SOME ASPECTS OF MAGNESIUM METABOLISM IN MAN

- by -

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ABSTRACT

Serum ionized magnesium was measured in 56 newborns and 64 older children using the ultramicrospectrophotometric method of Rice and Lapara. When cord blood levels are compared to serum levels no appreciable change was evident during the first week of life, although the cord blood levels tended to be slightly higher than the subsequent sera levels. No differences were noted in serum ionized magnesium in infants who were bottle fed as opposed to those who were breast fed. No significant differences could be found in levels of serum ionized magnesium between venous and capillary samples.

Serum ionized magnesium was determined during 33 exchange transfusions with citrated blood given to 16 infants. In each instance there was a reduction of ionized magnesium at the end of the transfusion with an average fall to less than 60 percent of the pre-exchange value. With repeated transfusions the pre-exchange level became lower, so that the lowest values occurred at the conclusion of multiple exchanges performed at close intervals. This effect is the result of citrate binding of magnesium, in vitro evidence for which is presented. No clinical effects are described, but electrocardiographic changes were seen at the lowest levels.

Serum ionized magnesium levels before, during, and after replacement transfusions were determined in 20 newborn infants.

In 10 infants exchanged with ACD blood, the level fell from 1.75 ± 0.16 mEq/L to 0.80 ± 0.16 mEq/L. By contrast, levels in 10 infants exchanged with two types of heparinized blood were unchanged: the pre-exchange values were 1.59 ± 0.11 mEq/L, and the post-exchange levels were 1.59 ± 0.08 mEq/L. Mean values for donor bloods were 0.42 ± 0.07 mEq/L with ACD blood, and 1.45 ± 0.03 mEq/L with heparinized blood.

In vitro studies involving the addition of known amounts of citrate to standard Mg^{++} solutions demonstrated that the citrate caused a reduction of ionic magnesium. It is proposed that the fall in serum ionized magnesium where ACD blood is used for exchange transfusion is the combined result of magnesium ion binding by the citrate, and the dilution effect of the relatively large proportion of anticoagulant to blood (1:3) used with the ACD mixture.

Free or non protein bound and protein bound serum magnesium levels have been determined in the blood from 10 normal healthy adults, by atomic absorption spectroscopy and by the Mann's Dye method. These in vitro studies indicate that 20 to 25% of the total serum magnesium is protein bound and that the remaining 75 to 80% comprises the ionized fraction. Evidence is also presented to indicate that the Mann's Dye method measures the free or non-protein bound fraction of the serum magnesium.

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LIST OF ABBREVIATIONS

Mann's Dye: Sodium 1-azo-2 hydroxy-3(2,4-dimethyl carboxanilide) naphthalene-1(2-hydroxy-benzene-4-sulfonate)

Obtained from the La Motte Chemical Products Company, Chestertown, Md.,

ANTICOAGULANT MIXTURES FOR COLLECTION OF DONOR BLOOD.

A.C.D. (N.I.H. Formula B): Acid-Citrate-Dextrose Solution. This is the Standard Canadian Red Cross mixture which contains; 4.4 grams citric acid, 13.2 grams trisodium citrate, and 14.7 grams glucose per liter of anticoagulant. Usually 380 ml of donor blood is added to 120 ml of this anticoagulant.

Obtained from Abbott Laboratories Chicago. Ill.

N.I.H. Formula A: This is the anticoagulant used by many centers in the United States. Formula A contains 7.3 grams citric acid, 22.0 grams trisodium citrate, and 24.5 grams glucose per liter of anticoagulant. Usually 450 ml of donor blood is added to 67.5 ml of anti-coagulant.

Obtained from Abbott Laboratories Chicago. Ill.

Panheprin 2115:	A commonly used anticoagulant employing sodium citrate as the buffer. Usually 470 ml of donor blood is added to 28.2 ml of this solution, containing 2115 USP units of sodium heparin and 84.6 mg. of sodium citrate. There is thus a total of 18 mg. of sodium citrate per 100 ml of donor blood. Obtained from Abbott Laboratories Chicago Ill.
Heparin-Saline:	An anticoagulant using only heparin in saline. Here 2.1 ml (2100 USP units) of sodium heparin are added to 26.1 ml of 0.9% saline solution. Usually 470 ml of donor blood is added to this solution.
THAM:	tris-(hydroxymethyl) aminomethane.
E.C.G.:	electrocardiogram.
R.P.M.:	revolutions per minute.
N.P.N.:	non protein nitrogen.

- LIST OF FIGURES -

SECTION IV

Mann's Dye: Sodium 1-azo-2-hydroxy-3(2,4-dimethycarboxanilide) naphthalene-1-(2-hydroxybenzene-4-sulfonate)

SECTION V

SECTION VI

SECTION VII

LIST OF TABLES

SECTION VI

SECTION VII

SECTION VIII

TABLE OF CONTENTS

- SECTION I -

- SECTION II -

REVIEW OF THE LITERATURE

- SECTION V -

SERUM MAGNESIUM IN EXCHANGE TRANSFUSION

- SECTION VI -

THE EFFECT ON SERUM MAGNESIUM OF EXCHANGE TRANSFUSION WITH CITRATED AS OPPOSED TO HEPARINIZED BLOOD.

- SECTION VII -

SERUM MAGNESIUM LEVELS IN THE NEWBORN AND OLDER CHILD

- SECTION VIII -

ULTRACENTRIFUGAL STUDIES OF PROTEIN-BOUND AND FREE MAGNESIUM IN NORMAL HUMAN SERUM

- SECTION I -

INTRODUCTION

It has been known for some time that citrate added to blood to be used in transfusion results in the formation of a poorly ionized calcium citrate complex. (12) Schachter and Rosen (3) have shown that the ionization constant for calcium citrate is 6.61×10^{-4}M. Whole blood is routinely added to the acid-citrate-dextrose solution (ACD) to complex the calcium ions needed for coagulation. The end result is therefore a decrease in the amount of circulating ionized calcium during and after transfusion. This decrease may be corrected by the intravenous administration of a ten percent solution of calcium gluconate to the patient during the course of the exchange. This is particularly necessary during an exchange transfusion because with this procedure ten mls of patient's blood is repeatedly withdrawn and replaced by an equal or slightly greater amount of donor blood until the level of toxic material in the patient's blood - for example, bilirubin in the newborn - has been significantly replaced. Hypocalcemic tetany is one of the undesirable side effects of the exchange transfusion with citrated blood. However, the administration of calcium gluconate at regular intervals tends to offset this. Despite this, there are relatively few studies of the actual changes involved because of the difficulty in measuring ionized calcium. Like calcium, magnesium is a divalent cation. Since both of these metals are found in Group II-A of the periodic chart of the elements, it could be anticipated that magnesium would also form poorly ionized complexes with citrate. (4) In 1959 Schachter and Rosen (3) showed that the ionization constant for magnesium citrate was identical to that of calcium citrate.

Although some data does exist regarding the behavior of calcium during exchange transfusion, (2) corresponding data on magnesium are relatively sparse and difficult to interpret. (5)

The present study was undertaken to provide information regarding the behavior of serum magnesium during exchange transfusion of citrated blood and to determine any related clinical effects thereof. (6) It was also deemed necessary to compare these results with those obtained from exchanges with heparinized blood. (7) The subjects studied may be, for the sake of clarity, divided into three groups as follows:

(1) Those being exchanged with citrated blood to which has been added the standard Canadian Red Cross mixture using the NIH Formula B anticoagulant.

(2) Those being exchanged with Heparinized blood in which the anticoagulant used was Panheprin 2115 or specially prepared blood using heparin in saline as the anticoagulant.

(3) A control group consisting of normal newborns taken from the newborn nursery of the Catherine Booth Hospital. (8)

Pre and post-exchange magnesium levels were measured as well as levels at regular intervals during the course of the exchange transfusion.

The method employed for the magnesium determination was that of Bohuon as modified for ultramicrospectrophotometric use by Rice and Lapara. (9) This method was chosen because it has the distinct advantage of requiring only 0.04ml of serum for duplicate determinations, making it ideally suited for use in a pediatric unit. The assay is unaffected by either calcium oxalate or calcium gluconate and protein. It is well known that gluconate interferes with the determination of magnesium by the

Titan Yellow Method; thereby severely limiting its usefulness. (5) In addition evidence is presented indicating that the proposed method is measuring ionized or non-protein bound magnesium as opposed to the other available methods that measure total magnesium.

Since it is the non-protein bound magnesium that is physiologically active, and of importance in exchange transfusions, this method lends itself readily to our purposes.

- SECTION II -

REVIEW OF THE LITERATURE

1. Biochemistry

Magnesium is definitely known to be an activator of many enzyme systems. In 1927 Erdtmann (10) discovered that magnesium ions increased the activity of mammalian alkaline phosphatase when added to the reaction mixture. Oxidative phosphorylation is also known to require the presence of ionized magnesium for activity. Ionized magnesium is necessary as an activator for the enzymes involved in intracellular catalysis, and is also involved in activation of ATP, in addition to other enzymes that cleave and transfer phosphate groups. ATP is required in such reactions as muscular contraction, protein, fat, nucleic acid and coenzyme synthesis. Particularily important enzymes requiring the presence of ATP and ionized magnesium are those in the Embden-Myerhof pathway, methyl group transfer, sulfate, acetate and formate transfer. Magnesium is also involved in decarboxylation reactions as a cofactor for thiamine pyrophosphate. All enzymes that catalyze the transfer of phosphate from ATP to a phosphate receptor or from a phosphorylated compound to ADP are activated by magnesium ions. This ion is an activator of all the enzymes that require thiamine pyrophosphate as a cofactor. These would include such enzymes as yeast carboxylase, (11) mammalian heart muscle carboxylase (12), and the pyruvic oxidase system of brain. (13) Enolase, the enzyme that catalyses the dehydration of the D-2-phosphoglyceric acid has been one of the most exhaustively studied magnesium ion-dependent systems. (14,15) Peptidases are also known to require ionized magnesium as a cofactor. The most intensively investigated one to date is LAP. (16,17) One can, therefore, infer that ionized magnesium is used in all major anabolic and catabolic processes in the human body. These

magnesium ion-dependent systems, and many others, too numerous to be detailed, have been completely reviewed by Lardy in 1951. (18)

Ionized magnesium has also been found to affect the oxidative phosphorylating ability of rat-liver mitochondria. In a recent experiment (19), mitochondria isolated from hypomagnesemic rats, were shown to be incapable of carrying out oxidative phosphorylation, suggesting that the magnesium ion in this isolated system may have a similar function in the in vivo system. Bain in 1955, plus Mudd and his coworkers in 1955, were able to demonstrate that the mitochondria themselves had a lower magnesium content relative to their normal controls. (20,21) Bartley and his coworkers (22) demonstrated that the mitochondrion may be the basic subcellular organelle responsible for the active transport processes in the cell, and Baltscheffsky (23) in 1957, found that rat-liver mitochondria quickly swelled when placed in a magnesium-free medium. He showed that uncoupling of oxidative phosphorylation had occured.

Magnesium is known to cross the cell membrane readily and is maintained at a high intracellular concentration by some active metabolic process other than by a mere passive transport phenomena. That this is so was clearly demonstrated in a series of experiments by several workers. (24-26)

There is much experimental evidence to show that magnesium deficiency causes changes in nerve conduction, transmission at the myoneural junction, and muscular contraction. The close association between magnesium concentration and phosphorylation reactions implies an effect on the energy-generating mechanisms of the mitochondria. (13,19)

As the magnesium concentration or the calcium concentration for that matter is lowered, the stimulation threshold of the motor nerve is lowered and conversely is raised by an increase. In muscle, because magnesium is active on a number of enzyme systems, the effects of magnesium are seen to be opposed to those of calcium. Low concentrations of magnesium enhance contractions, but low concentrations of calcium inhibit contractions. (27)

The effects of magnesium at the myoneural junction are the most difficult to explain. The magnesium concentration affects the quantity of acetylcholine liberated, the activity of the acetylcholinesterase, and also the excitability of the pre-synaptic nerve and muscle membrane. (28)

Vallee and coworkers (29) summarize these complex interactions by suggesting that an increase in transmission at the motor end plate may be the over-all result of a decrease in the concentration of either magnesium or calcium. Low concentrations of magnesium lower the excitatory threshold of pre-synaptic nerve and muscle membrane, but increase the release of acetylcholine. On the other hand low concentrations of calcium also lower the excitatory thresholds of the pre-synaptic nerve and the muscle membrane, but in contrast to magnesium, decrease the release of acetylcholine.

In conclusion it may be said that a summation of "enzymatic and charge effects" are required for activity of magnesium on the transmission of the nerve impulse while for calcium a "critically maintained balance between enzymatic and charge effects" is required.

If magnesium-28 is injected into either dog or man it is found to equilibrate rapidly into a volume greater than that of the extracellular space. The total rapidly exchangeable magnesium that is calculated from these data, however, is much smaller than the known body content. This, therefore, suggests that a large fraction of the total magnesium, perhaps in the bone does not equilibrate with the ingested dose. (30) Magnesium and potassium are quite similar in their distribution, since both are concentrated in the intracellular space. The highest concentration of magnesium is found in the liver and striated muscle where it reaches levels of approximately 20 mEq/L. The brain and the kidney are reported to have levels of 17 and 13 mEq/L respectively. (32) Erythrocytes, with a magnesium level of 6 mEq/L, have one of the lowest concentrations. (33) The reported magnesium concentration in normal

human serum varies depending upon the analytical method employed to measure it. The mean serum content is said to be 1.80 to 2.10 mEq/L. However, ranges from 1.40 to 2.50 mEq/L have been reported. (30,34,35) The serum magnesium content of infants is thought to be the same as that reported for children and adults. (36)

Cerebrospinal fluid contains more magnesium than serum, the mean values being 2.40 to 3.00 mEq/L. Therefore, this fluid cannot be considered as an ultrafiltrate of serum.

(a) Intake

Magnesium occurs abundantly in food stuffs. Green plants and leafy vegetables contain this cation in large amounts in the magnesium containing porphyrin, chlorophyll. The average daily dietary intake of magnesium has been reported to be 300 mg/day. Although the daily requirement is not accurately known, an intake of 220 mg/day is usually considered to be adequate for the average adult. This intake allows him ~~allows him~~ to maintain a positive magnesium balance. Infants require about 150 mg/day while the normal pregnant female appears to require 400 mg/day. During lactation even higher levels are needed. (37)

Soluble magnesium salts are readily absorbed from the small intestine. This absorption appears to be independent of pH variation.

(b) Excretion

The proportion of dietary magnesium which appears in the feces increases with intake. In man it is almost negligible when the intake is severely restricted. (38) Stool magnesium may be considered to be equivalent to that portion of ingested magnesium that has not been absorbed. The bowel does not represent a major excretory pathway for absorbed magnesium. This indicates that the ingested magnesium is excreted quantitatively in the urine.

