FREE CALCIUM IN THE PLASMA OF HEMODIALYSIS PATIENTS: A CLINICAL TRIAL OF A NEW METHOD OF QUANTITATING

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ABSTRACT

FREE CALCIUM IN THE PLASMA OF HEMODIALYSIS PATIENTS:

A CLINICAL TRIAL OF A NEW METHOD OF QUANTITATING

By

Carolyn Smith

The level of free calcium in the plasma of hemodialysis patients is influenced by several factors. If it is not carefully regulated, serious complications may develop.

Methods previously described for quantitating free calcium are not practical for use in the general clinical laboratory. They are time consuming, require large specimens, and some call for elaborate equipment.

The procedure used in this clinical evaluation has none of these limitations. It is rapid, reasonably accurate, requires no special equipment and comparatively small quantities of plasma are sufficient.

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INTRODUCTION

Patients undergoing long-term hemodialysis are subject to a great number of physiologic changes. Their blood must constantly be monitored for changes in hemoglobin, urea, creatinine and electrolytes, so that the efficiency of their dialysis may be evaluated. Occasionally, the dialysate solution must be adjusted to conform better to the physiology of a particular patient.

The plasma calcium concentration is frequently below normal in chronic renal failure for reasons not well understood. It may be due in part to decreased intestinal absorption. It may also be the consequence of the high concentration of plasma phosphorus, on the principle that the relationship between plasma concentrations of calcium and phosphorus are reciprocal. The uremic patient is somewhat protected from tetany by the presence of acidosis, since a higher proportion of the total calcium is ionized at a low pH (Wing, 1968).

Chronic renal failure is often associated with a variety of bone diseases, called "renal osteodystrophy." Some cases are also complicated by metastatic calcification due to secondary hyperparathyroidism (Black, 1967). With the dual risks of secondary hyperparathyroidism and metastatic calcification, it is of considerable importance to define the optimum calcium ion concentration of dialysis fluid for maintenance hemodialysis.

Direct measurements have shown that the external calcium balance during dialysis depends on the gradient between the plasma ultrafilterable (ionized) calcium and the calcium concentration in the dialysis fluid

(Wing, 1969). Since it is known that the removal of calcium by dialysis is a stimulus to parathyroid activity, it is concluded that the calcium concentration used should not be less than the ultrafilterable calcium (Wing, 1968).

The level of ionized calcium in hemodialysis patients is of considerable importance, and since its variation from the normal accurately reflects the status of calcium metabolism in the body, a method of its quantitation is needed. The methods currently in use for the quantitation of ionized calcium do not lend themselves readily to use in routine laboratory testing.

For these reasons, I have undertaken a clinical evaluation on hemodialysis patients, of a technique developed by a fellow graduate student (White, 1971). This method quantitates the calcium in an ultrafiltrate and appears to reflect the level of ionized calcium in the hemodialyzed patient.

HISTORICAL REVIEW

Calcium is present in the human body in greater quantities than any other cation. Approximately 1200 gms. are in the adult skeleton, and 0.3 gm. in the plasma (Hoffman, 1970). The calcium found in the plasma exists in 3 forms: protein-bound, ionized, and a small amount in complex with organic acids. These 3 portions exist in equilibrium. It is the ionized fraction of plasma calcium which is important in physiologic functions.

The regulation of neuromuscular excitability is calcium dependent. Membrane permeability is generally decreased by calcium ions; this effect balances the opposite action of sodium and potassium. If the level of ionized calcium falls sufficiently, both muscle and nerve excitability increase, resulting in tetany (Guyton, 1961). The calcium ion concentration also affects the myocardium. An increase tends to increase contractility and prolong systole; a decrease tends to weaken the strength of contraction. Many enzyme systems, such as adenosine triphosphate, lipase and succinic dehydrogenase, are dependent on calcium ions as co-factors (White, Handler and Smith, 1968). Adequate calcium is also necessary for the mechanism of blood coagulation, development of a healthy fetus, and effective lactation (Guyton, 1961). By far, the greatest amount of calcium in the body is involved with the formation and maintenance of the bony skeleton and the teeth.

Calcium is abundant in many foods, the primary source being milk and milk products. The major site of absorption of dietary calcium is the lower portion of the small intestine. Active transport of calcium ions occurs across the ileal mucosa; the system is capable of operating against a fivefold concentration gradient (White, Handler and Smith, 1968). The minimal passage of calcium across the duodenal membrane is largely by diffusion. The rate of absorption of calcium is dependent on several factors, including pH, fat absorption, and the concentration of phosphates in the intestinal contents.

