum pH was measured but was

not included in the report. However, Dr. Spencer stated (personal communication) that the mean serum pH was 7.4 and added that "Determinations of pH were also made on some, but not all ultrafiltrates, and we have noted that there was a slight tendency of increase of pH with ultrafiltration."

Ettori and Scoggan wrote three papers on the spectrophotometry of metal indicators at two wavelengths. In their first paper(13), they reported their calculated values for the absorptivities of free murexide and the calcium complex at both 480 and 520 mu and the value for the formation constant of calcium murexide. In determining these values, they used a calcium-free solution and solutions containing calcium ion concentrations ranging from 9.4×10^{-3} to 9.4×10^{-2} M. They assumed that the murexide in the most concentrated solution was completely bound by calcium. However, using their value for the formation constant, one may calculate that at this concentration of calcium the murexide is only about 96% bound. Applying this correction to the raw data, one finds a value for the formation constant that is about 20% lower than that reported in the paper. Furthermore, the ionic strength of this concentrated solution is far in excess of the 0.150 reported.

In their second paper(14) they reported that addition of bovine serum albumin to buffered murexide solutions caused no change in the absorption curve for murexide in the range used and concluded that ionic calcium concentration may be determined in the presence of albumin.

In their third paper(15), they proposed a method for the direct measurement of ionic calcium in plasma without prior removal of protein, citing the results of their earlier experiments as justification for the omission of this step. This seems to be insufficient justification, especially in view of the technical errors noted above and of the extreme paucity in their reports of substantiating experimental data. Furthermore, Raaflaub, in his methodology review(16), states in his discussion that murexide is adsorbed on the surface of the protein molecules, causing a shift of the murexide absorption curve to longer wavelengths and thus making removal of the proteins necessary. Unfortunately, he too failed to give supporting data.

The question of protein removal by ultrafiltration or dialysis deserves further comment. First, even with perfect technical performance, physicochemical factors exert forces which require estimation and necessitate introduction of correction factors in subsequent analysis. Among the most important of these is the Donnan effect, i.e., the influence of proteins on the distribution of ions on either side of the semipermeable membrane. Donnan factors are fairly well defined for dialysis but the unqualified application of these factors to ultrafiltration has been questioned(11). Secondly, as ultrafiltration proceeds the protein concentration of the residual solution rises, owing to loss of fluid through the membrane. One would expect the calcium-protein equilibrium to be upset as a result. Modifying influences must exist, however, since the calcium ion concentration was found(4,8)

to be the same in all fractions of ultrafiltrate collected throughout the procedure. Thirdly, since the calcium-protein equilibrium association depends on the temperature, the ultrafiltration or dialysis must be carried out at 37°C in studies simulating physiological conditions. With a minimum ultrafiltration time of about two hours, the possibility of a significant denaturation and breakdown of protein must be considered. The extent and effect of this breakdown would be hard to assess with accuracy. Finally, both procedures are lengthy, not only in terms of total elapsed time but in terms of actual handling time as well.

Elimination of the requirement of protein removal would be welcome indeed. On the other hand, the technique proposed by Ettori and Scoggan(15) for direct determination of calcium ion concentration without prior removal of protein should be adopted only if its limitations are defined, a point that seems arguable in this case. It was these considerations, then, that prompted the present investigation of the effect of protein on the calcium-murexide color reaction.

A second possible effect on the color reaction, that of sodium concentration, was included in the present study. A biological fluid such as plasma contains a relatively high concentration of sodium and this concentration is subject to normal fluctuations. In working with biological fluids, then, any interference by sodium with the calcium-murexide binding should be taken into account. The results of an earlier experiment in our laboratory, studying the effects of concentration of buffer ligands (binding groups) on the color reaction,

suggested that some sodium effect did indeed exist. In that earlier experiment the concentration of buffer, containing sodium as the principal cation, was reduced by dilution with either 150 mM NaCl or 150 mM KCl, the ionic strength thus being held constant. Murexide was added to measured samples of each dilution and the spectrophotometric absorbance was then measured. It was found that when the buffer was diluted with KCl, the absorbance decreased to the same extent as when the buffer was diluted with distilled water. The absorbance did not change, however, when the buffer was diluted with NaCl. These results suggested that the absorbance of the buffered, calcium-free murexide solutions was due entirely to the presence of sodium ions, most probably as a result of sodium-murexide interaction or complex formation.