(39,40) The kidney is now known to be the organ which regulates the body's content of magnesium. Infusions of magnesium salts, by increasing the filtered load presented to the tubule, result in a prompt increase in the rate of magnesium excretion. (41) Therefore, this indicates that an increased urinary output of magnesium follows an increased dietary intake, despite an absence of any detectable change in plasma magnesium levels. The mechanism whereby this renal tubular function is mediated is still poorly understood.

Normal adults in positive magnesium balance excrete about 60 to 120 mg/day in the urine. This is equal to approximately one third of the normal dietary intake. On a controlled diet of 260 mg/day, the normal adult female excretes approximately 96 mg/day, whereas the average twenty-four hour urine content of the normal male on the same intake was 162 mg/day. (42) The reason for this difference between the sexes is not apparent.

The level of protein in the diet has also been implicated as a determining factor in the amount of magnesium absorbed. Growth depression and other signs of hypomagnesemia were produced by increasing the protein level while maintaining normal magnesium intake. Increasing the calcium intake was also found to aggravate the magnesium deficiency, but the concomitant elevation of both calcium and protein intake produced no worse effect on magnesium deficiency than elevation of either one alone. (43) Tufts and Greenberg (44) found that by increasing the calcium content of a diet just adequate in magnesium, they could produce frank magnesium deficiency which could be corrected by simply increasing the magnesium intake. Alcock and MacIntyre (45,46) obtained somewhat similar results many years later. They found that the absorption of calcium was increased when the diet was deficient in magnesium and vice versa. Therefore, it appears that calcium and magnesium may possibly share a common pathway for intestinal absorption.

These workers speculate that since vitamin-D is known to increase plasma citrate levels, and since citrate is known to

chelate magnesium, a combination of these two effects could well explain the hypermagnesiuria in the presence of hypomagnesemia under the influence of this vitamin. Hanna, in 1961 (47) found that the absorption of magnesium from the gut of rats, given large doses of vitamin-D_2, was increased by about 80% over that of control animals, while urinary magnesium was increased by an almost equivalent amount. The fecal excretion of magnesium in the vitamin-D treated rats was significantly lower than in the control animals. The plasma magnesium was seen to fall in the vitamin-D treated group of rats.

(c) Endocrine Relationships

Disturbances of magnesium homeostasis have frequently been reported to occur in a wide variety of conditions involving an imbalance of hormone secretion. However, there is very little evidence to suggest that these disturbances are directly related to the effect of a particular hormone upon magnesium metabolism. These endocrine influences appear to represent an effect secondary to the main action of the hormone concerned; which nevertheless may be clinically important.

(i) Parathyroid

In 1933 Bulger and Gausmann (48) observed that the concentration of serum magnesium was little affected by parathormone, but Greenberg and Mackay (49) had reported that they had obtained rises of 0.4 mg/100 ml to 1.0 mg/100 ml of serum following intramuscular administration of 100 units of parathyroid extract. More recently, workers have reported that a negative magnesium balance usually accompanies hyperparathyroidism. Postoperatively, the balance is restored to normal. (50,51) Lifshitz and coworkers in 1967, (52) found that a fall in serum magnesium stimulates parathyroid hormone output. Elevated serum calcium and citric acid levels, hypocalciuria, hypophosphatemia and hyperphosphaturia were found consistently in magnesium-deficient

rats. They also found that the development of hypercalcemia and hypercitricemia was prevented by vitamin-D deficiency which appears to be in accord with the data indicating that full expression of parathyroid hormone activity does not occur in the absence of vitamin-D. Citrate excretion was also found to be reduced in the magnesium-deficient rat. These workers felt that magnesium deficiency is an additional example of the association of hypocitricuria and nephrocalcinosis.

Heaton and Anderson in 1965, (53) were able to prevent renal calcinosis in the rat by the removal of the parathyroid glands before the onset of magnesium deficiency, suggesting that the presence of parathormone was a necessary condition for the renal calcification.

The administration of parathormone or vitamin-D is known to increase the urinary output of citrate, but magnesium deficiency decreases urinary citrate. It is well known that citrate forms an undissociated complex with calcium and magnesium. Therefore, the urinary concentration of citrate may be of physiological importance in the stabilization of calcium phosphate in neutral or alkaline urine, and the absence of urinary citrate may well be a factor in the intratubular precipitation of calcium phosphate. However, the basic mechanism involved in the reduction of urinary citrate levels by magnesium deficiency is still quite unclear.

This apparent contradiction may be explained on the assumption of a common renal pathway for calcium and magnesium, with the two ions in competition. If there is a high filtered load of calcium presented to the renal tubule there may be a relative failure to reabsorb magnesium efficiently. (54) Roberts and coworkers, reported that in dogs, doses of parathyroid hormone produced increases in the urinary level of magnesium for the first three days. Thereafter it produced no detectable effect. (55)

(ii) Thyroid:

Hanna (56) has reported finding low plasma magnesium levels in severe cases of thyrotoxicosis. Three of these patients were found to have levels well below the lower limit of the normal range, and the remaining four were found to be well below the normal average. Two patients with myxedema were seen to have elevated levels of plasma magnesium. Tapley (57) proposes that the magnesium is responding to the level of triiodothyronine, and was able to produce a negative magnesium balance in myxedematous patients by the administration of triiodothyronine. He found that thyroxine caused swelling of isolated rat liver mitochondria. This effect was antagonized by $MgCl_2$ at a concentration of 10mM. He proposed that the mitochondrial structure, in some unknown way, is conditioned by critically located Mg^{++} sites on the mitochondrial membrane which are capable of binding magnesium. He further speculates that this phenomena is related to the known relationship between body temperature and plasma magnesium (58) the concentration of which is known to be elevated in hibernating animals. (59-61) Plasma magnesium has also been observed to rise in animals that have been experimentally cooled. This could very well represent a central nervous system depressing action necessary to maintain a state of hibernation. However, to date, this is still in the speculative stage. This rise in plasma magnesium, seen when animals are experimentally cooled, is not seen in humans.

Hyperthyroid patients are known to demonstrate significant increases in serum protein bound magnesium, even though their total serum magnesium remains normal. Conversely the bound magnesium was found to be low in patients with myxedema. The normal protein magnesium has been reported to be 14.5% of the total. In the hyperthyroid patient it has been reported to be about 20-40% of the total, and in patients with myxedema it is about 6% or less.(62) More refined measurements have indicated that the following values are probably more correct.

Fraction of bound magnesium in normal subjects	= 37%
Untreated hyperthyroidism	= 37.3%
After iodine therapy	= 37.3%
After thyroidectomy	= 35%

(63)

(iii) Pituitary

Hanna (56) reported an abnormally elevated concentration of plasma magnesium in a patient with renal dwarfism. At the same time he reported hypomagnesemic levels in a single case of acromegaly, and in two pituitary dwarfs who were treated with growth hormone. In a subsequent study (64) he reported on two additional patients, one who had been hypophysectomized, and the other who was a pituitary dwarf. Following hypophysectomy, calcium and magnesium absorption from the intestine was decreased, urinary excretion of calcium fell, but the level of urinary magnesium remained essentially unchanged.

Following intramuscular injection of growth hormone, intestinal absorption of calcium and magnesium rose as did the urinary excretion. The plasma magnesium fell, and plasma calcium was seen to increase. These changes then returned to normal within two days. These effects of growth hormone are thought to resemble those seen following administration of vitamin-D_2. Hanna suggested that both of these compounds may be exerting their known physiological effect by indirectly producing a rise in the plasma citrate levels.

(iv) Adrenal Cortex

Hanna (56) and Milne (65) have reported a lowered plasma magnesium accompanying primary aldosteronism. They divided their cases of aldosteronism into two main categories. The first of these included clinical conditions in which magnesium was lost from the

extracellular fluid space to bone and soft tissue. It also included patients who had undergone removal of a parathyroid adenoma and who were receiving vitamin-D or growth hormone. The patients in this group usually demonstrate a positive magnesium balance. The second group included patients who lost magnesium from the body by way of the gastrointestinal tract and the kidneys. In this category a negative magnesium balance is the cause of their hypomagnesemia.

In 1960 Hanna and MacIntyre (66) reported that, in a group of rats fed a liquid diet via a stomach tube and given daily doses of aldosterone ranging from 0.05 to 0.5 μg for three days there was a marked increase in both urinary and fecal magnesium. Furthermore, the increase was logarithmically related to the dose and was greater in the adrenalectomized than in the normal rats used as controls. A negative magnesium balance was observed in the adrenalectomized rats, with a depletion in the intracellular magnesium, as evidenced by a fall in the muscle magnesium concentration. Potassium level also fell in muscle and calcium increased. Primary potassium depletion in muscle does not cause secondary depletion of magnesium although the converse appears to be true. Therefore, secondary potassium depletion follows magnesium deficiency. (67) Hanna and MacIntyre (66) conclude that the action of aldosterone on cellular potassium may be secondary to its action on magnesium. The magnesium depletion found in primary aldosteronism should be attributed to a direct action of aldosterone itself and not to secondary renal damage.

Serum magnesium was found to be increased in human patients with Addison's disease. A similar elevation was also demonstrated in adrenalectomized dogs, cats and rats. (68) Dubois and his colleagues in 1943, had noted that the parenteral administration of magnesium salts caused alterations in the concentration of various phosphate esters. Phosphocreatine and ATP were increased, glucose-6-phosphate and fructose-6-phosphate levels were frequently decreased,

while changes were also seen in the muscle magnesium content and phosphate ester content. (69) Excessive levels of magnesium ions in muscle appear to inhibit the phosphokinase system responsible for formation of the hexose phosphates. (70)

(v) Pancreas

Decreased serum magnesium levels have been reported in patients with pancreatitis. The lowest level recorded was 0.95 mg/100 ml of serum, but no clinical symptomatology could be ascribed to this hypomagnesemia. Pancreatectomy does not produce any appreciable changes in serum magnesium levels. (71)

(d) Surgical Procedures

Patients undergoing a variety of surgical operations have been reported to have lowered plasma magnesium levels post-operatively (66) Heaton (72) reported that 56% of a group of patients undergoing surgery had lowered serum magnesium one day postoperatively. He observed moreover that these levels returned to normal on the second or third day postoperatively. Surgery was followed by a definite negative magnesium balance of about three days duration. Similar changes were observed after dietary restriction in their normal controls. The post-operative changes in urinary magnesium were also similar to the changes induced by dietary restriction in the normal controls. Although the urinary levels of magnesium were depressed in these surgical patients, they tended to return to normal in four or five days. Heaton suggested that the initial reduction in urinary excretion was due to the lowered ingestion of dietary magnesium, while the subsequent rise in magnesium excretion coincided with the increasing dietary intake of the patient.

(e) Magnesium Balance

Magnesium balance studies were first carried out as far

back as 1915. Magnesium intake and fecal excretion in infants with diarrhea was measured, demonstrating that in severe diarrhea, almost none of the ingested magnesium was absorbed. Magnesium metabolism has also been investigated in pathological states in which disturbances in the metabolism of other electrolytes occurs. Patients with idiopathic epilepsy have been reported to have low serum magnesium levels. (73) Lowered serum magnesium levels have also been reported in patients with chronic nephritis, when either muscular twitching or convulsions were present. Lowered levels have also been reported in patients with congestive heart failure who were treated with ammonium chloride and mercurial diuretics. (74-76)

(f) Diabetes

Large amounts of magnesium are found in the urine of diabetic patients during acidotic periods. Becket and Lewis (77) in a survey of 100 diabetic patients in acidosis, reported that their mean serum magnesium level was 2.64 $\pm$ 0.60 mg/100 ml, whereas thirty normal patients had a mean of 2.17 $\pm$ 0.33 mg/100 ml. The serum magnesium concentration usually follows a pattern similar to that of serum potassium. It is usually elevated before treatment, then during fluid and insulin therapy, the serum concentrations fall rapidly. Values as high as 9.3 mEq/L and as low as 0.56 mEq/L have been reported. (78) Results similar to the above have prompted Butler (79) to suggest adding magnesium as well as potassium to parenterally administered fluids. It would appear that in diabetic acidosis under treatment, magnesium like potassium is retained, indicating the existence of a deficit before treatment was initiated. (80) The deficit was ascribed to loss of intracellular ions as was shown by the fact that at the time this deficit existed, plasma levels of magnesium, potassium, and phosphate were high. Martin and coworkers (78) found that over a period of three and one half days of insulin deprivation, a loss

of 0.8 mEq/Kg of body weight was sustained. Approximately 40 mEq/Kg is retained during the first week of insulin therapy after an episode of acidosis. A renal loss of 23 mEq was noted during the initial fluid and insulin therapy of diabetic coma.

(g) Delerium Tremens

Magnesium salts have long been known to function as mild sedatives and were formerly used in the treatment of delerium tremens. It had been known for some time that patients suffering from chronic alcoholism had lowered serum magnesium levels. (80) That delirium tremens represented a state of hypomagnesemia was probably originally conceived because of the great similarity of symptoms demonstrated by patients with chronic alcoholism. That delirium tremours may be due to, or associated with, magnesium deficiency is based on the following experimental evidence. Initially thirty-three patients with chronic alcoholism were studied and divided into the following three categories. (81)

Category	Average Magnesium Conc.
Tremour Only	1.47 $\pm$ 0.2 mEq/L.
Tremour and Mild Delirium	1.46 $\pm$ 0.12 mEq/L.
Severe Delirium Tremens	1.29 $\pm$ 0.27 mEq/L.
Normal Subjects	1.91 $\pm$ 0.21 mEq/L.

When this group is compared to a group of sixteen patients with frank delirium tremens whose average magnesium level was 1.70 $\pm$ 0.20 mEq/L and to that of another group of twenty-seven alcoholic patients whose average levels were

2.05 ± 0.39 mEq/L, a considerable variation is seen. (82,30) These results tend to establish that the variation may be due to the difference in the methods employed and also that the decrease in serum magnesium concentration is not necessarily an absolute characteristic of this disease. (30) Serum magnesium levels were also found to be decreased in patients with clearly demonstrated alcoholic cirrhosis. (83,84) In one instance thirty-five patients were studied. These too, were divided into three categories according to the severity of their symptoms:

Category	Average Magnesium Conc.
Severe Confusion and Coma	1.37 ± 0.15 mEq/L.
Mild to Moderate Mental Symptoms	1.65 ± 0.25 mEq/L.
Asymptomatic	1.94 ± 0.30 mEq/L.

It may, therefore, be concluded that on the average, more severe impairment of liver function was present in the first group, although this derangement was not greater than that of many of the individuals comprising the other two groups. A lowered serum magnesium content in some patients with cirrhosis of the liver seems to be fairly well established although the degree of serum magnesium deficiency cannot be related to the severity of the disease process simply on the basis of the evidence presented.