The serum calcium concentration is homeostatically maintained. The mechanism includes a storage compartment, the skeleton, which may be drawn upon or into which an excess may be deposited; the kidneys which tend to conserve calcium, excretion through the bile and intestine; and 2 hormones, parathormone, acting to increase plasma calcium concentration, and calcitonin, which acts to decrease plasma calcium (Ferriman and Gilliland, 1968).

The total serum concentration of calcium is directly affected by the concentration of protein, especially albumin. In nutritional and nephrotic hypoalbuminemia, the total calcium may be reduced to a concentration of 6 to 7 mg./dl., but this is not sufficiently low to manifest symptoms of tetany (Hoffman, 1970). The extent of calcium binding by protein in the plasma is governed in part by pH. In the physiologic pH range of 7.35 to 7.45, 1 gm. of serum protein binds 0.84 mg. of calcium ion (West *et al.*, 1966). The binding of calcium seems to be very loose, since early attempts at quantitating the ionized fraction using dialysis drew all the calcium from the serum. Protein-binding does not impede the transcapillary movement of calcium, and the redistribution between protein-bound and ionized calcium is rapidly achieved as calcium ions are drawn from the plasma.

The level of total calcium in the blood is also regulated to some degree by the reciprocal relationship to phosphorus concentration. From the known concentration of all serum constituents concerned, the serum is supersaturated with calcium and phosphorus even when the calcium bound to protein is taken into account. Calcium phosphate might be expected to precipitate if a static equilibrium were established. But this is not the case within the body. Calcium and phosphorus are continually moving into and from the interstitial fluid and serum, and from regions of high concentration to regions of lower concentration. No spontaneous precipitation can take place other than the formation of a small amount of colloidal calcium phosphate in the serum. This is rapidly removed by reticuloendothelial cells and bones (Hoffman, 1970).

There are several important exceptions to the reciprocal arrangement, because other factors controlling the calcium and phosphorus concentrations prevent development of the equilibrium which is necessary for the reciprocal arrangement.

The calcium circulating in the plasma of an adult is not a nutritional constituent, but a functional one. Fluctuations outside the normal levels can have profound effects on the body; therefore, a regulatory mechanism is essential.

The normal range of calcium ion concentration in the extracellular fluids seldom vaires more than 10%. Therefore, it is evident that calcium ion regulation is one of the most highly developed homeostatic mechanisms of the body. This regulation is effected almost entirely by the parathyroid glands (Guyton, 1961).

Parathormone exerts its effect in the regulation of calcium by acting primarily at 3 sites. The principle rise in serum calcium is due to increased bone absorption. The decline in phosphate concentration is

due to the action of parathyroid hormone on the kidneys to increase the rate of phosphate excretion. This hormone also increases absorption of calcium ions from the gut and renal tubules (Copp, 1957).

Any decrease in calcium ion concentration in the extracellular fluid causes the parathyroid glands to increase their rate of secretion and, if prolonged disturbances exist, to hypertrophy. Conversely, increased calcium ion concentration inhibits the parathyroid glands and decreases serum calcium ion concentration (Copp, 1957).

The parathyroid control system is relatively slow to act, requiring many hours and occasionally days to reach maximum effect. Since the amount of calcium absorbed into or lost from body fluids can change greatly in a matter of hours, another more rapid mechanism of control must be available. This is the buffer function of the exchangeable calcium in bones (Barter, 1954). That portion of bone salts that has recently been deposited in the bone is still in reversible equilibrium with calcium and phosphorus in the extracellular fluids. That is, an increase in the product of extracellular calcium and phosphorus will cause an immediate deposition of more bone salts. A decrease in this product will cause an immediate absorption of some of the bone salts. This reaction is so rapid that a single passage of blood containing a high concentration of calcium through a bone removes almost all the excess calcium (Guyton, 1961).

When the parathyroid glands do not secrete sufficient parathormone, the osteoclasts of the bone become extremely inactive. As a result, bone resorption is so depressed that the level of calcium in body fluids decreases.

In hyperparathyroidism, extreme osteoclastic activity occurs in the bones; this elevates the calcium ion concentration in the extracellular

fluid while usually depressing slightly the concentration of phosphate ions (Bogdonoff *et al.*, 1956). When the plasma calcium and phosphate levels in extracellular fluids rise markedly as a result of hyperparathyroidism, the kidney cannot excrete the phosphorus rapidly enough. Therefore, the calcium and phosphate in the body fluids become supersaturated and calcium phosphate crystals begin to be deposited in the alveoli of the lungs, the tubules of the kidneys, the thyroid gland, the acid-producing area of the stomach mucosa, and in the walls of arteries throughout the body. This extensive metastatic deposition of calcium phosphate can develop within a few days.

