Method for Determination of Calcium in Serum, Parotid Fluid and Urine in the Weightless State

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Since the Manned Orbiting Laboratory program includes on-board monitoring of calcium metabolism, a method suitable for this determination under conditions of weightlessness has been investigated. Most techniques for calcium assay that are used at the present time have certain disadvantages that would preclude their use under conditions of weightlessness; whereas the nuclear fast red (NFR) technique of Baar, as modified by Kingsley and Robnett, is simple, and all steps in the procedure are compatible with performance in the weightless state. The possible interference of magnesium, hemolysis, and protein was investigated. These effects should be minimal unless drastic changes in magnesium and/or protein levels should occur. The interference by hemolysis may be obviated by the use of a proper blank. The method has been adapted to an apparatus to effect mixing in the weightless state and is regarded as a practicable first-generation method for monitoring calcium metabolism.

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UNCERTAINTY about the possible effects of weightlessness upon bone structure and calcium metabolism has prompted the desire to include on-board monitoring of calcium metabolism in the Manned Orbiting Laboratory Program.[2] It is the purpose of this paper to report experience with a method for quantitative determinations of calcium that appears to be acceptable for use in the weightless state. The method is the Kingsley-Robnett[6] modification of the procedure of Baar,[1] using nuclear fast red dye (hereinafter abbreviated NFR). This procedure was selected for use under conditions where the low number of determinations per day would not justify the orbiting of automated equipment. Favorable characteristics of the procedure include, in addition to suitability for the gravity-free state, freedom from volatile reagents likely to contaminate the cabin atmosphere, and the use of simple apparatus with low power requirement. The technique is simple, so that minimal laboratory training is required, and reproducibility and reliability of the method are acceptable.

Most techniques for calcium assay that have been used or that are used at the present time have certain disadvantages that would preclude their use under conditions of weightlessness; i.e., the classical Clark-Collip[4] procedure involves a precipitation of the calcium with subsequent washing of the precipitate prior to its dissolution and titration. The flame photometric[7] (p. 71) and atomic absorption[9] methods involve the use of an open flame which would be a hazard in a space cabin. Most spectrophotometric procedures, such as the Roe and Kahn[8] or Ferro and Ham[5] techniques, require the precipitation and washing of the calcium prior to the color development and are considerably more complex than the single-step NFR method. Procedures involving the use of chelating agents usually require a titration step which is not feasible in the weightless state.

METHOD

The NFR technique involves only the addition of the biological fluid to the working dye solution and the subsequent spectrophotometric measurement of the intensity of the dye color to determine the calcium content of the specimen. The stock dye solution consists of 100 mg. of nuclear fast red dye in 100 ml. of distilled water. The working dye solution is prepared by diluting 10 ml. of this solution to 50 ml. with 0.1 NaOH. The calcium content of a biological fluid is then determined by mixing 0.2 ml. of the fluid with 6 ml. of the working dye solution and reading the absorption of light at 575μ within 10 to 20 minutes after mixing.

For monitoring astronauts in the MOL program, determinations on urine and serum, and perhaps parotid fluid, would be required. Serum and parotid fluid can be used directly in the NFR method, but random urine specimens require dilution for accurate determination.

RESULTS AND DISCUSSION

Purification of dye—According to Kingsley and Robnett,[6] the NFR dye must be purified. To check this requirement, color development with a purified batch of dye was compared with that of an unpurified sample of dye obtained from Matheson, Coleman, and Bell. The comparison was made by measuring the intensity of color produced with various calcium standards added to working dye solutions prepared from purified and unpurified dye. As shown in Figure 1, the unpurified dye gave essentially the same results as the purified dye; therefore, the purification procedure does not seem necessary with this particular batch of dye.

Stability of color—The stability of the color developed was investigated. A standard calcium solution was pipetted, the color developed, and this solution was read at five-minute intervals over a period of one hour. As Figure 2 shows, the color faded slowly with time. In this instance there was a drop in apparent value from 5.3 to 4.6 mEq./l. over a period of one hour, which was calculated to be a decrease of 0.7 mEq., or 13 per cent of the original concentration. While the decrease was roughly linear with respect to time, the values read between 10 and 20 minutes after mixing were relatively constant. During that 10 to 20 minute interval, the decrease was less than 0.1 mEq./l., or less than 2 per cent of the original value. It appears, therefore, that colorimetric readings should be made between 10 and 20 minutes after mixing, and that all samples and standards should be read after the same elapsed time after mixing.