(h) Protein Binding:

Several workers (85,86) have reported that approximately 14-50% of the total serum magnesium is protein bound. In the normal physiological pH range they reported that 35% of the total magnesium is protein bound. Magnesium reacts

with serum proteins in a manner identical to that of calcium over a wide range of pH's. The amount of each cation bound to protein is, therefore, virtually the same. (86)

Numerous reports are now appearing in the literature, using a variety of methods to measure protein bound and unbound magnesium. (85, 87-91) These workers present evidence indicating that the fraction of protein-bound magnesium is approximately 20-40% of the total serum magnesium. Carr and coworkers (86,92) found that calcium and magnesium compete on an equal basis for binding sites on albumin and the other serum proteins. An empirical formula has been presented by Copeland and Sunderman (85) for the calculation of the free magnesium ion concentration from the total protein and total magnesium concentration. About 10-15% of the non-ultrafilterable magnesium appears to be independent of serum protein. (89)

Ionized magnesium has also been determined by ultrafiltration (85, 93-95) and by its property to activate isocitric dehydrogenase. (96,97)

(i) Hypermagnesemia

Hypermagnesemia had been reported in chronic renal failure as early as 1923. Acute renal failure is also known to be accompanied by increased levels of serum magnesium. Levels as high as 3.81 ± 0.6 mEq/L have been reported, as compared to the normal level of 2.08 ± 0.18 mEq/L. (98) Very few reports have appeared in the literature dealing with clearly defined cases of hypermagnesemia, other than those resulting from kidney disease.

(j) Diurnal Variation

There is no detectable variation of magnesium levels throughout the day provided that strenuous exercise is avoided (93,99) however, there appears to be a sine wave pattern to

the level of an individual throughout the year. (100)

In a more recent publication Briscoe and Ragan (101) have found what they believe to be a diurnal rhythm in the renal excretion of calcium and magnesium similar to that of sodium, potassium and creatinine. They detected a reduction in the excretion of calcium, magnesium, sodium and creatinine at night. Furthermore, the decrease in calcium was proportionately greater than in the other constituents studied. They also detected slight but consistent diurnal variations in serum concentrations of calcium and magnesium, with lower values in the morning than in the evening. They concluded that diet and physical activity play a dominant role in this diurnal fluctuation, but they also suggested that one should not discount the possibility that there may be a rhythm in parathyroid function as well.

Meals appear to be without effect on the serum magnesium level in the adult. The pre and post-prandial serum magnesium levels remain essentially unchanged. (102,103)

In women there appears to be a significant serum variation with the menstrual cycle with levels higher at the mensus than at the intermenses. (100) The significance of this observation is to date, quite unknown.

It is clear from the above presentation, that the exploration of the importance of magnesium metabolism and its relation to clinical medicine and biochemistry is still in its infancy. Magnesium will undoubtedly gain increased significance in the etiology and therapy of hitherto enigmatic diseases. Much progress can be expected from improved methods for determination of magnesium and from research to unravel this complex of relationships in the function of the cellular apparatus.

2. Pharmacology

The earliest recorded studies of the pharmacologic effects of magnesium date back to 1869, when Jolyet and Cahours (104) studied the effects of an intravenous injection of magnesium sulfate. They demonstrated that magnesium sulfate produced muscular weakness or paralysis in dogs and frogs, similar to the effect seen when curare was injected. Around the year 1906 the effects of magnesium on the central nervous system were discovered. (105,106) At that time magnesium was used as an anesthetic for general surgery, (107) and as a local anesthetic. The first effects of this induced hypermagnesemia were noticed on the respiratory system. A depression in the respiratory rate was noted first, followed by unconsciousness. Magnesium was observed to have two sites of action on the nervous system. (109)

The peripheral effect may be abolished by physostigmine or neostigmine. It was also noted that if neostigmine was administered in combination with an analeptic such as pentamethylenetetrazol or beta-phenyliscpropylamine, an instantaneous and complete reversal of the toxic effects of magnesium was achieved. The peripheral effect of magnesium has been extensively studied in animals. It was demonstrated that stimulation of motor nerves of animals that had been previously injected with magnesium sulfate caused no contraction, but direct stimulation of the muscle was followed by normal contraction. This implicated the neuromuscular junction as the site of the block. (110) In view of this the gross effects of magnesium may be considered to be similar to that of curare.

The mechanism of action of curare and magnesium, therefore, appears to be similar in that both produce decreased sensitivity of motor end plates to acetylcholine. (111) They have however been found to differ in regard to the stimulating effect of potassium. Magnesium opposes the effect of both potassium and acetylcholine on the sympathetic ganglion. Curare has no measurable effect on potassium. If potassium and magnesium are infused simultaneously,

there is a decreased muscular and respiratory paralysis, as demonstrated by Smith (111) in dogs. Magnesium is believed to cause a decreased liberation of acetylcholine at the neuromuscular junction and sympathetic ganglia, an effect which is antagonized by an excess of calcium. The amount of acetylcholine liberated varies as a function of the local concentration of calcium and magnesium ions in the region of the end plate or ganglion. That is to say, elevated levels of magnesium and decreased levels of calcium stimulate the release of less acetylcholine than if the relative concentrations of calcium and magnesium are reversed. (112,113)

Wacker and Vallee (42) in their recent review of magnesium metabolism, indicated that this cation can cause a fall in blood pressure by arresting the heart in diastole. However, levels ten times the normal physiological range were needed (27-44 mEq/L) to demonstrate this effect. They further demonstrated that if a dog were infused with a magnesium containing solution until a plasma level of 5 to 10 mEq/L was reached, marked changes were seen in the electrocardiograph. They observed an increased PR and QRS interval, accompanied by an increase in the height of the T wave. The overall effect was to depress the cardiac rate and the cardiac output.

Boen and his coworkers (114) produced a lengthened T-complex when they rendered dogs hypocalcemic and hypomagnesemic. However no attempt was made to distinguish between hypocalcemia and hypomagnesemia on this basis.

Magnesium is also known to potentiate the effects of the posterior pituitary hormones on the uterus in vitro. When magnesium was present in the medium in which uterine strips were suspended even Vasopressin demonstrated marked oxytocic properties. (115)

Because of the extensiveness of the literature concerned with the pharmacology of magnesium ions, this section has only dealt with the effects of an increase in magnesium on the neuromuscular and cardiovascular systems.

The following section will deal with magnesium deficiency states and some of the nutritional aspects of magnesium deficiency.

3. Magnesium Deficiency Syndrome

In 1926 (116) Leroy demonstrated that magnesium was essential for mammalian growth. Young mice maintained on magnesium deficient diets had their growth arrested after thirteen days on this regime. Death followed after twenty to thirty days. In 1932, Kruse and his coworkers (117) described the classical acute magnesium deficiency syndrome in rats. Vasodilatation, manifested by erythema, and hyperemia was seen within five days of removal of magnesium from the diet. Concomitantly the animals exhibited signs of increasing neuromuscular irritability culminating in seizures, with death often occuring during the first seizure. Chronic deficiency in animals surviving for long periods of time on magnesium deficient diets was manifested by alopecia, trophic skin lesions, hematomata of the ear lobes and swollen hyperemic gums. The symptoms of acute hypomagnesemia are similar to those of hypocalcemic tetany, including a lowered threshold sensitivity. (118) Pathological changes are also seen in heart muscle, the vascular system, the kidneys, liver and the brain.

In 1938, Tufts and his coworkers also produced magnesium deficiency in rats, thereby substantiating the experimental evidence of Kruse mentioned above. (119)

Wacker and Vallee (42) have further demonstrated that the serum magnesium levels of deficient rats fell from 3.00 mg/100 ml to 1.00 mg/100 ml (2.46 mEq/L to 0.82 mEq/L) in about seven to nine days. The erythrocyte magnesium levels fell from 7.0 mg/100 ml to 2.0 mg/100 ml in about eleven days. Soft tissue magnesium did not appear to change, although whole body magnesium and bone magnesium fell rapidly. There is a compensatory rise in whole body calcium during this time.

Hypomagnesemic rats fed a diet high in magnesium rapidly accumulate large quantities of magnesium in the bone. The above authors speculate that the bone may function as a reservoir to meet

the soft tissue demands during periods of hypomagnesemia. As little as 5.0 mg/100 ml of magnesium per 100 grams of food per day will prevent the symptoms of magnesium deficiency from appearing.

Large amounts of ingested calcium are known to aggravate the severity of magnesium deficiency. A greater dietary intake of magnesium is, therefore, needed to prevent symptoms from appearing. (48) High protein intake also increases the requirement for magnesium and causes the dificiency symptoms to appear. When on a diet of normal magnesium content, (43) magnesium deficient rats are known to be incapable of synthesizing protein normally.

Rats maintained on a high calcium, normal magnesium diet were shown to have an alkaline urine, calciuria, and renal calculi. They also showed a low serum magnesium and an increased serum calcium level.

Numerous attempts have been made to produce hypomagnesemia in man without too much success. Two normal healthy males were maintained on a low magnesium diet containing 1.0 mEq/day for twenty-four days. The total loss of magnesium during this period was 25.8 mEq and 71.6 mEq respectively.

Interestingly enough the serum levels did not change. The urinary losses were initially very high, but decreased sharply thereafter. The two test subjects then stabilized at a low level of 5.0 mEq/24 hours. The authors concluded that normal adults are apparently able to conserve magnesium. (87) All experimental attempts to induce a simple dietary magnesium deficiency in healthy subjects have, to date, failed. Vallee and coworkers (29) ascribe this failure to correlate deficiency with clinical symptoms ~~may be due~~, in part, to the inadequacy of the available methods used to determine magnesium. Vallee and Margoshes claim that the development of the multichannel flame spectrophotometer capable of determining magnesium simply, rapidly and accurately, has provided a means of overcoming this obstacle.(120,122)

In a recent paper Vallee and coworkers (29) described a new specific clinical entity which they called "Human Magnesium-Deficiency Tetany". This syndrome is virtually identical to that of hypocalcemic tetany. However, the two may be distinguished chemically. The symptoms are almost identical to those seen in magnesium-deficient animals. In each of the five patients they studied, the parenteral administration of magnesium sulfate promptly and completely reversed the symptoms, signs and chemical changes of the syndrome. Their patients included three males and two females ranging in age from thirty-eight to sixty years. All of these patients suffered from severe malnutrition secondary to intestinal malabsorption or chronic alcoholism. Conclusive proof that the syndrome in these patients is entirely due to an alteration of magnesium metabolism is afforded by the correlation of symptomatology, with the chemical changes observed in the serum, and of the concomitant restoration of both to the normal state by therapy. Intramuscular magnesium sulfate will relieve hypomagnesemic tetany within several hours with a simultaneous return to normal of the serum magnesium. However, no apparent correlation was found between the ratio or sum of magnesium concentration to calcium concentration in this syndrome. In all cases tetany is the primary sign of hypomagnesemia. In both man and animals the only way that it can be distinguished from hypocalcemia is by the measurement of the serum concentrations of these two cations. Simple dietary magnesium restrictions does not appear to produce tetany. (123)

Vallee, Wacker and Ulmer (29) in 1960 have also described an acquired type of magnesium deficiency which they term "Conditioned Magnesium Deficiency." A conditioned deficiency is involved in which even the normal intake fails to meet the requirements of the body on a daily basis, owing to the excessive losses from the gastrointestinal or urinary tracts or from a failure to absorb. The signs of this deficiency thus appear against a background of prolonged vomiting, intestinal malabsorption, prolonged acute infection,

severe malnutrition produced by alcoholism, gastritis, intestinal obstruction, or postoperative drainage. When severely debilitated patients undergo surgery, and are then given magnesium-free parenteral fluids for long periods of time, one would expect them to demonstrate signs of magnesium deficiency.

Hanna and coworkers (54) claim, however, that gross magnesium deficiency may produce convulsions but that tetany is not produced unless there is a concamitant hypocalcemia. Their suggestion finds partial support in a case described by MacIntyre and coworkers in 1961. (124) Their patient, suffering from persistent diarrhea had gross hypomagnesemia but demonstrated no sign of tetany. On a low-calcium diet the serum calcium fell to approximately 3.0 mEq/L, but the serum magnesium remained unchanged at around 0.9 mEq/L. Tetany subsequently developed with positive Chvostek's and Trousseau's signs. However, the tetany disappeared when the patient was placed on a high-calcium diet, and the serum calcium rose to normal levels with the magnesium remaining low.

At first glance these observations appear to contradict the findings of Vallee and coworkers. (29) However, Vallee clearly stated that in their patients the serum calcium was normal initially and remained normal, and that the appearance and disappearance of tetany was directly related to the lowering and raising of the serum magnesium concentration.

The investigations of magnesium metabolism in man thus far represent a limited number of observations performed on small numbers of patients. Although these preliminary studies have been encouraging, they have been carried out in only a few disease states. Many gaps remain in the knowledge concerning both the diseases in which a deficiency or an excess of magnesium is known. The large quantities of magnesium present in the human body, and its known biochemical functions, make it reasonable to suspect that new and clinically useful information will become available as simple and rapid methods are developed for the determination of magnesium in biological material.

4. Analytical Methods For The Determination Of Magnesium

An accurate determination of magnesium in biological material is beset with difficulties. The more prominent of these are the nonspecific nature of its precipitation reactions; the liability to interference of other naturally occuring ions, notably calcium, in its colourimetric or titrimetric measurement, and finally the relatively low intensity of its spectral lines.

The relative abundance of methods available only testifies to their inadequacy and most of the methods presently in use are either inaccurate or cumbersome.

Efforts to overcome these difficulties have resulted in a large number of methods being proposed for biochemical purposes. When large amounts of organic material are present as in food, feces, plasma or blood, preliminary ashing or protein precipitation usually proves to be a necessary first step.

The methods available for the determination of magnesium in biological materials are as follows:

a) Precipitation followed by weighing, titration, colourimetry or flame spectrophotometry.
b) Direct colourimetry of magnesium complexes.
c) Complexiметric titration.
d) Direct flame emission spectrophotometry.
e) Atomic absorption spectrophotometry.
f) Fluorometry.
g) Polarography.

The principle methods in each of the seven categories listed above will be reviewed.

(a) Precipitation Methods

In 1910 McCrudden (125) Mendel and Benedict (126) introduced a precipitation technique based on the precipitation of calcium as calcium oxalate, followed by precipitation of magnesium as the ammonium phosphate salt. The precipitates obtained are ashed

and weighed. This method has been found to be applicable to the analysis of food, feces and urine.

McCrudden showed that in inorganic solutions the coprecipitation of magnesium with calcium oxalate could be avoided if conditions were properly chosen, and that addition of citrate reduced the interference from iron. Several modifications of this technique are presently in use. (127-131)

The determination of magnesium by precipitation as magnesium ammonium phosphate actually can be traced back to 1877, (132) when Stalba found that he could quantitate the double salt by acidimetric titration. If calcium were present, as in serum, urine, etc., the calcium must first be removed by precipitation as the oxalate. Then the magnesium in the supernate can be precipitated as the magnesium ammonium phosphate. To prevent the precipitation of the magnesium as magnesium hydroxide ($Mg(OH)_2$) or ($Mg_3(PO_4)_2$), the magnesium must first be precipitated in slightly acid solution as $MgHPO_4$. This salt was then converted to the $MgNH_4PO_4$ by the addition of ammonium hydroxide. The precipitate was then quantitated gravimetrically.