Some patients with mild hyperparathyroidism show few signs of bone disease. They have instead a tendency to form kidney stones, because most of the excess calcium and phosphate mobilized from the bones by hyperparathyroidism is excreted via the kidney (Black, 1967).

Because a low level of calcium ions in the body fluids directly increases the secretion of parathormone, any factor that causes a low level of calcium initiates the condition known as secondary hyperparathyroidism. This may result from a low calcium diet, pregnancy, lactation, rickets or osteomalacia. Hyperplasia of the parathyroid glands is almost certainly a corrective measure for maintaining the level of calcium in the body fluids at a nearly normal value.

"Renal rickets" is a type of osteomalacia resulting from prolonged kidney disease. The kidney disease prevents normal excretion of acids and leads to acidosis. This in turn converts most of the $HPO_4^{=}$ ions of the blood into $H_2PO_4^{-}$ ions which do not precipitate in the bones. The excretion of calcium in combination with acid radicals and diminished calcium absorption tends to produce hypocalcemia. Secondary hyperparathyroidism corrects this tendency but leads to demineralization of the

skeleton. This change resembles osteitis fibrosa (Ferriman and Gilliland, 1968).

During the past few years, investigators of calcium metabolism have felt that the tight control of serum calcium concentration involves something more than the secretion of parathormone, which acts to increase the calcium level. In 1963, Hirsch, Gautier and Munson extracted and purified a protein from the thyroid gland which acts as a calcium-lowering substance. This hormone-like substance is called calcitonin. Its sole action is to counteract the effects of hypercalcemia.

Calcitonin accelerates the transfer of calcium from blood to bone; it does not increase the loss from the body, nor is its action mediated by the kidney, gastrointestinal tract, hypophysis or parathyroid. Calcitonin exerts a direct effect on the bone, accelerating the rate of calcium deposition and inhibiting calcium resorption (Hoffman, 1970).

Another important factor in calcium metabolism is vitamin D. It is the most important single factor in the absorption of calcium (Irving, 1957). One direct effect of vitamin D is to increase the permeability of the intestinal mucosa to calcium or alter the calcium ion in such a way that it can diffuse more readily or be actively transported through the membrane. Because of vitamin D's effect in increasing calcium absorbability, it also tends to increase very slightly the calcium ion concentration in extracellular fluids. The absorption of phosphorus increases secondarily.

Whether vitamin D has a direct action on bone to promote deposition of calcium phosphate is unknown. Theoretically, all the favorable action of vitamin D in rickets can be explained by its promotion of better absorption of calcium and phosphorus. Yet there is much evidence that vitamin D does affect bone directly. It may help in the action of

parathormone on bone (Hoffman, 1970). Excessive doses of vitamin D over prolonged periods may produce disturbances comparable to those of hyperparathyroidism.

Treatment of metabolic bone disease in patients undergoing long-term hemodialysis is similar to that in other patients with chronic uremia. Working out a plan of treatment, especially with respect to dosage of vitamin D, is complicated by the added variables of calcium and phosphorus exchange across the hemodialyzer (Black, 1967).

The most common bone abnormality in chronic azotemic renal failure is a defect of mineral deposition which is indistinguishable in its morphological features from other types of osteomalacia (Ball, 1960). In other cases, the bone appears normally mineralized and the changes so closely resemble those of primary hyperparathyroidism as to warrant their description as generalized renal osteitis fibrosa (Albright and Reifenstein, 1954). Its presence implies the erosive destruction of whole bone substances with the release of the minerals contained in the bone. Such dissolved salts may either be retained within the body or lost with the production of negative external balances of calcium and phosphorus. The erosive process does not appreciably involve the surface layers of osteoid, which tissue appears to resist the normal processes of bone removal; it occurs as a burrowing or tunneling excavation of the mineralized core of bone beneath the osteoid seams (Wolbach, 1947).

The more extensive transformation of the skeleton, which is implied by the term "osteitis fibrosa renalis generalisata", is invariably associated with renal failure of long standing (Black, 1967). In this form of azotemic osteodystrophy, which is indistinguishable from the bone disease of primary hyperparathyroidism, there is a greatly increased skeletal turnover with progressive replacement of lamellae by woven bone which calcifies normally.

It is likely that the osteitis fibrosa associated with renal rickets will become generalized with the passage of time; and it is sometimes possible to witness this transformation clinically (Gilmore, 1947).