EXPERIMENT

Materials

All solutions were prepared from reagent grade chemicals as described below, using distilled water which had been passed through a mixed-bed deionizer. All glassware used in the investigation was cleaned in chromic acid cleaning solution and thoroughly rinsed with distilled water and then with distilled, deionized water.

A 2 mM solution of murexide[1] was prepared by dissolving 30.2 mg of the indicator in water and diluting to 50 ml. This solution is unstable and must be refrigerated during storage and kept in an ice bath during use. Even at refrigerator temperatures it breaks down slowly and must be made up each week.

Calcium chloride standard solution was prepared by dissolving 5.005 g $CaCO_3$ in HCl, evaporating to dryness to remove the excess HCl, dissolving the calcium chloride in water and diluting to one liter. The final concentration was 50 mM.

The stock protein solution employed was Chemvarion E-2[2], a commercial preparation of pooled human serum from which the inorganic ions had been removed. The protein concentration in this solution as it is supplied is about 6%.

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[1] Murexide (C.I. 56085), J. T. Baker Chemical Co., Phillipsburg, N. J.

[2] Kindly supplied by Clinton Laboratories, Los Angeles, Calif.

The stock solution of sodium barbital buffer was prepared by dissolving 14.714 g sodium barbital, 9.714 g sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$), and 13.896 g sodium chloride in water and diluting to 500 ml.

The stock solution of potassium barbital buffer was prepared by dissolving 13.147 g barbital, 7.005 g potassium acetate, and 17.726 g potassium chloride in water to which had been added 71.38 ml 1 N KOH, and then diluting to 500 ml with water.

Four working buffer solutions with pH 7.35 and ionic strength 0.152 were prepared by addition of HCl and either water or stock protein solution. Two protein-free solutions were prepared by adding 2.6 ml of 2 N HCl to 50 ml stock buffer and diluting to 250 ml with water, using stock potassium buffer for the first (Soln. A, containing neither sodium nor protein) and stock sodium buffer for the second (Soln. B, containing sodium but not protein). The protein-containing solutions were made up just before use in quantities sufficient for only that run. These solutions consisted of 10 ml

Table 1. Ionic Concentrations in Working Barbital Buffer Solutions

Substance	Conc., mM
Sodium ion, Na^+, or potassium ion, K^+	152.2
Chloride ion, Cl^-	115.9
Acetate and barbital anions, AcO^- + $Barb^-$	36.3
Undissociated acid, AcOH + BarbH	20.8

Table 2. Method of Preparation of Test Solutions

Sodium ion conc., mM			0			70			140		
Protein Fraction			0	½	1	0	½	1	0	½	1
Working buffer solns., ml / $CaCl_2$ (a) and KCl (b), ml			4.6 A	2.3 A 2.3 C	4.6 C	2.3 A 2.3 B	2.3 B 2.3 C	2.3 C 2.3 D	4.6 B	2.3 B 2.3 D	4.6 D
Calcium Concentration, mM	0	0.4 b	1	2	3	4	5	6	7	8	9
	1	0.1 a 0.3 b	10	11	12	13	14	15	16	17	18
	2	0.2 a 0.2 b	19	20	21	22	23	24	25	26	27
	4	0.4 a	28	29	30	31	32	33	34	35	36

Numbers in the body of the table are code numbers assigned combinations of constituents.

stock buffer and 0.52 ml 2 N HCl diluted to 50 ml with stock protein solution, using stock potassium buffer for [illegible]e (Soln. C, containing protein but not sodium) and stock sodium buffer for the other (Soln. D, containing both sodium and protein). The pH of each working solution was checked with a Beckman Zeromatic pH meter and adjusted to 7.35 when necessary by addition of 0.1 N HCl. The ionic strength of each solution, disregarding protein effects, was 0.152, with the ionic concentrations as shown in Table 1. The sodium and potassium solutions were identical in every respect except that potassium ion was substituted for sodium ion in the latter solutions.

Method

The procedure for studying the effects discussed earlier involved measuring the absorbances of murexide solutions containing the combinations of calcium, sodium and protein that are shown in Table 2. Thus, each solution had a calcium concentration of 0, 1, 2 or 4 mM, a sodium ion concentration of 0, 70 or 140 mM, and a protein fractional strength of 0, ½ or 1. The designation of fractional strength of protein was used because the exact percentage concentration was not known, being determined in a later stage of the investigation.