McCrudden's and Stalba's techniques have been extended for serum or plasma, but require a minimum of two ml of material. In 1917 Marriott (133) suggested that the magnesium concentration could be related to the ferric thiocyanate decolourization of the $MgNH_4PO_4$ precipitate. However, this method was never widely used because of the poor precision.

Denis measured the magnesium ammonium phosphate nephelometrically using strychinine molybdate. (134) Briggs (135) and Denis (136) later introduced the technique of determining the phosphate in the precipitate colourimetrically by the molybdenum blue method, using hydroquinone as the reducing agent. Variants of this latter technique are still being used, despite the numerous modifications that are still being proposed. The aminonapthosulfonic acid reagent of Fiske and Subbarow has been

used to replace the hydroquinone (137), and the molybdivanadate method for phosphate determination has also been applied. (138,139) This latter method has the advantage of colour stability. The major problem with all of these methods is the elimination of phosphate contamination during the precipitation step without sustaining a lose of some of the magnesium precipitate. There, therefore, remains the uncertainty of the completeness of the separation of calcium from magnesium as well as the difficulty of eliminating contamination of the precipitate without losing some of the material.

A second and widely used precipitating agent for magnesium is the 8-hydroxyquinoline method introduced by Yoshimatsu in 1929. The hydroxyquinoline in the precipitate has been quantitated photometrically with Folin's phenol reagent, (140,141) and by the blue-green colour formed with ferric iron in an acid solution. (142) It has also been quantitated photometrically by bromination followed by iodometric titration of excess bromide. (130,142)

In serum, calcium interferes to some extent, but this can be ignored if oxalated plasma is used initially. However, the precipitate of magnesium quinoline is light and fluffy, which may give rise to large errors from loss of the precipitate. Zinc and copper have often been reported as contaminants.

This method, in spite of its drawbacks, is rapid and requires no special equipment. It has, therefore, found quite wide acceptance for less critical applications.

b) Direct Colourimetry

The search for simple but rapid techniques to be used for biochemical purposes led to the application of the colour reactions of magnesium with the dye Titan Yellow (Clayton Yellow; Thiazole Yellow), first described by Kolthoff (143) in 1927. The method depends on the formation of a red magnesium hydroxide-dye

lake in an alkaline solution. The lake consists of a dye absorbed on the surface of colloidal particles of magnesium hydroxide ($Mg(OH)_2$). The earlier methods did not call for protein precipitation but later modifications demonstrated that this was a desirable preliminary step. (144) The lake so formed is suitable for colourimetry, although its colloidal nature requires the addition of gum ghatti or polyvinyl alcohol as a stabilizing agent. (144)

In 1934, Hirschfelder and coworkers introduced a modification of the basic procedure of Kolthoff. This was further modified in 1951 by Orange and Rhein. (36) Their technique permitted the use of a smaller sample and obtained greater sensitivity by using long path length colourimeter cuvettes. This procedure has been used especially where small sample size is desirable. Although the method is reported to be specific for magnesium it is fraught with difficulties, some of which are:

(1) As noted above the red lake has a tendency to precipitate out of solution. To stabilize this lake and thereby give an optically clear solution one must add gum ghatti, gum arabic, polyvinyl alcohol or a polyvinyl alcohol-sodium lauryl sulfate mixture. Mixtures of glycerol and starch have also been used by some workers, but none of these agents has been entirely satisfactory.

(2) The colour tends to fade. Hydroxylamine added to the reaction mixture prevents this to some extent.

(3) The colour reaction is not too sensitive, although polyvinyl alcohol tends to potentiate the colour development.

(4) Calcium and iron tend to interfere giving falsely high results. Calcium appears to potentiate the colour development.

(5) Gluconate is also known to interefere, especially at the levels normally used in infants undergoing exchange transfusion.

Bohuon (147) recently described a simple and direct colourimetric method for the determination of magnesium in serum, urine and feces. This method is based on the reagent, sodium 1-azo-2-hydroxy-3-(2,4-dimethylcarboxanilide)-naphthalene-1-(2-hydroxybenzene-5-sulfonate) which was introduced by Mann and Yoe (148) in 1956. At a pH of about 9.0 the blue colour of the reagent becomes pink in the presence of magnesium. When the colour is developed in an aqueous ethanol solution, the complex remains adequately stable for photometric measurement. Interferences from calcium at the concentrations normally found in serum is negligible. In most cases protein precipitation is unnecessary. It has also been noted that there is no interference from calcium gluconate or from drugs.

In 1964, Rice and Lapara (9) introduced a modification of the method of Bohuon. This procedure, employing a 0.02 ml sample, renders the method truly ultramicro in quantity. The method, besides being specific for magnesium, is not affected by the presence of oxalate, gluconate, citrate or calcium, a problem that has limited the usefulness of the other available methods. The small sample size has the added advantage of making this an ideal method for use in a pediatric unit.

The azo dye, Chrome Fast Blue BG has also been used in a photometric method for serum magnesium. (149) The azo dye 1,8-dihydroxy-2-(3'-chloro-6'-dihydroxybenzene-azo) naphthalene-3,6-disulfonic acid, requires no removal of calcium, no waiting for colour development and no stabilizing agents. It is sensitive to quantities of magnesium as small as 0.01 μEq/ml of solution. This method represents a definite advantage over methods such as the Titan Yellow.

(c) Complexometric Titration Methods

Most titrometric methods are based on the chelating agent disodium ethylenediaminetetraacetate (EDTA; "VERSENE"), which has been used extensively during the past ten years. Preliminary deproteinization of the serum is not necessary. Usually two titrations are required using different indicators, one to give the total of magnesium plus calcium, and the other calcium alone. Therefore, magnesium is determined by difference. The dye Eriochrome Black T, gives the concentration of calcium and magnesium, and ammonium purpurate yields the calcium concentration alone. To date, many modifications and a variety of indicators have been suggested. (149-156) High protein and phosphate concentrations tend to slow down the colour change of the indicator. Phosphate should be removed previously as the morpholinephosphotungstate. Other divalent cations, notably Zn and Fe are known to interfere with the colour development. The method, as employed today, requires 0.1 ml of serum, making it suitable for use in a pediatric hospital. Although fast, it is unreliable.

(d) Emission Spectrophotometry

Quantitative emission spectrophotometry has now found wide acceptance. Flame spectrophotometric methods are more accurate, but at the same time tend to be more difficult than the currently available colourimetric methods. The main difficulty is in the emission characteristics of the element itself. Flame emission spectrophotometry uses two main wavelengths for the determination of magnesium. The magnesium oxide emission band at 370mμ is easily excited, but unfortunately considerable spectral interference can occur here. The atomic emission band at 285.2mμ is sharp, but requires a high flame temperature for its effective excitation. It should be noted, however, that this latter emission line is very close to the sodium line at 285.3mμ.

Davis (131) used 8-hydroxyquinoline to precipitate the magnesium thus freeing the magnesium from other interfering ions such as calcium, sodium, potassium and phosphate. He then measured the magnesium concentration at 285.2 mμ. Dissolving the precipitate in an acetone-acetic acid mixture improved the emission characteristics. Oxalate in the original sample was found to depress the flame emission. Kapuscinski and coworkers (157) used the oxide bands for magnesium and calcium. They ashed the samples and then sprayed a concentrated extract of this into the flame. Their method proved to be subject to interference from sodium, potassium, and phosphate.

Teloch (158) introduced a modification of the method of Davis, (131) suggesting that magnesium should be measured at 372 mμ. However sodium, potassium, glucose and phosphate still caused a significant amount of interference.

Alcock and coworkers (159) used an instrument with an oxyacetylene flame, a narrow slit width, a single resolution double monochromator and a wavelength of 285.2 mμ. Interference from sodium, potassium and phosphate was largely overcome by including appropriate concentrations of these ions in the standard solutions used. They found that it was necessary to deproteinize the sample before analysis.

Fawcett, Wynn (160) and Montgomery (161) have devised the most practical and most widely used method to date. They used a commercially available single monochromator instrument, an air-acetylene flame, and a wavelength of 285.2 mμ. The emission, at this wavelength, due to the magnesium was small by comparison with the nonspecific flame background. However, other factors such as monochromator band width, wavelength stability during a series of measurements, and flame temperature plus background stability made this method one of the most reliable so far in this category.

Many flame spectrophotometers now in use are capable of measuring magnesium in serum, urine, feces and other biological

fluids including tissue homogenates. (10,162) However, in most cases it is advisable to remove protein and interfering ions such as sodium prior to analysis.

(e) Atomic Absorption Spectrophotometry

Atomic absorption spectrophotometry is an interesting and new approach to the determination of elements such as calcium and magnesium. This method, originally developed by Willis (163), and later modified by Walsh (164) for biochemical use, is based on the estimation of the light absorbed by the excited atoms of the element at the wavelength of the resonance line of the element. Light emitted from a quartz-ended hollow discharge tube using a magnesium cathode, is passed through a flame in which the sample is vapourized. The emerging light passes through a single monochromator to a phototube. The higher the concentration sprayed, the more light of the magnesium resonance wavelength emitted by the lamp is absorbed in the flame, and the lower is the phototube current. The lamp's emission is specific for magnesium and non-specific absorption in the flame is small, and can be easily corrected for. Therefore a high resolution optical system is unnecessary. (165-167)

Dawson and Heaton (165) have constructed an instrument that has won wide acceptance in hospital laboratories. To date, this would appear to be the method of choice for magnesium determinations for clinical purposes.

(f) Fluorometry

Schacter (168) has showed that at the concentration of magnesium likely to be encountered in biological materials, the fluorescence of the magnesium-8-hydroxyquinoline complex in ethanolic solution is almost specific for magnesium. This method fortunately avoids precipitation of magnesium, a step usually responsible for the relatively large errors inherent in other methods. No interference from other cations has been reported to date. However,

few reports describing the use of this instrument in clinical biochemistry have appeared in the literature, making it rather difficult to assess the worthiness of this technique.

(g) Polarography

Direct polarographic measurement of magnesium in serum and urine has not as yet been achieved. However, an indirect method has been reported by Irving. (169) This method is based on the polarographic determination of zinc liberated by calcium and magnesium from an ammoniacal zinc-EDTA-complex. It is noteworthy that this procedure has not yet been widely used in clinical biochemistry, because of the cost of the equipment.

—SECTION III—

EXPERIMENTAL STUDIES

In the experimental section of this thesis, the general methods used, and the procedures common to the various studies undertaken will be described first. The results will then be presented under Sections V to VIII inclusive.

(1) Serum Ionic Magnesium In Exchange Transfusion.

(2) The Effect On Serum Ionic Magnesium Of Exchange Transfusion With Citrated As Opposed To Heparinized Blood.

(3) Serum Magnesium Levels In The Newborn And Older Child.

(4) Ultracentrifugal Studies Of Protein-Bound And Free Magnesium In Normal Human Serum.

In each of these four sections the aim of the particular experiment will be defined, and a discussion relating the findings to those of other investigators will follow the presentation of the results.

- SECTION IV -

GENERAL METHODS

1. Reagents and Materials.

(a) Glassware:

All glassware used in these experiments was thoroughly washed with detergent and water, soaked overnight in 1:1 nitric acid, and rinsed several times in deionized glass distilled water. This glassware was reserved for magnesium determinations only and was protected from trace metal contamination after it had been cleaned. The blood was collected in new unused "vacutainers."

(b) Water:

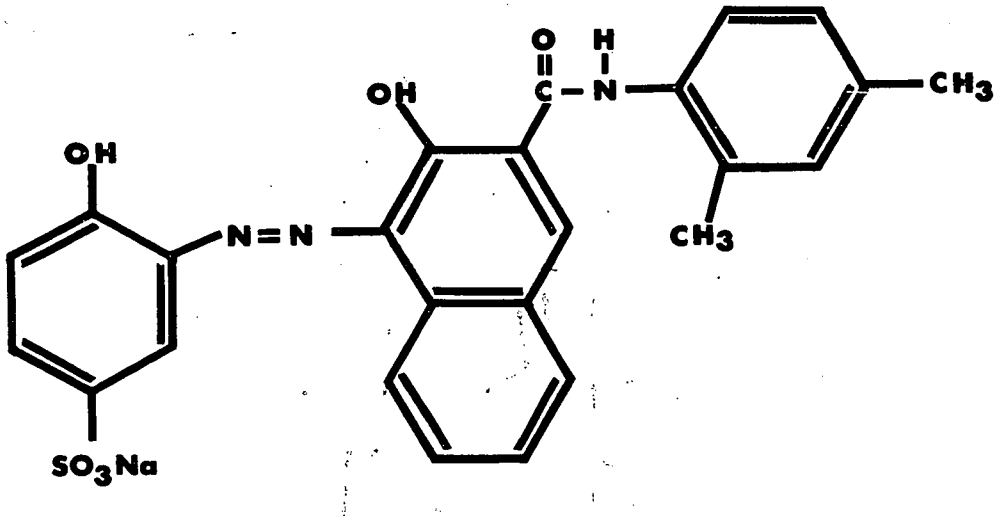
Only resin-deionized glass distilled water was used for the preparations of reagents and solutions used in the determinations.

2. Reagents.

(a) Mann's Dye Reagent:

Dissolve 25.0 mg of Mann's Dye, Sodium 1-azo-2-hydroxy-3(2,4-dimethylcarboxanilide) naphthalene-1(2-hydroxybenzene-4-sulfonate) in 200 ml of redistilled 95% ethanol in a 250 ml volumetric flask. Fifty ml of water was then added and the contents of the flask were mixed to ensure homogeneity. This reagent, if stored in a cool dark area, is stable for at least two months.

- MANN'S DYE -



Sodium 1-azo-2-hydroxy-3-(2, 4-dimethylcarboxanilide)-naphthalene-1'-(2-hydroxybenzene-4-sulfonate)

(b) Sodium Borate, 0.08M.:

Dissolve 30.51 grams of $Na_2B_4O_7.10H_2O$ in 500 ml of hot water. The resulting solution is allowed to cool to room temperature before being diluted to 1.0 liter.

(c) Blank

Mix 44.0 ml of water; 48.0 ml of redistilled ethanol; and 8.0 ml of 0.08M sodium borate.

(d) Working Reagent.

This solution is prepared as follows; 50 ml of water; 10 ml of sodium borate; and 40 ml of redistilled 95% ethanol are mixed together.

Solutions b,c, and d, are stable indefinitely if stored in a cool dark area.

The appropriate volume of working reagent is prepared fresh daily by mixing accurately, in a volumetric flask, four volumes of the alcoholic borate solution with one volume of the Mann's Dye reagent. This solution is stable for approximately twenty four hours if kept away from direct sunlight.

(e) Magnesium Standard Solution, 10.00 mEq/L:

0.1233 grams of $MgSO_4.7H_2O$ is accurately weighed out, dissolved, and made up to 100 ml with water. Working standards of 1,2,3, and 4 mEq/L are prepared fresh daily by accurately diluting the stock standard solution 1:10; 2:10; 3:10; 4:10 with water.

3. Procedure.

Using a 20 µl Beckman Micropipette exactly 20 µl of unhaemolyzed serum was added to a test tube containing 5.0 ml of working reagent. The tube was covered with Parafilm and the

contents were mixed thoroughly.