Although it is the rule to find hyperplasia of the parathyroid glands in cases of azotemic osteodystrophy, opinion as to its significance is based on somewhat limited evidence. This association of parathyroid bone disease with massive parathyroid hyperplasia has been designated "azotemic hyperparathyroidism" (Stanbury and Lumb, 1966).

The development of clinical bone disease appears to be determined by the rate of skeletal growth and by the chronicity of the renal failure. Consequently, renal osteodystrophy is more common in children and in patients with either congenital renal anomalies or with slowly progressive renal disease such as chronic pyelonephritis (Black, 1967).

In the clinical features of renal bone disease, the symptoms are those to be expected of any generalized skeletal disease, but sometimes they are overshadowed by the more obvious clinical features of uremia. Metabolic studies suggest that the fundamental defect in azotemic osteodystrophy is the inability to absorb and utilize dietary minerals.

The concept of an acquired resistance or insensitivity to vitamin D is attractive. The idea is supported by the demonstration that when calcium is absorbed under the influence of large doses of vitamin D, it is retained in the body with phosphate in proportions appropriate for the deposition of bone mineral. This is followed by healing of rickets and osteomalacia and reversal of changes due to secondary hyperparathyroidism (Black, 1967).

Once the intestinal malabsorption of calcium is overcome by appropriately large doses of vitamin D, the plasma calcium may rise, but there is no predictable or quantitative relationship between the amount absorbed and the change in plasma calcium (Stanbury and Lumb, 1962).

As a group, patients with azotemic hyperparathyroidism or generalized renal osteitis fibrosa maintain normal levels of plasma calcium even when levels of phosphate are greatly elevated. Their parathyroid glands are effective in maintaining plasma ionic calcium, although the external mineral balances are similar to those of patients with defective mineralization and probably indicative of a similar state of vitamin D resistance (Stanbury and Lumb, 1966). It has been suggested that overcoming the abnormal response of bone produced by the apparent vitamin D resistance of renal failure and to maintain a normal level of plasma calcium ion may sometimes entail an increased endogenous production of parathyroid hormone sufficient to cause "parathyroid bone disease."

Uremic patients with florid bone disease have predictable hyperphosphatemia and normal or near-normal levels of plasma calcium, with generalized osteitis fibrosa, normally mineralized bone and massive parathyroid hyperplasia. It must be regarded as established that parathyroid glands subjected to prolonged stimulation and hyperplasia by chronic renal failure can develop functional autonomy (McIntosh, Peterson and McPhail, 1966).

If intestinal malabsorption of calcium in azotemic patients does indeed reflect a resistance to vitamin D, the demonstration of significant net absorption during treatment with a particular dose of vitamin D should signify that this resistance has been overcome. If the requirement of the osteocyte for vitamin D is the same as that of the intestinal epithelial cell, this therapeutic provision should restore responsiveness to the calcemic action of parathyroid hormone (Stanbury and Lumb, 1962).

Although resistance to vitamin D seems to account satisfactorily for many biochemical and histologic features of azotemic osteodystrophy, it alone will not explain the severity of the secondary hyperparathyroidism.

In patients with advanced renal failure, the renal lesion prevents correction of the hyperphosphatemia and this potential stimulus to the parathyroids persists. It is not known whether the high plasma phosphate can itself directly stimulate the parathyroid glands or whether it is the longer duration of the renal failure that accounts for the massive glandular hyperplasia in azotemia (Black, 1967).

In all the foregoing conditions, the level of ionized calcium is the determining factor. The total calcium may fall in the normal range, but this may not be an accurate indication of the status of calcium metabolism in the body.

The realization that ionized calcium is of primary physiologic importance brought about numerous attempts to measure directly its concentration in body fluids.

The isolated frog heart preparation of McClean and Hastings (1934) met the conditions of being sensitive to calcium in the ionized form, and being quantitative. Using this technique, it was found that protein-free fluids such as cerebrospinal fluid contain all or nearly all their calcium in the ionized form. The analysis of cerebrospinal fluid was then considered a means of assessing calcium metabolism.

The calcium-protein relationship was next investigated. This led to the discovery that the ionization of calcium in protein-containing fluids is determined by a chemical equilibrium between calcium and protein.

Using this relationship, McClean and Hastings (1935) developed a nomogram from which ionized calcium could be calculated, given the total calcium and total protein. However, specific conditions have been enumerated which limit the application of such a nomogram (Moore, 1970).