The four working solutions, prepared as described earlier, were dispensed from separate 5 ml burets into tubes to give four 4.6 ml samples of each of the nine combinations of sodium and protein (Table 2). All tubes were placed in an ice bath immediately after dispensing. To each tube was added

x ml 50 mM $CaCl_2$ and (0.4 - x) ml 150 mM KCl, where x is 0, 0.1, 0.2 or 0.4. From Table 2, one may ascertain how each sample was prepared. The dilution of the buffers with calcium and potassium chloride solutions caused a reduction of sodium ion concentration from 152 to 140 mM. It should be noted that the only function of the potassium ion, substituted for both sodium and calcium ions, was maintenance of ionic strength.

Three tubes at a time were then transferred from the ice bath to a 39°C water bath, allowed to equilibrate, and 3 ml of each sample was pipetted into a separate 1 cm square cuvette. The absorbance of each was read against a water blank in a Beckman Model DU spectrophotometer at 470 mu, after which the cuvette holder with the samples was returned to the water bath. Following addition of 0.1 ml murexide solution to each sample, the absorbance was read again. While the absorbances were being read a new series of three tubes was put into the bath to warm. These steps were repeated until the absorbances of all 36 tubes (Table 2) had been measured. The elevated temperature of the water bath was maintained to correct for the slight cooling of the samples which occurred during measurement of the absorbances. The temperature at the time of measurement was 37°C.

The procedure described was replicated four times. To remove possible bias owing to murexide or protein degradation, the order in which the absorbances of the combinations of constituents were measured was randomized for each replication.

The total protein concentration was determined by the biuret method(17) in six samples of fractional strength 1 from

each replicate, using the 2 ml remaining in each tube after withdrawal of the 3 ml sample for absorbance measurements. Paper electrophoresis of the stock protein solution revealed normal percentages of the various protein fractions.

Results

Since the initial absorbance reading in each case represented the reagent blank, the true absorbance of murexide and its calcium complex is expressed by the difference between the final and initial absorbance readings. These corrected absorbances were calculated for each tube and are given in App. 2.

The formula of Raaflaub(16) relating absorbance to calcium ion concentration is given in Eq. 5, App. 3. A slightly more convenient function is $A/A_0 - 1$, where A is the absorbance of the sample and A_0 is the absorbance of Tube 1. The calculated functions are shown in Appendix 3.

In the statistical analysis the hypotheses tested, stated in null form, were:

1. There is no significant difference among the ratios of absorbances of solutions which vary only in protein concentration.
2. There is no significant difference among the ratios of absorbances of solutions which vary only in sodium ion concentration.

Analysis of variance applied to the data also tested the significance of calcium concentration, of the various interactions, and of the reproducibility. The results of this analysis of variance are given in Table 3. Thus it can be seen that significant differences were found for calcium and sodium, for

Table 3. Results of Analysis of Variance

Source	df	s.s.	m.s.	F	p[1]
Calcium (Ca)	3	7.0337	2.3446	48	.001
Protein (Pr)	2	0.1915	0.09575	2.0	>.10
Sodium (Na)	2	0.1008	0.0504	112	.001
Ca x Pr	6	0.2860	0.0477	106	.001
Ca x Na	6	0.0027	0.00045	<1	
Pr x Na	4	0.0015	0.00038	<1	
Ca x Pr x Na	12	0.0042	0.00035	<1	
Replicates	3	0.0234	0.0078	17.3	.001
Within repl.	105	0.0482	0.00046		
Total	143	7.6920			

[1] Values of 0.05 or less are significant.

Table 4. Results of Regression Analysis

Calcium ion level, mM	0	1	2	4
Mean sodium level, $\bar{x}$	70	70	70	70
Mean sum of absorbance ratio functions, $\bar{y}$	0.5007	1.0962	1.5922	2.3073
Variance about regression, $s_{y \cdot x}^2$	0.0007	0.0018	0.0042	0.0057
Regression coefficient, b	0.00154	0.00148	0.00148	0.00105
Test for linearity F[1]	0.51	0.58	0.09	0.14

[1] Values of F less than 1.0 are not significant.

the first order interaction between calcium and protein, and for the replicate means. These results will be discussed in the following section.

Regression analysis was applied to the data to determine the nature of the sodium effect. Absorbance ratio functions for the three levels of protein at each level of sodium were summed and y values were determined, y being the mean for the four replicates of the sum of the functions. The value for the regression coefficient, b, in the regression equation, $y' = \bar{y} + b(x - \bar{x})$, was calculated for each level of calcium. In the equation, y' is the estimated value of y for a given value of x, $\bar{y}$ is the mean of y for the three levels of sodium, x is the sodium ion concentration, and $\bar{x}$ is the mean sodium ion concentration. The values for b are given in Table 4 with the other pertinent data. The regression curve for each level of calcium was tested for linearity, with no significant deviation from linearity found.