With each set of unknowns, a reagent blank tube and a standard tube were prepared by adding 20 µl of H_2O or 20 µl magnesium standard solution respectively to 5.0 ml of working reagent.

After waiting ten minutes, to allow the reaction to go to completion, the absorbance of each unknown was determined at 600 mµ against a respective serum blank consisting of 20 µl of serum mixed with 5.0 ml of blank. A Beckman DB Spectrophotometer, with a cuvette having a 1.0 cm light path, was used throughout.

The absorbance of the reagent blank and the standards (1-4 mEq/L) were determined at 600 mµ against water.

This colour is stable for twenty-four hours. It is unaffected by temperature variations, but appears to be light sensitive. Therefore, it is advisable to keep the solutions out of direct sunlight.

The Mann's Dye is specific and very sensitive for the determination of magnesium. In the pH range of 8 - 11 magnesium ions cause a colour change from blue to purple. As this colour change occurs there is a simultaneous decrease in the absorbance at 600 mµ. Because absorbance at 600 mµ represents the amount of unreacted dye, it would be anticipated that the difference between the absorbance at 600 mµ of a reagent blank and an unknown would measure the amount of pink magnesium-dye complex formed. Moreover, this absorbance difference should be proportional to the magnesium concentration of the serum.

4. Calculations:

$$\frac{(\text{Absorbance of Reagent Blank})-(\text{Absorbance of Unknown})}{(\text{Absorbance of Reagent Blank})-(\text{Absorbance of Standard})} \times 2.00 = \text{mEq Mg/L}$$

Because of the sensitivity of the method the following restrictions are imposed:

(1) The difference between the Reagent Blank and the 2.00 mEq/L Standard must never exceed 0.105 O.D. Units, and must never be less than 0.095 O.D. Units.

(2) The difference between the duplicate samples must never be more than 0.005 O.D. Units.

(3) A 1.00 mEq/L and a 2.00 mEq/L Standard must be prepared for each series of determinations. If the standard curve is to obey Beer's Law then the difference between these two standards must be 0.050 O.D. Units.

(4) Lipemic serum must be cleared of its chylomicrons by an initial centrifugation at high speed.

If any of these conditions were not met, particularly numbers one and three, the entire series of determinations was repeated.

- SECTION V -

SERUM MAGNESIUM IN EXCHANGE TRANSFUSION

Citrated blood used for the purposes of exchange transfusion results in the formation of an incompletely ionized calcium citrate complex and consequently a decrease in the amount of ionized calcium in the circulation. (1,2). In routine clinical practice this is anticipated, and an attempt is made to correct it by the intravenous administration of calcium, in the form of a 10% calcium gluconate solution, at regular intervals during the exchange procedure. Like calcium, magnesium is also a divalent cation, and is also known to be bound by citrate. (4) The salts of bivalent ions in solution are not completely dissociated, but form chelation complexes. (170) While data are available regarding the behavior of calcium during exchange transfusions (2), corresponding data on magnesium are inadequate and difficult to interpret. (5) The present study was undertaken to provide information regarding the behavior of non-protein bound serum magnesium levels during exchange transfusions with citrated blood and to determine any related clinical symptoms thereof .

- Materials and Methods -

Biological Material

Studies have been carried out during thirty-three exchange transfusions given to a total of sixteen infants within the first week of life. In all instances the indication for exchange transfusion was hyperbilirubinemia. Of the sixteen infants, six had erythroblastosis due to Rh incompatibility, ABO incompatibility was diagnosed in five of the patients, and the remaining five had idiopathic hyperbilirubinemia presumably resulting from a delay in the maturation of hepatic enzyme systems.

Of this last group, four infants were premature by weight and gestational age, and the fifth was a full-term infant. All of the blood used in the transfusions was prepared in a similar manner, so that each 500 ml represented a mixture of 120 ml of acid-citrate-dextrose complex and 380 ml of donor blood.

Preparation Of The Anticoagulant:

Acid-citrate-dextrose * complex is the standard Canadian Red Cross mixture using the NIH Formula B anticoagulant. This contains 4.4 grams citric acid, 13.2 grams trisodium citrate and 14.7 grams glucose per liter of anticoagulant. Each bottle of donor blood contains 120 ml of this solution plus 380 ml of blood. Therefore, each bottle of donor blood would contain 0.528 grams citric acid plus 1.584 grams trisodium citrate.

Many transfusion centers in the United States use the NIH Formula A anticoagulant in a ratio of 450 ml of donor blood to 67.5 ml of anticoagulant. Formula A contains 7.3 grams citric acid, 22.0 grams trisodium citrate and 24.5 grams glucose per liter of anticoagulant. Therefore, each bottle of donor blood so constituted will contain 0.493 grams citric acid and 1.485 grams of trisodium citrate. The citrate levels are then of a similar order of magnitude for the two preparations, and the results of the present studies are, therefore, applicable to either of the anticoagulant mixtures.

Methodology:

In each case the blood transfused was less than three days old, and the total amount exchanged varied from 125 to 150 ml per kilogram of body weight. Calcium in the form of a 10% solution of calcium gluconate ** was administered at regular intervals during the exchange procedure.

In all instances the pre and post-transfusion blood was sampled, and when possible, samples were taken at 100 ml intervals

* Abbott Laboratories, Chicago, Ill.
** Sandoz Pharmaceuticals, Hanover, N.J.

during the course of the exchange transfusion. Care was taken to discard the first two ml aliquot of blood drawn, in order to avoid adulteration with donor blood trapped in the catheter. All samples obtained were of venous blood via the umbilical vein. In addition, samples of the donor blood taken from the bottle, were also analyzed. In each instance blood was taken from a glass syringe into a sterile dry test tube and the serum separated after centrifuging at 2,000 r.p.m. for 10 minutes. The serum was then kept at a temperature of 0° to 4°C and analysis performed within twenty-four hours. All estimations were carried out in duplicate and agreement between paired samples was obtained to within five per cent.

Serum magnesium determinations were carried out as detailed in the General Methods, Section IV of this thesis.

- Results -

The results of this study will be presented under four major headings.

1. Effect Of Exchange Transfusion On Ionized Serum Magnesium.

In all the cases studied there was a marked fall in the level of ionized serum magnesium as a direct result of the exchange transfusion. (Figure 1) Because the pre-exchange level of magnesium, with succeeding exchange transfusions, tends to be lower, mean values have been calculated for pre and post-exchange levels for the initial exchange only (Figure 2). For the sixteen infants studied, ionized magnesium pre-exchange levels showed a range of 1.62 to 2.00 mEq/L with a mean value of 1.81 mEq/L. These values are in the range of the reported normal. (171) Corresponding post-exchange levels ranged from 0.8 to 1.3 mEq/L with a mean value of 1.07 mEq/L.

There was a progressive fall in the serum ionic magnesium during the course of the exchange transfusion as increasing amounts of blood were replaced, followed by a return

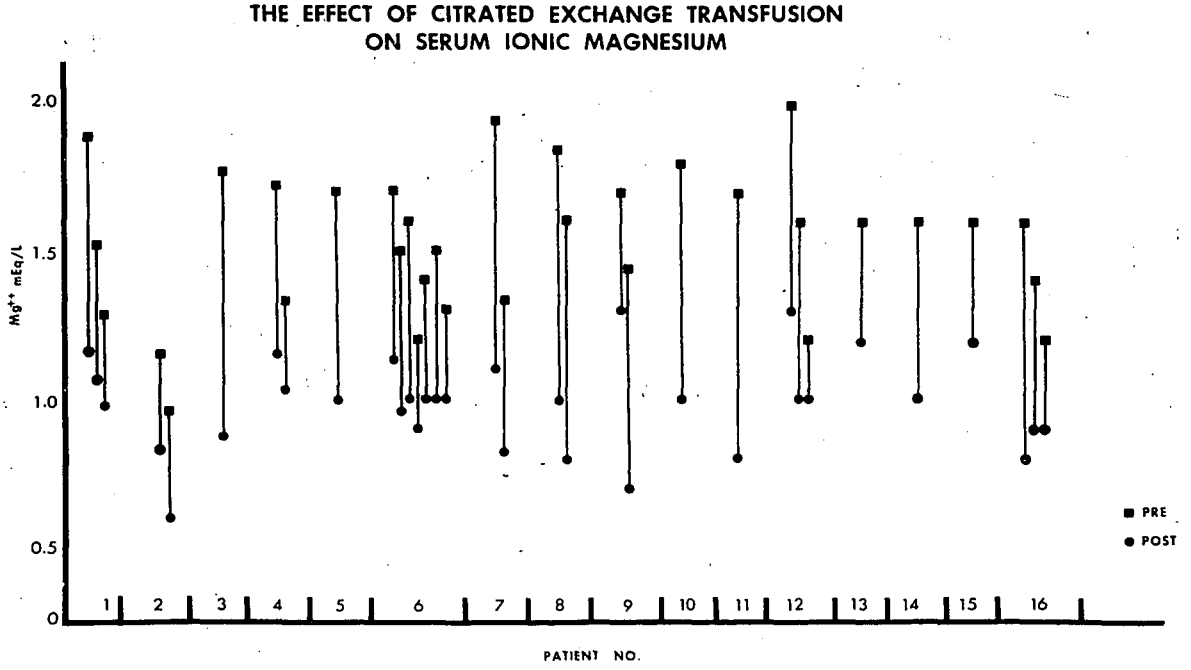


Fig. 1 The effect of citrated exchange transfusion on serum ionic magnesium. In every instance there is a fall in ionized serum magnesium at the end of the transfusion. Note the effect of repeated exchanges (cases No. 1,2, 4,6,7,8,12 and 16) in which the pre-exchange magnesium levels are lower than before the first exchange.

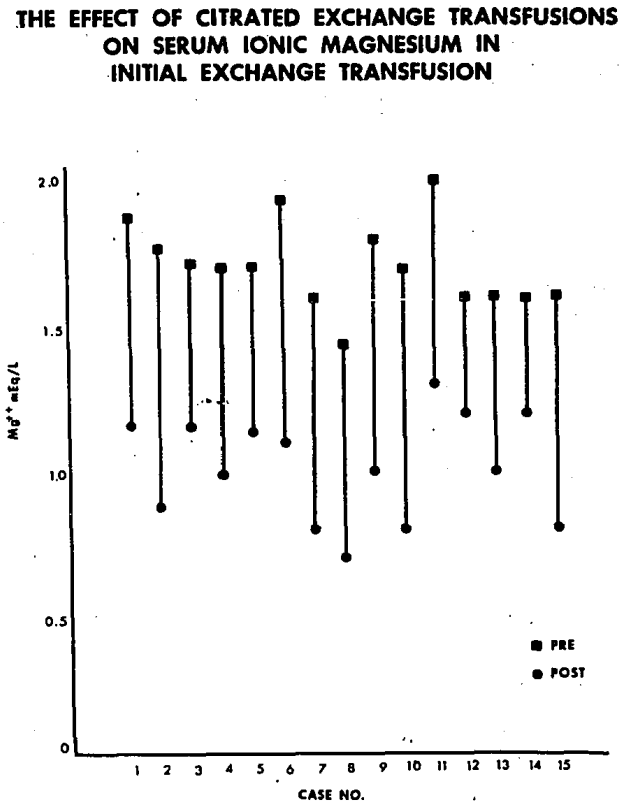


Fig. 2 The effect of citrated exchange transfusions on serum ionic magnesium in initial exchange transfusion. Only pre and post-exchange levels of ionized serum magnesium for the initial exchange are reported. In all cases the pre-exchange levels are greater than the post-exchange levels.

toward the pre-exchange levels once the exchange had been completed (Figures 3 and 4). However, with repeated exchange transfusions, the initial pre-exchange level tended to be lower with each subsequent exchange (Figure 5) so that while the resultant drop was not as great, the lowest levels occurred following multiple exchanges. This relationship may also be appreciated from Figure 1.

2. Effect Of The In Vitro Addition Of Citrate:

To 2.1 ml of a standard Mg^{++} solution, prepared as previously described, was added 1.2 ml of acid citrate dextrose (ACD) complex identical to that used in preserved blood. The proportions are identical to the ratio of ACD to the plasma present in the standard Canadian Red Cross transfusion bottle. Figure 6, demonstrates that the addition of ACD results in a profound reduction in the amount of recoverable ionic magnesium. This effect is not produced by the addition of dextrose alone, therefore, suggesting that it is the citrate portion of the ACD mixture which is responsible for this reduction.

3. Effect Of Hypomagnesemia On The Electrocardiogram:

No definite clinical effects attributable to hypomagnesemia were observed in any of the infants under study. However, electrocardiographic monitoring demonstrated a flattening of the T waves in one of the 16 patients when the serum ionized magnesium fell below 0.8 mEq/L. (Figure 7) The time interval between tracings was 1 hour thirty minutes. The patient demonstrating this phenomenon had a pre-exchange magnesium level of 1.7 mEq/L, and a post-exchange level of 0.8 mEq/L. Two other babies actually had lower magnesium levels without ECG changes while one infant (No. 6) had seven exchange transfusions without a depression of magnesium below 0.9 mEq/L (Figure 1).