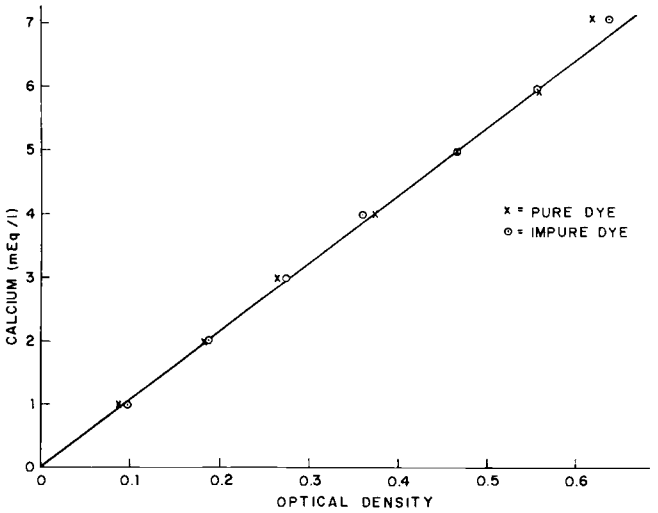

Fig. 1. Standard calcium curves using purified and non-purified NFR dye.

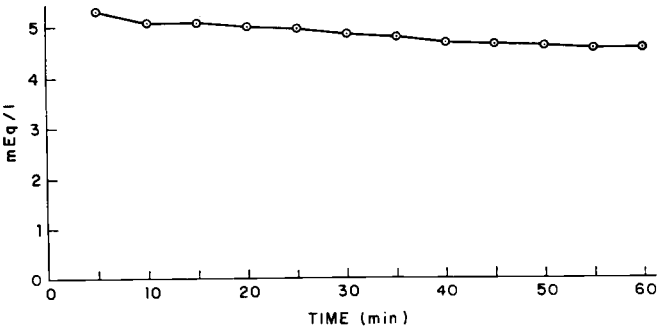

Fig. 2. Changes in color intensity after mixing working NFR dye solution with calcium standard.

Normal values—Data obtained from duplicate analyses of 75 samples of serum, 75 samples of parotid fluid, and 20 samples of urine gave average values of 5.7, 1.9, and 4.4 mEq./l., respectively. The corresponding ranges and standard deviations are shown in Table I. The standard deviations shown do not apply to the distribution of the values about the mean, but they are the standard deviations or replicate determinations on the same sample. As such, they are a measure of the pre-

TABLE I. NORMAL CALCIUM VALUES OBTAINED WITH NFR TECHNIQUE APPLIED TO VARIOUS BIOLOGICAL FLUIDS

Sample	Number of Samples	Mean Value of Replicates: mEq/l	Range: mEq/l	Standard Deviation
Serum	75	5.7	5.1-6.2	0.09
Parotid Fluid	75	1.9	1.3-2.8	0.06
*Urine	20	4.4	2.6-5.9	0.05

*All urine samples were random samples diluted fivefold prior to analysis.

cision of the method. Accepted values for serum calcium levels are 4.5 to 5.8 mEq./l. by standard methods such as the Clark-Collip[4] procedure and 4.5 to 5.7 mEq./l. by the EDTA titration method[7] (p. 75). Our values are slightly higher, probably because the dye is sensitive to magnesium. Although one equivalent of magnesium ion develops less color than one equivalent of calcium ion, in our hands, the total serum calcium measured includes some contribution by serum magnesium. Presumably, the same would be true of parotid fluid and urine levels.

It should be emphasized that the urinary levels reported here are not 24-hour excretion levels; they are levels measured in random samples supplied during working hours by laboratory personnel. Furthermore, in order to read results of the urine samples from the standard curve, the random urine was diluted fivefold. The values recorded, therefore, should be multiplied by five to obtain the actual urine concentration. These values appear reasonable, but the purpose of their measurement was to assess the reproducibility of the determination instead of to measure the 24-hour excretion.