Application of Duncan's Multiple Range test to the data for replications showed that the means for the first, second and fourth replicates did not differ significantly from each other but all of these differed from the mean for the third.

The data for biuret determination of protein concentration were tested for differences in mean protein concentration among the four replicates. Since analysis of variance showed no significant difference among the means, the data for the four replications were pooled. Calculation of the mean protein concentration from the pooled data gave values of 4.35% and 0.028% for the mean and standard deviation respectively.

DISCUSSION

Two comments regarding the technique are in order. First, the choice of 470 instead of 480 mu (the absorption maximum for calcium murexide) for the measurements gives a greater ratio of absorbances, allowing more precise measurement of the calcium concentration. Secondly, since Raaflaub's formula (Eq. 5, App. 3) eliminates the necessity of knowing the total murexide concentration, a fresh solution need not be prepared every day. Weekly preparation of a fresh solution is deemed sufficient. The formulation has the additional advantage that the ratio of sample volume to murexide volume may be changed at will so long as both standards and unknowns are brought to the same final volume.

When protein is added to a calcium-containing solution, it may bind varying amounts of calcium to a maximum of about 50% of the calcium in the solution. As a result of this phenomenon the calcium ion concentration per se in most of the tubes was not known. However, since the experiment sought to measure only the change in apparent concentration rather than the absolute calcium ion concentration, relative information was deemed sufficient. The use of relative measurements also made it unnecessary to calculate the reciprocals of the absorbance ratio functions.

The question of the interference of the calcium-protein interaction with measurement of protein effect on the color

reaction may be mentioned. The interaction variance is made up of a component from the second order interaction, a component from the Ca x Pr interaction alone, and an error component. The protein effect has in addition to these three components a component from the Na x Pr interaction and a component from protein alone. Because the Ca x Pr mean square (m.s.) differed very significantly from the error m.s., the Ca x Pr m.s. was used to test the protein effect (and the calcium effect). Since the Na x Pr interaction had already been shown to be insignificant ($F<1$), all that remained to be tested was the component owing to protein alone.

The experimental results, confirming previous reports, indicate that: 1) A significant effect of calcium shows that there actually is a calcium-murexide color reaction, and 2) a significant calcium-protein interaction verifies that protein binds calcium.

The finding of a significant difference among replicate means points out the difficulty of the test procedure. Since the difference was in the third replicate, it seemed obvious that the source of error might well be the absorbance, A_o, of Tube 1, since A_o is used for calculating the absorbance ratio functions of all the 35 other tubes. Inspection of the data showed that had the absorbance of Tube 1 been 0.410 instead of the recorded 0.412 the significant difference would not have occurred. Had the experiment been conducted on repeated observations instead of replications, this source of error would have been included in the experimental error, thus making the experiment less sensitive. In the present study the

effect of replications is taken out. The significant replicate mean square does not influence the experimental error.

The existence of a sodium effect was one of the principal hypotheses tested. The finding of a linear regression for sodium at all levels of calcium concentration suggests that its action is not a binding of murexide represented by the reaction, $m\ Na^{+} + Mu^{-n} = Na_mMu^{m-n}$, with its associated equilibrium equation, $K = \frac{(Na_mMu^{m-n})}{(Na^{+})^m(Mu^{-n})}$, since this reaction would result in a nonlinear plot. While the precise reaction remains undefined, relevant conclusions can be drawn from the results. Insertion into the regression equation,

$$y' = \bar{y} + 0.00148(x - 70),$$

of $\bar{y} = 1.0962$ (The value of $\bar{y}$ for a calcium level of 1 mM) and x values of 120, 140 and 160 gives values of 0.3901, 0.3999 and 0.4098 for $y'/3$, the estimated ratio of absorbances. The change of ratio produced by a 20 mM change in sodium ion concentration is equivalent to that produced by changing the calcium ion concentration by about 0.04 mM. Thus the usual variation of plasma sodium concentration will cause only a negligible change in the apparent calcium ion concentration. However, the effect is of sufficient magnitude that some defined level of sodium ion approximating that of the plasma should be included in the buffer used in the test procedure.

Walser in 1961(18) reported finding no change in absorbance when either potassium or ammonium salts were substituted for sodium salts in barbital buffer. However, he reported

more recently (in press, 1964) that a sodium-murexide interaction does exist.