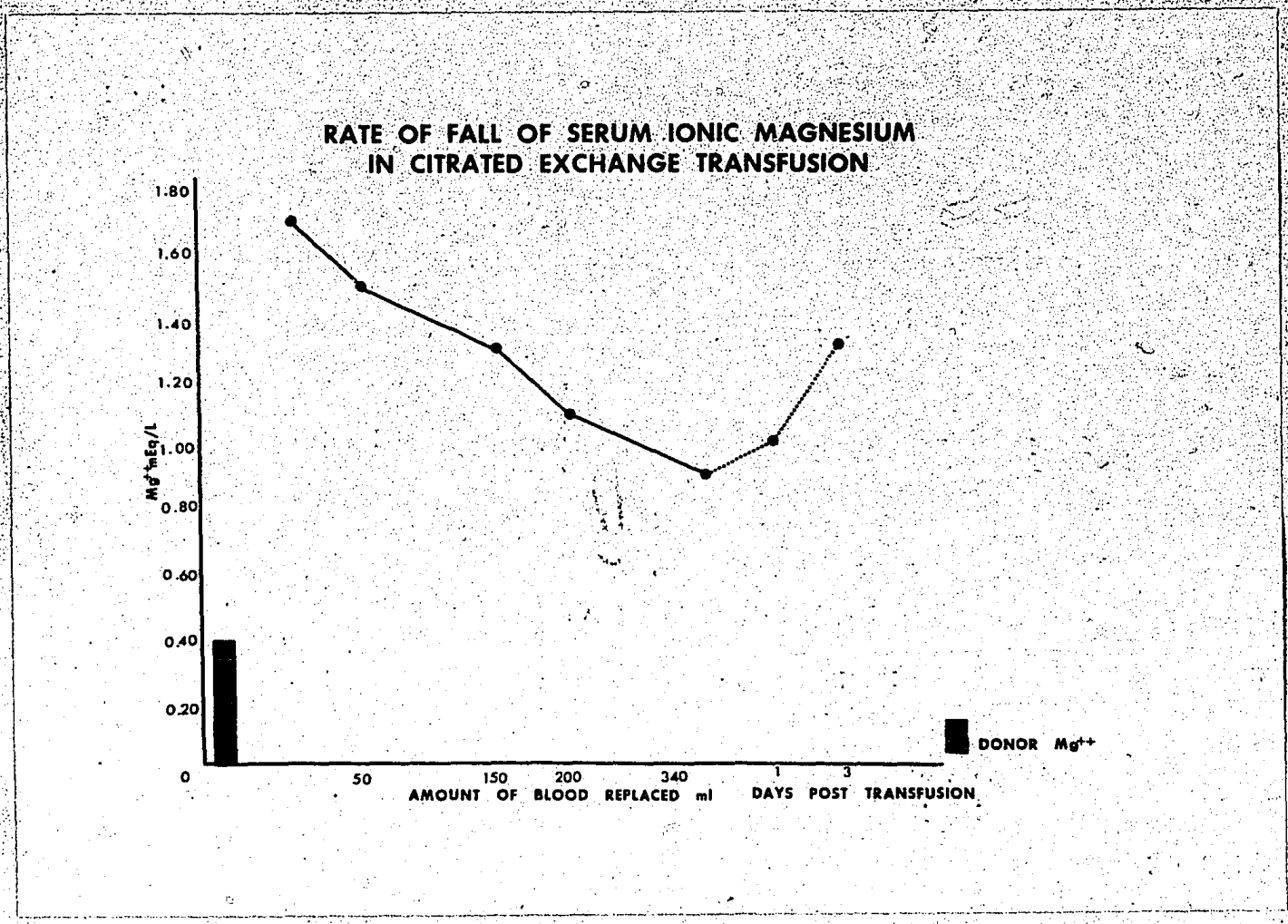


Fig. 3 Rate of fall of serum ionic magnesium in citrated exchange transfusion. The points on the solid line show the progressive fall in ionized serum magnesium as increasing amounts of blood are exchanged. The points on the dotted line following this show a progressive rise in the days following the exchange. The level of ionized serum magnesium in the donor blood is shown in the block at the lower left.

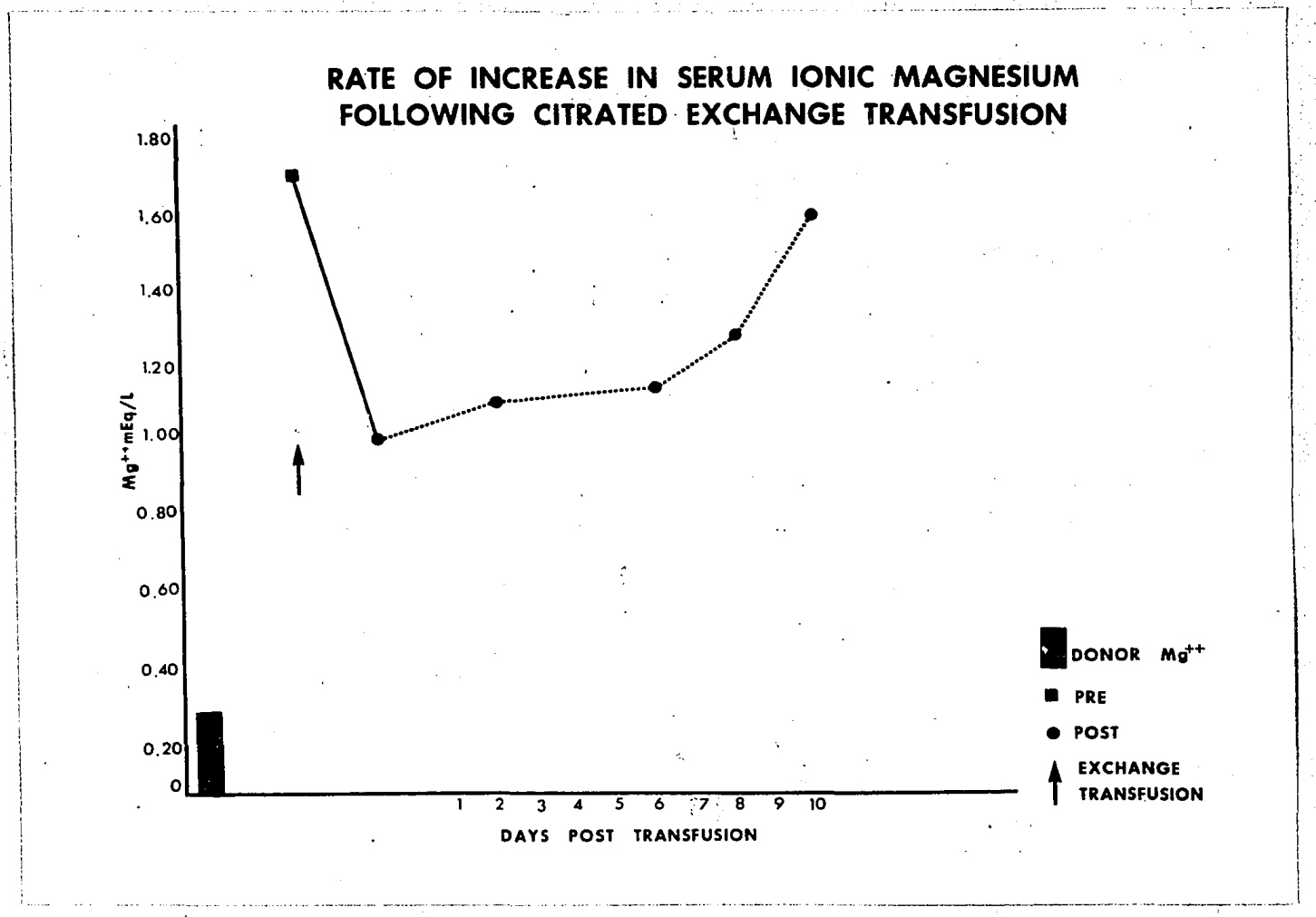


Fig. 4 Following a single exchange transfusion, the dotted line shows a progressive rise in serum ionized magnesium in the days following the transfusion. The level of ionized serum magnesium in the donor blood is indicated in the block at the lower left.

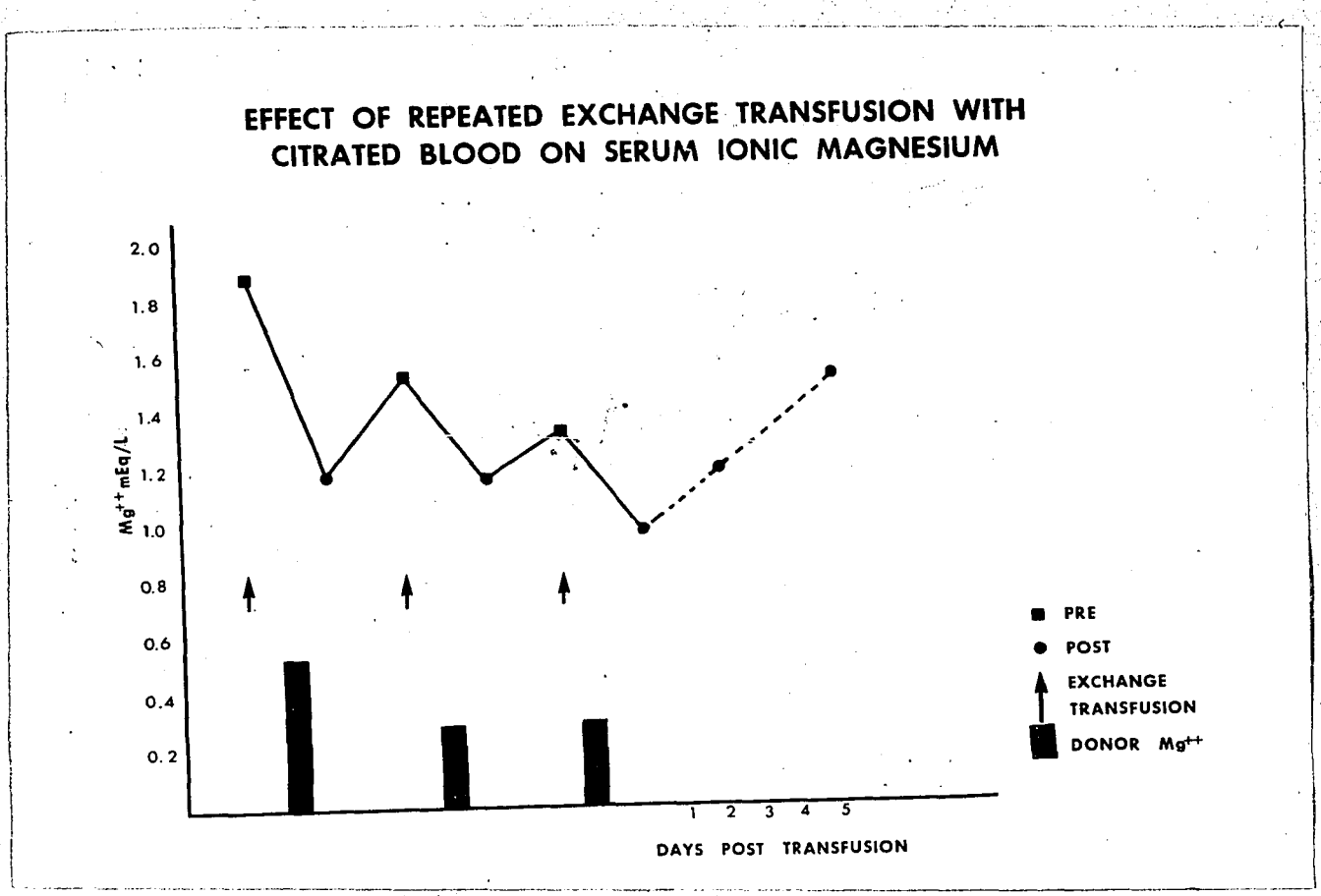


Fig. 5 With repeated transfusions the pre-exchange level is progressively less and the resultant post-exchange level lower. Ionized serum magnesium content of the donor blood is shown in blocks for each exchange. The dotted line indicates the rise in serum ionized magnesium in the days following the last (third) exchange.

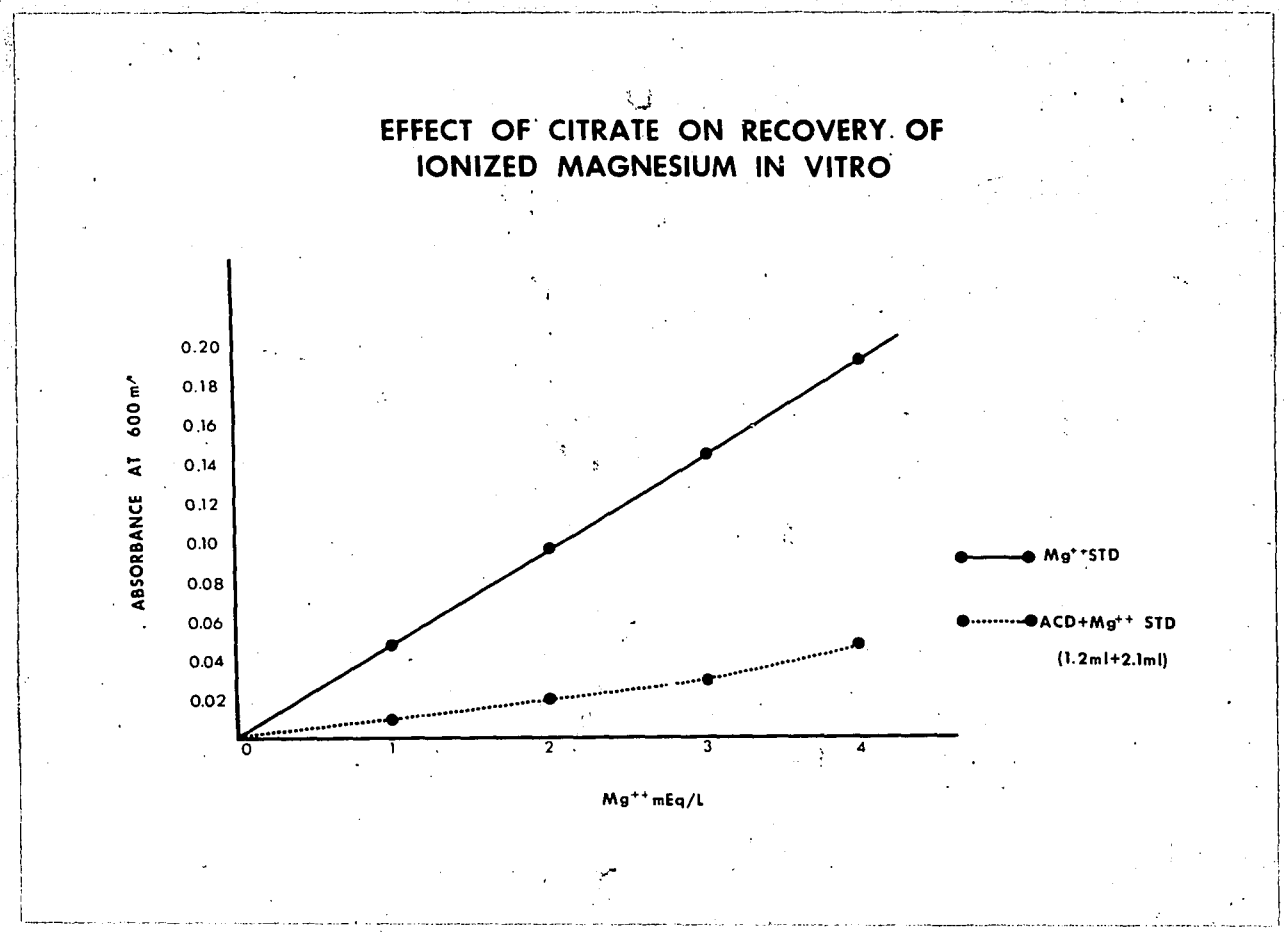


Fig. 6 The standard recovery curve is shown in black line. The dotted line indicates the marked depression of recovery when citrate is added to the medium. The effect is not seen if dextrose alone is added.