A series of calcium selective electrodes has been developed, each somewhat improved over the preceding models. The most contemporary model

has 2 main disadvantages: the pH cannot be readily monitored, and it has not been equipped with a thermostat. Since protein-binding of calcium is pH- and temperature-dependent, a difference between calcium ion concentration measured at 37 C. and 25 C. is to be expected (Moore, 1970).

Ultrafiltration methods have been developed as a means of measuring unbound calcium. These procedures involve the centrifugation of serum in special apparatus for 75 minutes at 2500 rpm (Prasad and Flink, 1957). Approximately 10 ml. of serum are required to produce 2.5 to 3 ml. of filtrate.

Also, ultracentrifugation methods have been used for quantitating unbound calcium. These involve centrifugation of plasma for 8 hours at an average of 115,000 x g. (Loken *et al.*, 1960). A protein concentrate accumulates in the bottom of the tube, with a clear supernate on top.

Other methods, utilizing ion exchange resins and removing plasma waters with dextran gel, have been described; but of all techniques, the one most widely used is that using ammonium purpurate (murexide) for the measurement of calcium in an ultrafiltrate (Rose, 1957).

The techniques for quantitating ionized or free calcium that have been developed to date have aspects that limit their application to special situations. In view of the value of following free calcium levels during the course of many diseases, a method requiring relatively small amounts of blood and no special equipment is needed. The object of this experiment was to test the efficacy and clinical applicability of a new method suggested by White (1971).

METHODS AND MATERIALS

Source of Specimens

Predialysis and postdialysis specimens of heparinized blood were obtained from 16 patients undergoing long term hemodialysis. "Normal" specimens were obtained from laboratory personnel, and plasma from patients with various disease processes was selected from the hospital population. The plasma was separated from the cells and kept in tightly stoppered 12 x 75 mm. tubes until initiation of testing.

Total Calcium

The total calcium was determined on each specimen using the automated procedure of Kessler and Wolfmann.* The samples were mixed with 1N HCl to release protein-bound calcium. These mixtures were dialyzed into a recipient stream of cresolphthalein complexone. Colored complexes between calcium and dye were formed upon the addition of diethylamine, which resulted in an alkaline mixture. The solutions were warmed to 37 C. before entering the colorimeter. The developed color in the sample was read at 580 nm.

Free Calcium

Calcium was measured in each sample after thermoprecipitation of plasma proteins according to the method of White (1971). The calcium of

*Technicon method file N-36 I/II; Technicon Corp., Chauncey, N.Y., 1965.

this supernate was isolated from the protein-bound calcium by heating the aliquots at 100 C. for 3 to 5 minutes. The coagulums were then whipped with a stirring rod and spun at 3500 rpm for 10 to 15 minutes. The resulting supernatant fluids contained the free or unbound calcium, which was quantitated in the same manner as the total calcium.

Albumin

Albumin was measured in all the test sera using the method of the American Monitor Corporation.* The patient's serum (0.2 ml.) was added to 3.0 ml. of a 3,3',5,5' tetrabrom-m-cresol-sulfonphthalein-buffered detergent solution. The dye-albumin combination produced an intense blue color with a maximum absorption at 600 nm. Standards (2.5-gm. and 5.0-gm.) were run with each group of patients and a standard curve was plotted for each run.

Total Protein

The total protein was measured on each patient using the biuret method: 0.1 ml. of patient serum is added to 8.0 ml. of biuret reagent. The purple color is produced by the formation of a colored-complex of copper in an alkaline solution, with 2 or more carbamyl groups (-CO-NH-) which are joined directly together or through a single atom of N or C (Weichselbaum, 1946). Reading was made on a spectrophotometer at 540 nm using Monitrol XLT-33 as the standard and Monitrol XPT-55 as a control.**

Calculations:

$$\text{total protein (gm./dl.)} = \frac{\text{(O.D. of X)(T.P. value of std.)}}{\text{O.D. of std.}}$$

O.D. = optical density
T.P. = total protein

*American Monitor Corp., Indianapolis, Ind.