The second hypothesis tested was the effect of changing protein concentration. The experimental results allow one to conclude only that no significant effect has been demonstrated. More extensive testing may prove conclusively that the effect either does or does not exist. An experiment to test this will be undertaken in the near future.

Since proteins are relatively insoluble in water, the protein concentration in the stock solution was slightly lower than that in normal human plasma. This concentration was reduced still further by the addition of the buffer concentrate, HCl, and the calcium and potassium chloride solutions. As a result the final protein concentration was at most only about 60-80% of the protein concentration of human plasma.

Summary

The effects of sodium and protein on the calcium-murexide reaction were studied by measuring the absorbance of murexide solutions with sodium levels of 0, 70 and 140 mM, calcium levels of 0, 1, 2 and 4 mM, and protein levels of 0, 2.18 and 4.35%. Four replications were made, with the order of measurement randomized for each replication. In addition to the effect of calcium concentration and the calcium-protein interaction, significant differences were found among the means for replications ($p<.001$) and sodium concentration ($p<.001$). The regression of absorbance on sodium ion concentration did not deviate significantly from linearity. A significant protein effect was not demonstrated.

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APPENDIX 1

GLOSSARY OF TERMS

Ionic Strength

The ionic strength, μ, of a solution is defined as one-half the sum of the products of the molality and the square of the charge, summed over all ionic species; i.e.,

$$\mu = \tfrac{1}{2}\sum m_i z_i^2,$$

where m_i is the molal concentration of the ith ionic species and z_i is its valence or charge. In dilute solutions, the molarity, c, may be substituted for the molality.

Spectrophotometric Terms

The Beer-Lambert Law states that when a solution contains a solute which absorbs energy from power radiated through the solution, the ratio of the emerging power, P, to the incident power, P_o, is $P/P_o = e^{-k'cl}$, where k' is a constant for a given solute, c is the concentration of the solute, and l is the length of the path through the absorbing solution. If a reference solution is used which is identical in constitution to the unknown solution except that it contains none of the solute in question, and if the concentration and length of path are given in moles per liter and centimeters respectively, then k will be the molar absorptivity and the absorbance, A, will be given by the equation, $A = \log(P_o/P) = kcl$.

If one is familiar with the older terminology, it can be seen from the above discussion and equations that the absorbance is the same as the extinction, the absorptivity is the same as the extinction coefficient, and the power is the same as the intensity.

APPENDIX 2

RAW DATA

Replicate / Tube	Absorbance				Ratio, $(A/A_0 - 1)$			
	1	2	3	4	1	2	3	4
1	.426	.417	.412	.406	.0000	.0000	.0000	.0000
2	.495	.480	.471	.462	.1620	.1511	.1432	.1379
3	.529	.513	.507	.524	.2418	.2302	.2306	.2906
4	.436	.430	.437	.421	.0235	.0312	.0607	.0369
5	.509	.495	.471	.477	.1948	.1870	.1432	.1749
6	.554	.533	.528	.511	.3005	.2782	.2816	.2586
7	.461	.456	.451	.437	.0822	.0935	.0947	.0764
8	.561	.507	.500	.494	.2300	.2158	.2136	.2167
9	.565	.557	.524	.525	.3263	.3357	.2718	.2931
10	.539	.530	.516	.517	.2652	.2710	.2524	.2734
11	.572	.560	.545	.535	.3427	.3429	.3228	.3177
12	.597	.583	.572	.558	.4014	.3981	.3883	.3744
13	.563	.547	.526	.540	.3216	.3118	.2767	.3300
14	.586	.565	.554	.543	.3756	.3549	.3447	.3374
15	.619	.599	.593	.586	.4530	.4364	.4393	.4433
16	.577	.565	.546	.545	.3545	.3549	.3252	.3424
17	.567	.591	.570	.566	.3310	.4173	.3835	.3941
18	.639	.618	.599	.585	.5000	.4820	.4539	.4409
19	.626	.614	.595	.610	.4695	.4724	.4442	.5025
20	.641	.622	.602	.607	.5047	.4916	.4612	.4951
21	.671	.639	.607	.629	.5751	.5324	.4733	.5493
22	.650	.634	.615	.614	.5258	.5204	.4927	.5123
23	.661	.621	.620	.611	.5516	.4892	.5048	.5049
24	.686	.656	.622	.625	.6103	.5731	.5097	.5394
25	.656	.654	.639	.624	.5399	.5683	.5510	.5369
26	.669	.662	.634	.633	.5704	.5875	.5388	.5591
27	.690	.661	.646	.640	.6197	.5851	.5680	.5764
28	.764	.743	.728	.719	.7934	.7818	.7670	.7709
29	.754	.732	.687	.705	.7700	.7554	.6675	.7364
30	.739	.729	.702	.703	.7347	.7482	.7039	.7315
31	.776	.750	.743	.740	.8216	.7986	.8034	.8227
32	.764	.733	.708	.710	.7934	.7578	.7184	.7488
33	.749	.743	.682	.698	.7582	.7818	.6552	.7192
34	.789	.759	.743	.739	.8521	.8201	.8034	.8202
35	.774	.731	.709	.738	.8169	.7530	.7209	.8177
36	.777	.749	.733	.708	.8239	.7962	.7791	.7438