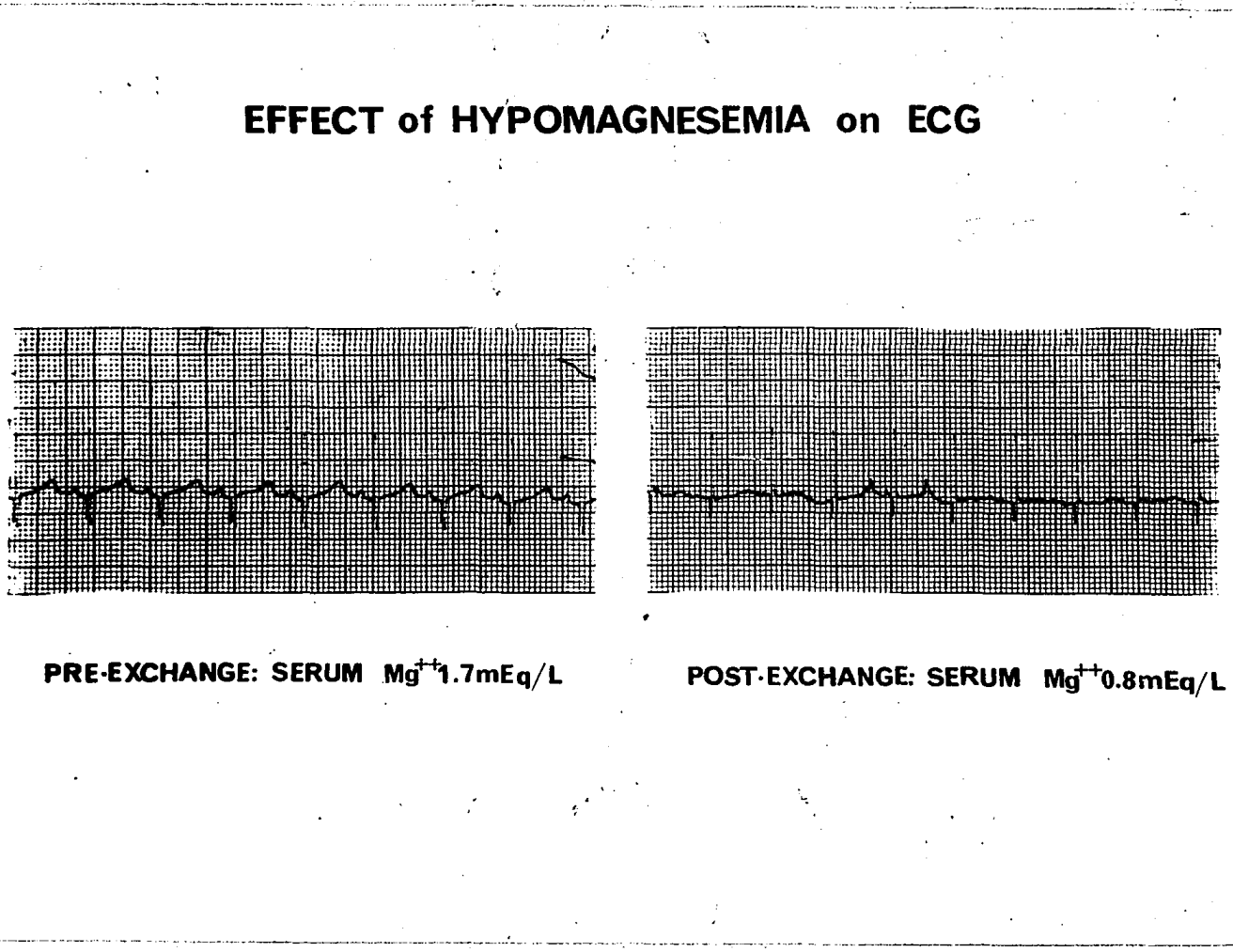


Fig. 7 Effect of hypomagnesemia on electrocardiograph, Lead II. Note flattening of T waves on post-exchange tracing. Time interval between tracings was 1 hour, 30 minutes.
Left, Pre-exchange: serum ionized magnesium = 1.7 mEq/L.
Right, Post-exchange: serum ionized magnesium = 0.8 mEq/L.

- Discussion -

During the course of an exchange transfusion with citrated blood, Farquhar and Smith (2) indicated that one must consider the following four biochemical situations relative to the clinical situation of the infant:

(1) The influence of high citrate levels.
(2) The influence of Ca ion depression by citrate.
(3) The influence of high serum potassium levels.
(4) The influence of the mutually reinforcing influence of high potassium ion and low calcium ion on the heart.

They demonstrated large excesses of citrate in the infant's serum as the exchange progressed. When considering point #2; if the formation of the calcium citrate complex, in vivo, were dependent only upon chemical kinetic factors, then all of the available calcium ion in these infants should have been combined as calcium citrate, except for the small amount liberated in the primary dissociation of the calcium citrate complex. This dissociation is not sufficient to restore the calcium ion to normal levels. The administration of calcium, in the form of the readily ionizable calcium gluconate, is necessary to offset the calcium lowering effect of the citrate.

During the exchange transfusion the rate of exchange is the most important factor in the development of hypercitremia. This induced hypercitremia develops as the result of the infusion of approximately 120 ml of ACD solution with every 380 ml of donor

blood. In fact, Wexler and his colleagues(1949) (1) showed that the level of citrate rose to a value that was from ten to to thirty times greater than the pre-transfusion value. In 1956, Mollison suggested a maximum rate of infusion of 260 mg of citrate per Kg of body weight per hour. (172) However, Wexler admitted to using rates far in excess of this without clinical incident.

Mackay and his colleagues in 1940, (173) showed that rabbits injected with sodium citrate intravenously exhibited an increase in the size of their livers. This increase in liver size was shown to be due, in part, to an increase in liver glycogen following administration of the citrate. It would appear, therefore, that in the case of exchange transfusion, the citrate may be rapidly converted to liver glycogen and perhaps even lipid. It should be borne in mind, however, that reports describing the rapid metabolism of citrate during the infusion of citrated blood are based on the observation of plasma citrate concentration during a period when rapid diffusion out of the plasma compartment is occurring (1,2, 174). The transfer of citrate to the other compartments of the body water could account, at least in part, for the disappearance of exogenous citrate from the plasma water.

A combination of these two facts (i.e. metabolism and diffusion), could account for the rapid removal of citrate out of the plasma compartment.

Each bottle of donor blood used in this study contained 0.528 grams of citric acid plus 1.584 grams of trisodium citrate. Citrated blood is, therefore, weakly acid in reaction (pH = 6.7-7.0). Such a medium would favor the production of a metabolic acidosis. This increase in hydrogen ion concentration tends to compensate for the existing hypocalcemia and hypokalemia in terms of neuromuscular irritability.

The results of this present study clearly demonstrate a marked and consistent depletion of ionized serum magnesium as a result of exchange transfusion with citrated blood. This conclusion

is at variance with that found by Anast, (5) who reported reduced levels of serum magnesium but attributed this to an artifact due to interference by gluconate from calcium gluconate administered during the exchange transfusion. As pointed out by Rice and Lapara (9), the values obtained with the method employed in the present study are unaffected by the presence of calcium gluconate. Thus, these results do not give rise to the same uncertainty of interpretation.

Citric acid forms a weakly dissociated salt with divalent cations such as Mg^{++} and Ca^{++} (4). In fact Robinson and Stokes in 1955, (170) pointed out that the salts of divalent cations in solution are not completely dissociated but form chelation complexes with anions such as citrate. It would, therefore, seem reasonable that the rise in serum ionized magnesium which occurs after completion of the exchange transfusion (Figures 3 and 4) results from the release of magnesium from this chelate as the citrate is metabolized. Other possible mechanisms to account for this rebound effect would be the release of protein-bound magnesium to the unbound form and/or a shift of intracellular magnesium to the extracellular compartment. From the figures presented, it would seem that unless sufficient time elapses for this to occur between exchanges, the initial pre-exchange levels are likely to be lower with each subsequent exchange, so that the absolute level tends to be lowest following repeated exchange transfusion (Figure 1).

The in vitro evidence of the effect of the addition of citrate on the recovery of Mg^{++} correlated well with the in vivo findings of Bunker, Benedixen, and Murphy (174) after the infusion of sodium citrate into an adult male. They were able to demonstrate a fall in serum magnesium as the citrate level rose. Further support for this effect can be seen from the low levels of serum magnesium in the donor citrated plasma (indicated in blocks on Figures 3,4, and 5). The mean ionized serum magnesium in thirty bottles of donor blood

used in this study was 0.5 mEq/L, as compared with the value for normal adult males of 1.5 to 2.0 mEq/L. (175) This level is lower than one would expect simply on the basis of the dilution effect of the ACD mixture in the bottle. The failure of the addition of dextrose or calcium gluconate alone to affect the recovery of Mg^{++} supports the contention that citrate is the responsible agent. Therefore, hypomagnesemia after exchange transfusion with citrated blood is a reflection both of the in vivo citrate effect and the use of donor blood of low magnesium content.

Symptomatic hypomagnesemia has been reported in both adults (176) and newborn infants (177). The effects have been mainly manifested through the central nervous system, with lethargy, disturbances of consciousness, muscular irritability, tremulousness, and sudden severe epileptiform seizures. Because many of the effects of low serum magnesium may be suppressed by the presence of calcium (29), it is possible that clinical effects during exchange transfusions with citrated blood are masked by the regular administration of calcium in the form of calcium gluconate. In addition, the relatively rapid rebound in magnesium levels may prevent effects at the cellular level.

Even though no definite clinical effects of hypomagnesemia were noted in any of the infants studied, electrocardiographic monitoring demonstrated a flattening of the T wave when the serum ionic magnesium fell below 0.8 mEq/L (Figure 7). Hanna and coworkers (176) reported a similar effect in adults, which was reversed by the administration of Mg^{++}. It is interesting to note that similar T wave changes were demonstrated by Robinson and Barrie (178) in thirteen of thirty infants continuously monitored during exchange transfusion. Unfortunately, no accompanying serum magnesium levels were reported.

Finally, it should be pointed out that exchange transfusion still carries with it an incidence, variously reported as

1.0 to 7.5 percent, of unexplained and often sudden death ascribed to the procedure itself. (179-182) Further studies of the state of ionized serum magnesium may provide additional information on this phenomenon.

- SECTION VI -

THE EFFECT ON SERUM MAGNESIUM OF EXCHANGE TRANSFUSION WITH CITRATED AS OPPOSED TO HEPARINIZED BLOOD

In blood transfusions the use of citrate as the anticoagulant has become almost traditional. For most purposes this anticoagulant is quite satisfactory. Blood treated with this acid-citrate-dextrose complex may be kept for long periods of time against any emergency. However, the use of citrated blood for purposes of exchange transfusion must be accompanied by the routine administration of calcium, in the form of a 10% calcium gluconate solution, to combat the calcium-binding effect of the citrate. Despite this there are relatively few studies of the actual changes involved, because of the difficulty in the measurement of ionized calcium. Like calcium, magnesium is also a divalent cation and is similarly bound to citrate. (1,4) Unlike calcium, however, methods for the estimation of ionized magnesium are more readily available (9,36). In Section V of this thesis, evidence has been presented, demonstrating a profound fall in serum ionized magnesium when citrate is used as the anticoagulant for the donor blood in exchange transfusion. (6) The present study was undertaken to evaluate this finding, using heparinized instead of citrated donor blood.

- Methods and Materials -

Biological Material

Serum ionized magnesium levels before, during, and after replacement transfusion were determined in twenty infants. In all instances the indication for exchange transfusion was hyperbilirubinemia. Rh incompatability with erythroblastosis foetalis was present in eight, and ABO incompatability was suspected as a causal factor in five cases. The remaining seven infants were

classified as having "idiopathic" hyperbilirubinemia presumably resulting from a delay in maturation of liver enzyme systems.

In all instances fresh blood less than 24 hours old was used. The amount exchanged varied from 125 to 150 ml per Kg body weight.

Preparation Of Anticoagulant Solutions Used

Of the twenty infants studied, ten were exchanged with Canadian Red Cross citrated blood. The usual mode of collection involves the addition of 380 ml of donor blood to 120 ml of an acid-citrate-dextrose (ACD) solution (NIH Formula B). The ACD mixture contains 0.94 mM of sodium citrate and 0.55 mM of anhydrous citric acid per ml of anticoagulant. There is, therefore, a total of 32.4 mM of citrate per 100 ml of donor blood.

Five infants were exchanged with blood in which the anticoagulant used was Panheprin 2115*. Sodium citrate is used as a buffer in this solution. Usually 470 ml of donor blood is added to 28.2 ml of the anticoagulant.

The Panheprin mixture contains 1.95 mM of sodium citrate per ml of anticoagulant. There is thus a total of 11.0 mM of citrate per 100 ml of donor blood.

Five further infants were transfused with specially prepared blood using only heparin in saline as the anticoagulant. Here 470 ml of donor blood is added to 2.1 ml (2100 units) of sodium heparin plus 26.1 ml of 0.9% saline. Thus no citrate whatsoever is present in the donor bloods used in the last five infants under study.

In Vitro Effects Of Citrate On Ionized Magnesium

To determine the in vitro effect of citrate on ionized magnesium the following studies were carried out. Each of the three anticoagulants used, (ACD mixture, Panheprin, and Heparin-saline) was added to four different concentrations of a Mg^{++}

* Panheprin 2115: Abbott Laboratories, Chicago, Ill.

standard solution. The proportions were 0.5 ml of anti-coagulant to 4.5 ml of Mg^{++} standard. The Mg^{++} standard was fixed at 1, 2, 3, and 4 mEq/L. Aliquots of this mixture were then added to 5.0 ml of Mann's Dye in the usual manner. The effect of the addition of each of the anticoagulants on the standard Mg^{++} recovery curve measured as absorbance at 600 mμ on a Beckman model DB spectrophotometer was then determined.

To determine the quantitative effect of citrate on the recovery of Mg^{++} increasing amounts of citrate in the form of a progressively more concentrated sodium citrate solution were added to 2.0 mEq/L (4.0 mM/L) of Mg^{++} and the absorbance was read as above. The maximum citrate concentration used was 80 mM/L. The 2.0 mEq/L standard was chosen because it represents the closer approximation to the normal serum values in the newborn infant (171,8).

Methodology

Serum ionized magnesium was determined by the method of Bohuon, using Mann's Dye as modified for ultramicrospectrophotometric use by Rice and Lapara (9). This method has been fully described in General Methods; Section IV of this thesis.

This method has the advantage that its accuracy is unaffected by the presence of calcium, gluconate or citrate, a problem which has limited the usefulness of other commonly used methods in these circumstances (5,8). In each case both pre and post-transfusion specimens were obtained. In addition, intermediate samples were taken at 100 ml intervals during the course of the exchange transfusion. A sample of donor blood was analyzed in each case. All samples were venous blood taken via a catheter inserted into the umbilical vein. With samples taken during the procedure itself care was taken to discard the initial 2.0 ml to avoid contamination of the sample by donor blood trapped in the catheter. The blood was taken from a glass syringe into a sterile dry test tube and the serum was separated after centrifuging at

2000 r.p.m. for 10 minutes. The serum was kept in the cold at a temperature of 0°-4°C until analysis which was always performed within twenty-four hours. All estimations were carried out in duplicate, and agreement between paired samples was obtained to within 5%.