**Dade Division, Amer. Hosp. Supply Corp., Miami, Fla.

Table 1. Results of calcium (mg./dl.) and protein (gm./dl.) determinations in patients undergoing hemodialysis

Patient #	Total Calcium		Free Calcium		Total Protein		Albumin	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	8.0	8.3	4.0	4.0	6.9	7.0	2.6	2.4
2	11.3	10.3	6.5	6.1	6.5	6.6	4.2	4.3
3	8.8	8.0	4.5	4.8	6.4	6.6	3.9	3.6
4	9.2	10.7	4.3	5.5	6.6	6.5	3.6	3.4
5	9.0	9.0	5.8	5.3	5.9	6.0	2.9	2.8
6	10.0	9.7	5.0	4.5	8.0	7.8	3.2	3.3
7	9.0	8.6	5.2	6.2	5.5	5.7	3.0	3.2
8	8.6	9.8	5.4	6.5	6.8	6.9	3.5	3.8
9	9.7	10.5	5.7	6.0	7.6	7.5	3.8	3.9
10	10.1	10.1	5.9	5.9	6.4	6.6	3.6	3.7
11	10.6	11.2	5.6	5.7	5.9	6.2	3.0	3.5
12	11.3	9.3	5.0	4.2	6.2	5.2	2.9	2.6
13	9.0	9.5	5.0	5.6	5.8	5.7	2.4	2.4
14	10.6	9.8	4.8	5.0	7.2	7.3	3.9	3.9
15	10.3	10.3	5.0	5.3	7.3	7.4	3.8	3.8
16	10.1	9.7	6.5	6.8	6.1	6.5	3.6	3.8

Table 2. Results of calcium (mg./dl.) and protein (gm./dl.) determinations in patients with other diseases

Patient #	Disease	Total Calcium	Free Calcium	Total Protein	Albumin
1	Cirrhosis	8.0	5.3	6.0	3.0
2	Leukemia	7.8	5.7	4.1	2.5
3	Prethyroidect.	9.6	4.2	7.8	4.1
4 pt. 3	Postthyroidect.	5.8	2.6	7.1	3.9
5	Multiple myeloma	9.4	6.8	10.2	2.6
6	Hodgkin's Disease	8.4	5.3	6.4	3.2
7	Postpartum	9.8	5.4	6.4	3.2

Table 3. Results of calcium (mg./dl.) and protein (gm./dl.) determinations in normal "patients"

Patient #	Total Calcium	Free Calcium	Total Protein	Albumin
1	9.2	6.0	6.1	3.3
2	9.8	6.2	7.2	4.2
3	9.3	6.0	5.8	3.2
4	9.8	6.4	6.4	4.1

Table 4. Tabulation of the mean and standard deviation of plasma calcium (mg./dl.) and protein (gm./dl.) concentrations in normal and hemodialyzed patients

	Mean	S.D.
Dialysis Patients		
Total calcium (pre)	9.7	0.8
Total calcium (post)	9.7	0.8
Free calcium (pre)	5.2	0.6
Free calcium (post)	5.4	0.8
Total protein (pre)	6.6	0.7
Total protein (post)	6.6	0.7
Albumin (pre)	3.4	0.5
Albumin (post)	3.4	0.5
Normal Patients		
Total calcium	9.5	0.1
Free calcium	6.1	0.6

Table 5. Results of the t-test for unpaired data comparing the calcium values from normal dialysis patients and abnormal dialysis patients

total calcium (pre)	$p = <10 > 5$
total calcium (post)	$p = > 50$
free calcium (pre)	$p = > 2.5 > 1$
free calcium (post)	$p = > 10 > 25$

DISCUSSION

A clinical trial of a new technique for quantitating free calcium was undertaken. Since long-term hemodialysis patients are subject to abnormal calcium metabolism, a group of 16 such patients was selected for the study.

The results show that this method accurately reflects the level of free calcium in the plasma. The mean value, 5.4 mg./dl. free calcium in the postdialysis specimens, corresponds to the mean value obtained by Kaye *et al.* (1967), who also tested postdialysis specimens, using the murexide method.

The level of free calcium in the patient undergoing hemodialysis is directly dependent on the calcium level in the dialysate solution; since the concentration of calcium ions in the dialysate solution is 6 mg./dl., the postdialysis value of ionized calcium should be somewhat less than 6 mg./dl., reaching an equilibrium depending on the level of free calcium in the patient's plasma (Wing, 1968).

The results from 9 of the patients in this study followed this relationship; however, in 7 of the patients, they did not. In reviewing the histories of these 7, it was found that 2 expired within 24 hours of this dialysis; 1 other was in critical condition and received multiple, rapid infusions and transfusions. Two others were diagnosed as having probable secondary hyperparathyroidism; and the remaining 2 were showing early signs of bone changes associated with secondary hyperparathyroidism.

Ideally, a measure of the level of parathyroid hormone or vitamin D should be used to evaluate the status of calcium metabolism in these patients. The techniques presently available for these assays are beyond the scope of routine testing; consequently, indirect methods have been substituted. Since free calcium has been shown to vary in conditions of abnormal calcium metabolism, a test for this parameter, suitable for use in the routine clinical laboratory, can be a useful tool in managing dialysis patients. The results of applying the t-test for unpaired data in this study ("normal" dialysis patients and those with secondary hyperparathyroidism) only show a significant difference on the predialysis free-calcium data (Table 5).