The standard deviations shown in Table I are least for the serum and greatest for the urine samples; but, when per cent deviation is considered, the precision of the method is greater with urine than with serum. Experiments to be described later in this paper indicate that protein affects color development. Probably, the protein in serum causes the decrease in precision of levels measured in that fluid. Parotid fluid, containing only about 50 per cent of the protein in serum, gives somewhat less precision but contains less calcium. Urine, containing no protein, gave the most precise results.

Recovery studies—Experiments were performed to determine whether calcium ion added to serum could be recovered. The calcium levels in pooled human serum samples were determined. Varying amounts of solutions of known calcium content were then added to aliquots of these pools, and the new calcium levels were measured. In the case of human serum, approximately 87 per cent of the calcium was recovered when 1.5 mEq./l. was added, and about 71 per cent when 2.0 mEq./l. was added. The recoveries from ion-free serum averaged 95 per cent upon initial addition of calcium, and 88 per cent when another small increment of calcium was added. Although recoveries were better from ion-free serum than with serum, they were not complete. With mixtures of aqueous solutions, however, recoveries are quantitative. In other words, equal volumes of 4.0 and 2.0 mEq./l. standards will read the same as a 3.0 mEq./l. standard. Incomplete recovery of added calcium, therefore, appears to be caused by serum constituents, presumably protein.

This inference is supported by the following experiment. Water was added to ion-free serum to dilute the later to ½, ¼, and ⅛ of its original concentration. Water was used as an infinite dilution (zero concentration) of the ion-free serum. To 4.75 ml. of each of these dilutions was added 0.25 ml. of a 100 mEq./l. calcium standard in water. The concentration of calcium ion in each of these solutions, therefore, should have been 5.0

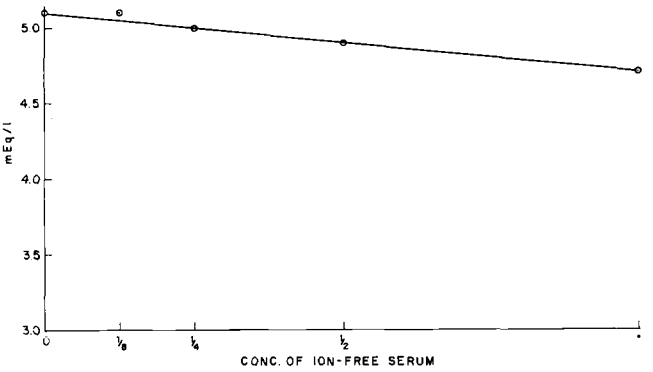

Fig. 3. Addition of a known quantity of calcium to dilutions of ion-free serum.

mEq./l. The actual concentrations measured are shown in Figure 3. The observed recovery in this instance is 4.73/5.10, or 93 per cent in the undiluted ion-free serum. It is clear that part of the calcium ion added is bound by some serum constituent (presumably protein) so that incomplete recovery is observed and that recoveries approach 100 per cent with increasing dilution of the ion-free serum. These findings are interpreted as evidence for interference of protein in the quantitative determination.

Magnesium interference—In their original article, Kingsley and Robnett[6] described results indicating that low levels of magnesium ion did not interfere with the calcium determination. In our hands, however, there is an increment in the color intensity when magnesium is added to a standard serum (Versatol). Table II shows

TABLE II. MAGNESIUM INTERFERENCE AS DEMONSTRATED BY THE ADDITION OF KNOWN QUANTITIES OF MAGNESIUM TO CONTROL SERA

Magnesium Added mEq/l	Calculated Concentration: mEq/l	Observed Concentration: mEq/l	% of Expected Increase
0.0	4.3	4.3	0.0
0.8	5.1	4.7	50.0
1.7	6.0	4.8	29.4
2.5	6.8	5.0	28.0
3.3	7.6	5.6	39.4
4.2	8.5	5.8	35.7

this effect. The measured increment in apparent calcium content averaged approximately 37 per cent of the number of equivalents of magnesium ion added. This figure is to be compared with the 93 per cent recovery of calcium ion added to ion-free serum and 88 per cent recovery of calcium added to Versatol. Variations in magnesium level of serum, therefore, would affect the apparent calcium level measured; but the actual changes measured would be less than half of the true change in magnesium level. The contribution of magnesium should not seriously affect the usefulness of the method for monitoring calcium metabolism in astronauts, since changes in the level would be the significant information to be obtained. Changes in serum magnesium level are measured only about one-half as efficiently as changes in serum calcium. Since the serum magnesium level is normally slightly less than 2 mg. per cent, a 100

per cent change in this level would change the serum calcium level only about 0.5 mEq./l. Such an error in the calcium level would be undesirable, but it is rather unlikely that such a variation in the serum magnesium level in an astronaut would occur.