APPENDIX 3

DERIVATION OF A FORMULA FOR DETERMINATION OF CALCIUM ION CONCENTRATION

For the equilibrium, Ca + Mu = CaMu, the law of mass action states that the formation constant,

$$K = \frac{(CaMu)}{(Ca)(Mu)} , \qquad (1)$$

where Ca represents calcium ion, Mu is free murexide, CaMu is the complex, and brackets denote concentration. In a solution containing calcium and murexide, the total murexide concentration is shown by $(Mu)_t = (Mu) + (CaMu)$. Rearranging Eq. 1 and substituting for (Mu) by $(Mu) = (Mu)_t - (CaMu)$ gives: $K(Ca)\left[(Mu)_t - (CaMu)\right] = (CaMu)$, which may be rearranged to give:

$$(CaMu) = \frac{K(Ca)(Mu)_t}{1 + K(Ca)} . \qquad (2)$$

In the solution the absorbance,

$$A = a_0(Mu) + a_1(CaMu)$$
$$= a_0\left[(Mu)_t - (CaMu)\right] + a_1(CaMu) \qquad (3)$$

where a_0 is the absorptivity of the free murexide and a_1 is the absorptivity of the complex.

Combination of Eqs. 2 and 3 gives:

$$A = a_0\left[(Mu)_t - \frac{K(Ca)(Mu)_t}{1+K(Ca)}\right] + a_1 \cdot \frac{K(Ca)(Mu)_t}{1 + K(Ca)}$$

or:
$$A = (Mu)_t\left[a_0 + (a_1 - a_0)\frac{K(Ca)}{1 + K(Ca)}\right] . \qquad (4)$$

Since the murexide concentration gradually declines owing to degradation, it is advantageous to remove it from the equation. Since in a calcium-free solution, the absorbance, $A_o = (Mu)_t a_o$, $(Mu)_t$ may be removed from Eq. 4 by dividing Eq. 4 by the above, yielding:

$$\frac{A}{A_o} = 1 + \frac{a_1 - a_o}{a_o} \cdot \frac{K(Ca)}{1 + K(Ca)} . \qquad (5)$$

If we now represent the constant, $(a_1 - a_o)/a_o$, by ϕ, rearrange and take the reciprocal of both sides of the equation, we obtain:

$$(A/A_o - 1)^{-1} = \frac{1 + K(Ca)}{\phi K(Ca)} = \frac{1}{\phi K} \cdot \frac{1}{(Ca)} + \frac{1}{\phi} . \qquad (6)$$

Plotting the left side of Eq. 6 on the ordinate and $(Ca)^{-1}$ on the abscissa gives a linear plot with slope $(\phi K)^{-1}$ and Y-intercept $1/\phi$. Thus, Eq. 6 also serves as a simple graphical method for the determination of the formation constant.

Table 2. Method of Preparation of Test Solutions

Sodium ion conc., mM			0			70			140		
Protein Fraction			0	½	1	0	½	1	0	½	1
Working buffer solns., ml / $CaCl_2$ (a) and KCl (b), ml			4.6 A	2.3 A 2.3 C	4.6 C	2.3 A 2.3 B	2.3 B 2.3 C	2.3 C 2.3 D	4.6 B	2.3 B 2.3 D	4.6 D
Calcium Concentration, mM	0	0.4 b	1	2	3	4	5	6	7	8	9
	1	0.1 a 0.3 b	10	11	12	13	14	15	16	17	18
	2	0.2 a 0.2 b	19	20	21	22	23	24	25	26	27
	4	0.4 a	28	29	30	31	32	33	34	35	36

Numbers in the body of the table are code numbers assigned combinations of constituents.