A group of normal petients was also included in the study. The mean level of free calcium in their sera was 6.1 mg./dl., which falls within the normal range of 5.4 to 6.5 mg./dl. quoted by White (1971).

Also, a group of patients with various other diseases usually showing apparently abnormal levels of total calcium was included. Most of these were shown to have normal levels of free calcium.

SUMMARY AND CONCLUSIONS

Methods previously described for quantitating free calcium are not practical for use in the general clinical laboratory. They are time consuming, require large specimens, and some call for elaborate equipment.

The procedure used in this clinical evaluation has none of these limitations. It is rapid, requires no special equipment and comparatively small quantities of plasma are sufficient.

This new method (White, 1971), using thermal precipitation of proteins from undiluted serum or plasma, has been subjected to clinical trial in this study. Observations on a series of 16 patients undergoing hemodialysis demonstrate the clinical value of this procedure as an aid in estimating the range of calcium ion concentration. An assortment of patients with conditions usually associated with abnormalities of total calcium was also studied; most of these were shown by this method to have normal levels of free calcium.

It can be concluded that the technique used in this study is applicable as a "routine" test, and can be an aid in evaluating the level of ionized calcium in plasma.

BIBLIOGRAPHY

BIBLIOGRAPHY

Albright, F., and Reifenstein, E. C.: *The Parathyroid Glands and Bone Disease.* Williams and Wilkins Pub., Baltimore, Md., 1948.

Ball, J.: Diseases of bone. Recent Adv. in Path., (1960): 293.

Bartter, F. C.: The parathyroids. Am. Rev. Physiol., 16, (1954): 429.

Black, D. A. K. (ed.): *Renal Disease*, 2nd ed. F. A. Davis Co., Philadelphia, Pa., 1968.

Bogdonoff, M. D., Woods, A. H., White, J. E., and Engel, F. L.: Hyperparathyroidism. Am. J. Med., 21, (1956): 583.

Copp, D. H.: Calcium and phosphorus metabolism. Am. J. Med., 22, (1965): 275.

Danesh, B. J. N. Z., Walker, C. H. M., and Mathers, N. P.: The relationship of post dialysis plasma calcium and magnesium to the dialysate levels and to changes in pH. New Eng. J. Med., 22, (1970): 771.

Ferriman, D. G., and Gilliland, I. C.: *A Synopsis of Endocrinology and Metabolism*, The Williams and Wilkins Co., 1968: 72-85.

Gilmore, J. R.: *The Parathyroid Glands and Skeleton in Renal Disease*, Oxford Med. Publishers, London, 1961.

Guyton, A. C.: *Textbook of Medical Physiology*, 2nd ed., W. B. Saunders Co., Philadelphia and London, 1961: 1047-1065.

Hoffman, W. S.: *The Biochemistry of Clinical Medicine*, 4th ed., Yearbook Med. Pub., Chicago, 1970: chapter 12.

Irving, J. T.: *Calcium Metabolism*, John Wiley and Sons Pub., Inc., New York, 1957.

Kaye, M. G., Cohen, G. F., Chatterjee, R., and Mangel, R.: Regulation of the plasma ionized calcium and its therapeutic control in patients treated by regular hemodialysis. Trans. Am. Soc. Int. Org., 13, (1967): 341.

Kaye, M. G., and Silverman, M.: Calcium metabolism in chronic renal failure. J. Lab. Clin. Med., 66, (1965): 535.

Kessler, G., and Wolfmann, M.: An automated procedure for the determination of calcium and phosphorus. Clin. Chem., 10, (1964): 686.

Loken, H. F., Hand, R. J., Gordon, G. S., and Whittington, J. J.: Ultracentrifugal analysis of protein bound and free calcium in human serum. J. Biol. Chem., 235, (1960): 3654.

Lloyd, H. M., and Rose, G. A.: Ionized, protein bound and complexed calcium in the plasma in primary hyperparathyroidism. Lancet, 2, (19): 1258.

McIntosh, D. A., Peterson, E. W., and McPhail, J. J.: Autonomy of parathyroid function after renal transplantation. Ann. Int. Med., 65, (19): 1133.

McClean, F. C., and Hastings, A. B.: A biologic method for the estimation of calcium ion concentration. J. Biol. Chem., 107, (1934): 337.