Effect of hemolysis—The effect of hemolysis upon the calcium level measured by the nuclear fast red method was investigated. Samples of blood were obtained and centrifuged to separate serum and clot. Half of the clear serum was withdrawn. The other half was left in contact with the clot, which was then macerated by vigorous mechanical agitation. After standing an additional 18 hours, the tubes were centrifuged and the markedly hemolyzed serum separated. The apparent calcium content of the original and hemolyzed serum was then determined in the usual way. Results are shown in Table III. It is obvious that the hemolyzed samples all

TABLE III. EFFECT OF HEMOLYSIS UPON THE CALCIUM LEVEL MEASURED BY THE NFR METHOD

Sample	Nonhemolyzed	Hemolyzed	Hemolyzed Blank	Hemolyzed Minus Blank
1	5.8	6.3	0.6	5.7
2	5.9	6.4	0.7	5.7
3	5.5	6.1	0.6	5.5
4	5.7	6.2	0.7	5.5
5	5.5	6.0	0.6	5.4

have a higher apparent calcium content than do the nonhemolyzed samples. Blanks were prepared using 0.2 ml. of clear or hemolyzed serum in 6.0 ml. of 0.1 NaOH (not containing the NFR dye). The increment of this hemolyzed blank over the nonhemolyzed blank was taken as the apparent calcium content due to hemolysis. This value is shown in the fourth column of Table III. When this figure is subtracted from the corresponding value for calcium content of the hemolyzed sample, the resulting calcium level is strikingly similar to that for the nonhemolyzed sample. In other words, the apparent increase in calcium in the hemolyzed sample must have been caused by the color of the hemoglobin. A suitable blank corrected for this chromogen, and the resulting value was very close to that for the nonhemolyzed sample. Apparently, the presence of iron as a result of hemolysis did not affect the calcium determination.

Evaluation—Certain conclusions can be reached regarding the usefulness of the NFR method for projected use in on-board monitoring of calcium metabolism in astronauts in a military orbiting laboratory.

First of all, the method does not measure calcium ion exclusively. Magnesium ion is also measured, although only ½ to ⅓ as efficiently as calcium.

In addition, protein apparently interferes with the determination to cause incomplete recoveries. The magnitude of this effect was approximately 0.4 mEq./l. for undiluted serum versus the protein-free solution. This quantity, therefore, should represent the maximum error in apparent calcium level that could result from a 100 per cent change in protein level. In other words, the effect of variations in serum protein concentrations should not be very marked unless drastic changes in protein levels occurred.

Hemolysis will cause an increase in apparent calcium, but this can be corrected by an appropriate blank.

Added to this appraisal of acceptability are the positive virtues that (a) all steps in the procedure are compatible with performance in the weightless state and (b) the procedure is extremely simple. The method is therefore being adapted to an apparatus[3] to effect mixing in the weightless state. This apparatus, in turn, is being adapted to the Bio-Courier apparatus already in being for quantitative readout and telemetry of results. This determination is therefore regarded as a practicable first-generation method for on-board monitoring of calcium metabolism under the conditions of weightlessness which will be experienced in the Manned Orbiting Laboratory.

SUMMARY

The nuclear fast red method for measuring calcium concentrations in biological fluids was investigated.

Purification of a batch of dye proved unnecessary.

Color developed was relatively stable between 10 and 20 minutes after mixing.

Normal values obtained were slightly higher than those reported for classical procedures.

Recoveries of calcium ion added to serum averaged 90 per cent but increased with decreasing protein concentration.

Addition of magnesium ion increased the color intensity but, on a molar basis, only ½ to ⅓ as efficiently as calcium ion.

The color interference resulting from hemolysis can be corrected by an appropriate blank.

All steps in this method appear to be compatible with performance in the weightless state.

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