McClean, F. C., and Hastings, A. B.: The state of calcium in the fluids of the body. J. Biol. Chem., 108, (1935): 285.

Moore, E. W.: Ionized calcium in normal sera and whole blood filtrates with the ion selective electrode. J. Clin. Invest., 49, (1970): 318.

Ogden, D. A., and Holmes, J. H.: Changes in total and ultrafilterable plasma calcium and magnesium during hemodialysis. Trans. Am. Soc. Art. Org., 12, (1966): 200.

Prasad, A. S., and Flink, E. B.: Effects of carbon dioxide on the concentration of calcium in an ultrafiltrate of serum obtained by centrifugation. J. Appl. Physiol., 10, (1957): 103.

Rose, G. A.: Determination of ionized and ultrafilterable calcium of normal human plasma. Clin. Chem. Acta, 2, (1957): 227.

Stanbury, J. W., and Lumb, G. A.: Metabolic studies, renal osteodystrophy, ionized calcium, phosphorus and nitrogen metabolism in rickets, osteomalacia and hyperparathyroidism complicating chronic uremia in the osteomalacia of adult Fonconi Syndrome. Quart. J. Med., 41, (1962): 1.

Stanbury, S. W., and Lumb, G. A.: Parathyroid function in chronic renal failure. Quart. J. Med., 35, (1966): 1.

West, E. W., Todd, W. R., Mason, H. S., and Van Bruggen, J. T.: *Textbook of Biochemistry*, 4th ed., McMillan Co., New York, 1966.

White, A., Handler, P., and Smith, E. L.: *Principles of Biochemistry*, McGraw-Hill Pub., New York, 1968: 887-896, 932-935.

White, F. J.: Thesis, Michigan State University, 1971.

Wilson, R. E., Bernstein, D. S., Murray, J. E., and Moore, F. D.: Effects of parathyroidectomy and kidney transplantation on renal osteodystrophy. Amer. J. Surg., 110, (1965): 384.

Wing, A. J.: Optimum calcium concentration of dialysate fluid for maintenance hemodialysis. Brit. Med. J., 4, (1968): 145.

Wolbach, S. B.: Vitamin A deficiency and excess in relation to skeletal growth. J. Bone & Joint Surg., 29A, (1947): 171.

Wiechelbaum, T. E.: Determination of proteins in blood serum and plasma. Am. J. Clin. Path., 16, (1946): 32.

APPENDIX

APPENDIX

1. Reagents for automated calcium determinations:

   1N HCl - 83 ml. conc. HCl q.s. to 1000 ml. with distilled water,
   + 0.5 ml. Brij-35/L.

   Cresolphthalein complexone 0.007%
   cresolphthalein complexone 0.070 gm.
   sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) 50 gms.
   HCl 1N q.s. to 1000 ml. + 0.5 ml. Brij-35/L*

   Diethylamine - 150 ml. diethylamine + 0.5 gm. potassium cyanide
   q.s. to 1000 ml. with distilled water.

2. Standards for automated calcium determinations:

   Stock Calcium Standard - (50 mg./100 ml.)
   1.25 gm. calcium carbonate
   7.0 ml. conc. HCl
   q.s. to 1000 ml. with distilled water

   Stock Magnesium Standard - (100 mg./100 ml.)
   8.36 gm. Magnesium Chloride ($MgCl_2 \cdot 6H_2O$)
   q.s. to 1000 ml. with distilled water

   Working Calcium Std.:

ml. Stock Ca.	ml. Stock Mg.	mg. Ca./100 ml.	mg. Mg./100 ml.
10	2	5.0	2
15	2	7.5	2
20	2	10.0	2
25	2	12.5	2
30	2	15.0	2

   q.s. each std. to 100 ml. Add 1 drop conc. HCl

*Technicon Corp. Chauncey, N.Y.

3. Biuret Reagent for Total Protein:

Dissolve the following in 1000 ml. distilled water:

$CuSO_4 \cdot 5H_2O$	3 gms.
$KNaC_4H_4O_6 \cdot 5H_2O$	12 gms.
KI	2 gms.

With constant swirling, add 600 ml. 10% NaOH. Dilute to 2000 ml.

VITA

The author was born in South Lyon, Michigan, on August 1, 1943. She lived there and graduated from South Lyon Community High School in 1961. After graduation from high school, the author entered Michigan State University, and in 1965, she received a B.S. degree in Medical Technology. After receiving her B.S. degree, she completed a 12-month internship at Swedish-American Hospital in Rockford, Illinois. Later that same year, she became registered with the Registry of Medical Technologists of the American Society of Clinical Pathologists.

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