- Results -

The results of the present study are to be presented under three main headings:

1. Ionic Magnesium Content Of Donor Bloods

As shown in Table 1, there is a striking difference in ionized serum magnesium levels of citrated as compared to heparinized donor blood. Thus, the mean ionized serum magnesium level of the donor blood for the ten infants in whom ACD blood was used was 0.42 ± 0.07 mEq/L. By contrast, the ten heparinized donor bloods (five Panheprin and five Heparin-saline) showed a mean serum ionic magnesium of 1.45 ± 0.03 mEq/L. These differences are highly significant ($P < 0.001$)

2. Effect Of Citrated Versus Heparinized Donor Blood On Ionized Serum Magnesium Levels In Vivo

The mean serum ionized magnesium for the ten infants subsequently exchanged with citrated blood was 1.75 ± 0.16 mEq/L with a range of 1.44 to 2.00 mEq/L, as compared to a mean of 1.59 ± 0.11 mEq/L with a range of 1.40 to 1.80 mEq/L, for the ten infants subsequently exchanged with heparinized blood. The differences are not significant ($P > 0.05$).

It can be seen in Table 1 that after 100 ml of blood had been exchanged there was a drop in serum ionized magnesium in the ACD group infants in each case. The fall varied from 0.56 to 0.30 mEq/L with a mean of 0.41 mEq/L. The five Panheprin exchanged infants show a similar but less severe drop (range: 0.21 to 0.44 mEq/L.) There is no change at all in the heparin-saline group.

TABLE 1

SERUM Mg^{++} LEVELS IN EXCHANGE TRANSFUSION

TYPE OF EXCHANGE	CASE NUMBER	PRE TRANS Mg^{++}mEq/L	AFTER 100 ml Mg^{++}mEq/L	POST TRANS Mg^{++}mEq/L	DONOR BLOOD Mg^{++}mEq/L
ACD	1	1.44	1.09	0.70	0.40
	2	1.80	1.24	1.00	0.40
	3	1.70	1.30	0.80	0.50
	4	1.60	1.20	0.74	0.30
	5	1.80	1.50	1.00	0.50
	6	1.88	1.54	1.16	0.48
	7	1.70	1.25	0.99	0.30
	8	1.94	1.50	1.10	0.47
	9	2.00	1.50	1.30	0.40
	10	1.60	1.30	1.20	0.40
PANHEPRIN R_*	1	1.73	1.52	1.65	1.40
	2	1.55	1.40	1.60	1.35
	3	1.50	1.35	1.55	1.40
	4	1.60	1.40	1.55	1.38
	5	1.50	1.36	1.50	1.40
SODIUM HEPARIN	1	1.40	1.44	1.44	1.40
	2	1.56	1.60	1.60	1.58
	3	1.60	1.56	1.60	1.40
	4	1.80	1.84	1.80	1.60
	5	1.60	1.60	1.60	1.58

R_* Abbott Laboratories

Serum ionized magnesium levels in exchange transfusion. Sodium heparin is referred to as heparin-saline in the text.

When one considera the post-exchange levels, the differences between Panheprin and Heparin-saline seen at the 100 ml mark have disappeared. All five of the Panheprin-exchanged infants show serum ionic magnesium levels which have returned to the pre-exchange level. The post-exchange levels are now similar for both heparin groups of infants. The mean ACD group post-exchange value is 0.99 $\pm$ 0.16 mEq/L, while the mean heparin (Panheprin + Heparin-saline) exchanged values are 1.59 $\pm$ 0.08 mEq/L. There is therefore a striking difference ($P<0.001$) between the pre- and post-exchange serum ionized magnesium levels in the ACD group. For the heparin groups the pre- and the post-exchange values are essentially unchanged. This finding is shown graphically in Figure 1.

In Figure 2, the serum ionic magnesium is plotted against the increasing volume of blood exchanged. A longitudinal study of three replacements using respectively ACD, Panheprin and Heparin-saline is shown. Aside from the slight, transient, rapidly recoverable drop seen with the Panheprin, there is no real change during the procedure in the heparinized group. By contrast the ACD exchange group shows a rapid and severe decline in ionized serum magnesium. When this is plotted logarithmically, the changing rate of the decline shows a progressive disappearance which one would expect with the progressively changing relationship of patient-to-donor blood.

3. <u>The In Vitro Effect Of Citrate On Mg^{++}</u>

The addition of the ACD mixture results in a marked depression of the recoverable ionic magnesium at all four concentrations of magnesium. Neither Panheprin nor Heparin-saline appreciably lowers the curve. (Figure 3). The slight depression seen at higher magnesium concentrations is thought to be the result of some degree of magnesium heparinate complexing. The complex so formed ionizes relatively easily (183).

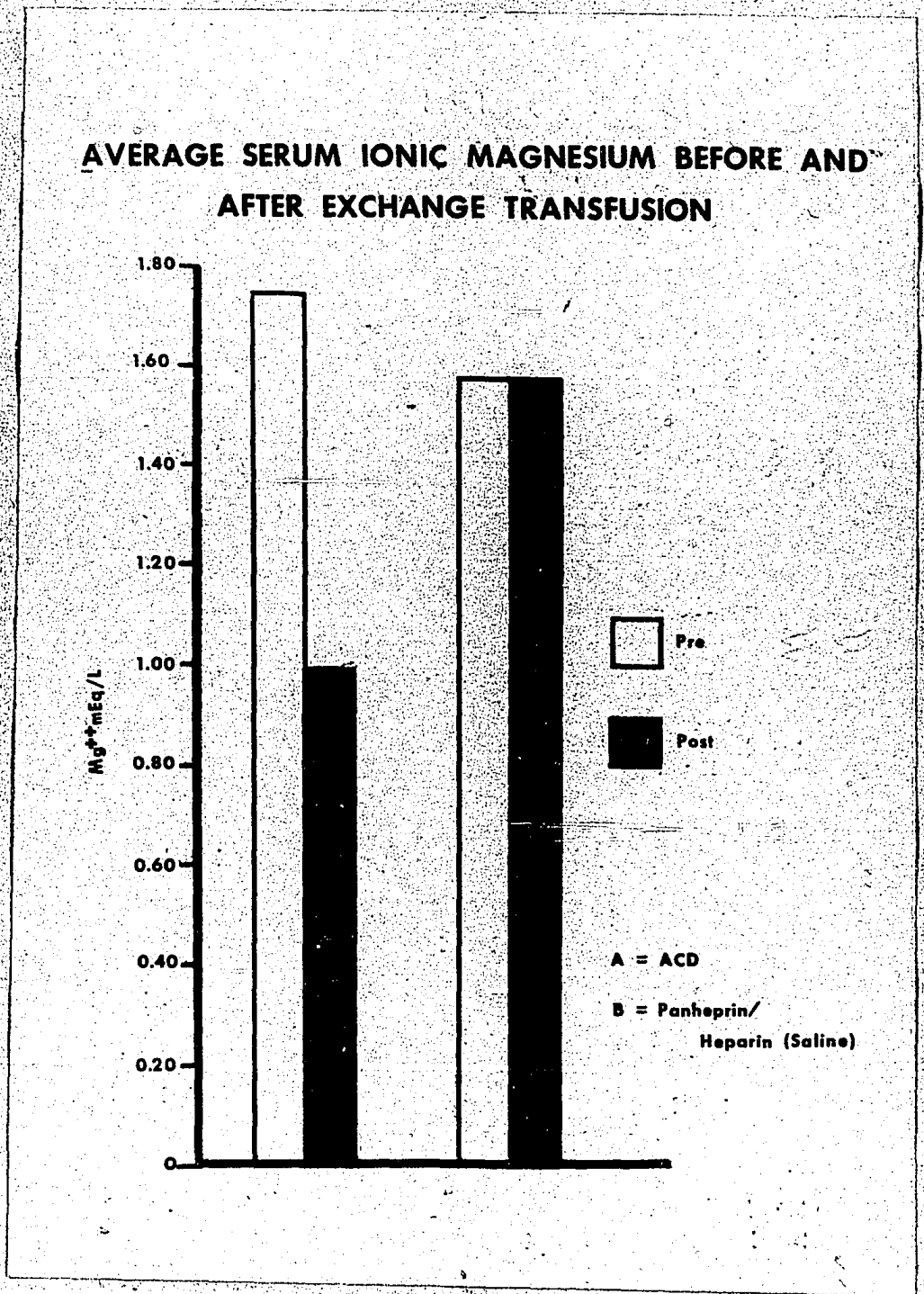
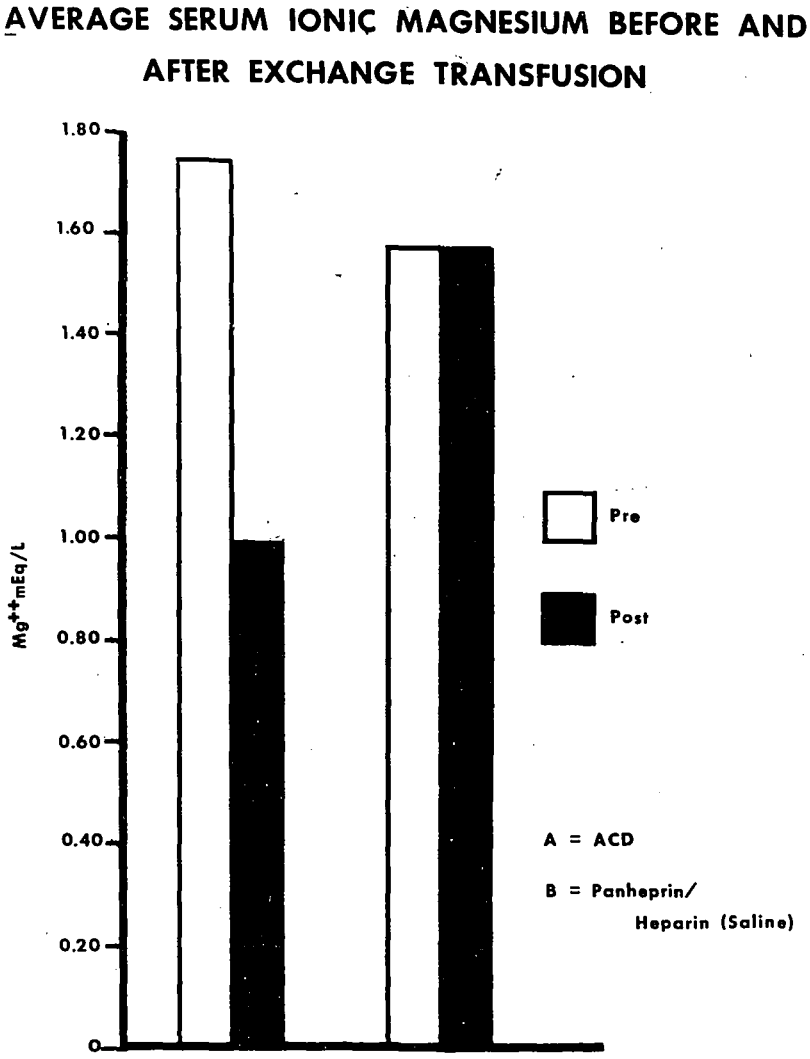


Fig. 1 Average serum ionized magnesium before and after exchange transfusion. The clear bars represent pre-exchange levels; the dark bars represent the post-exchange values. The two heparin groups have been combined (B). Note the fall in the serum ionic magnesium in the citrate group (A).



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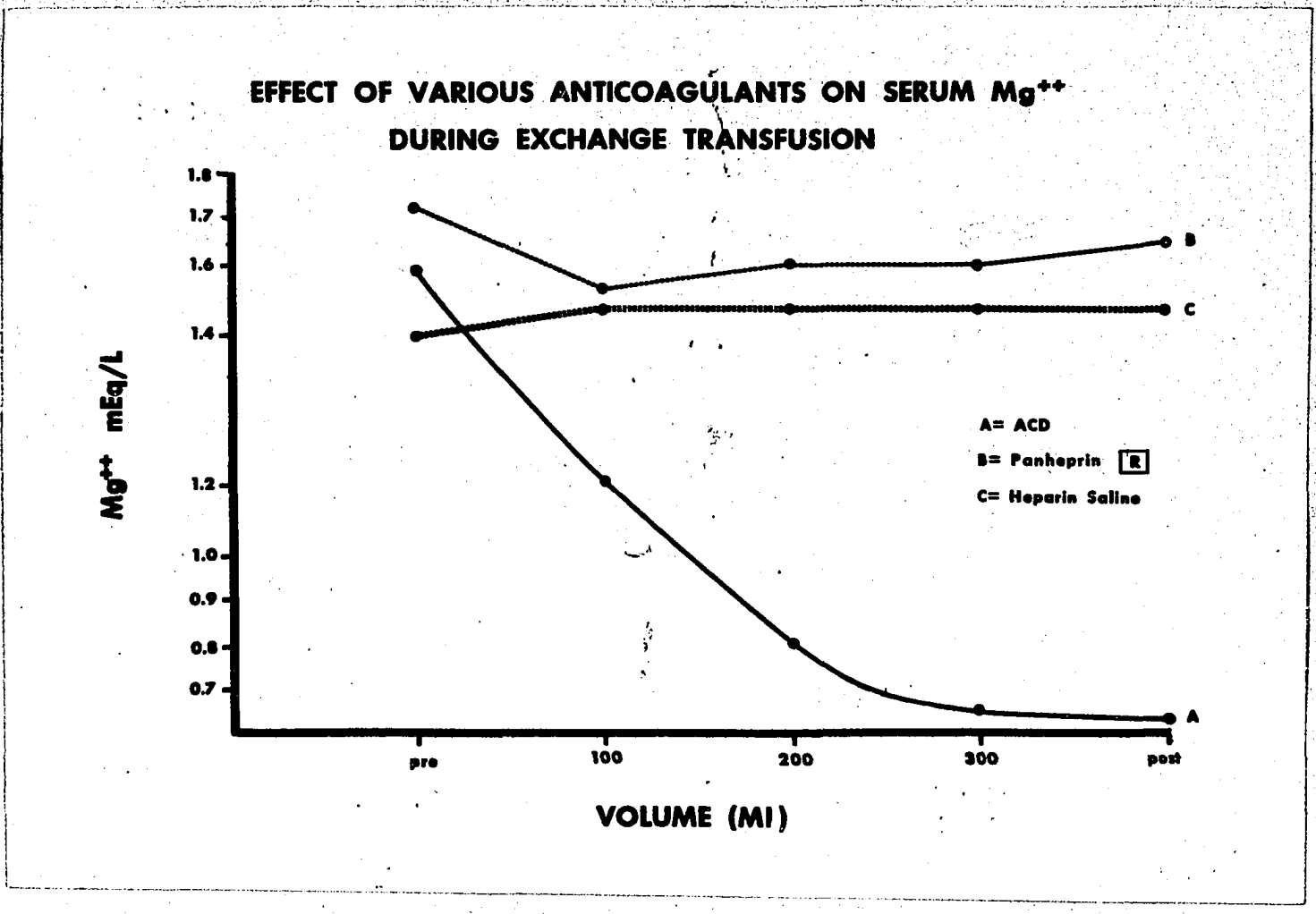

Fig. 2 Effect of various anticoagulants on ionized serum magnesium during exchange transfusion. Changes in serum ionic magnesium are plotted against the increasing volume of blood exchanged. There is no real change with heparin-saline (C), and a transient early fall which subsequently rises with panheprin (B). The values obtained during ACD exchange are plotted on a logarithmic scale (A).

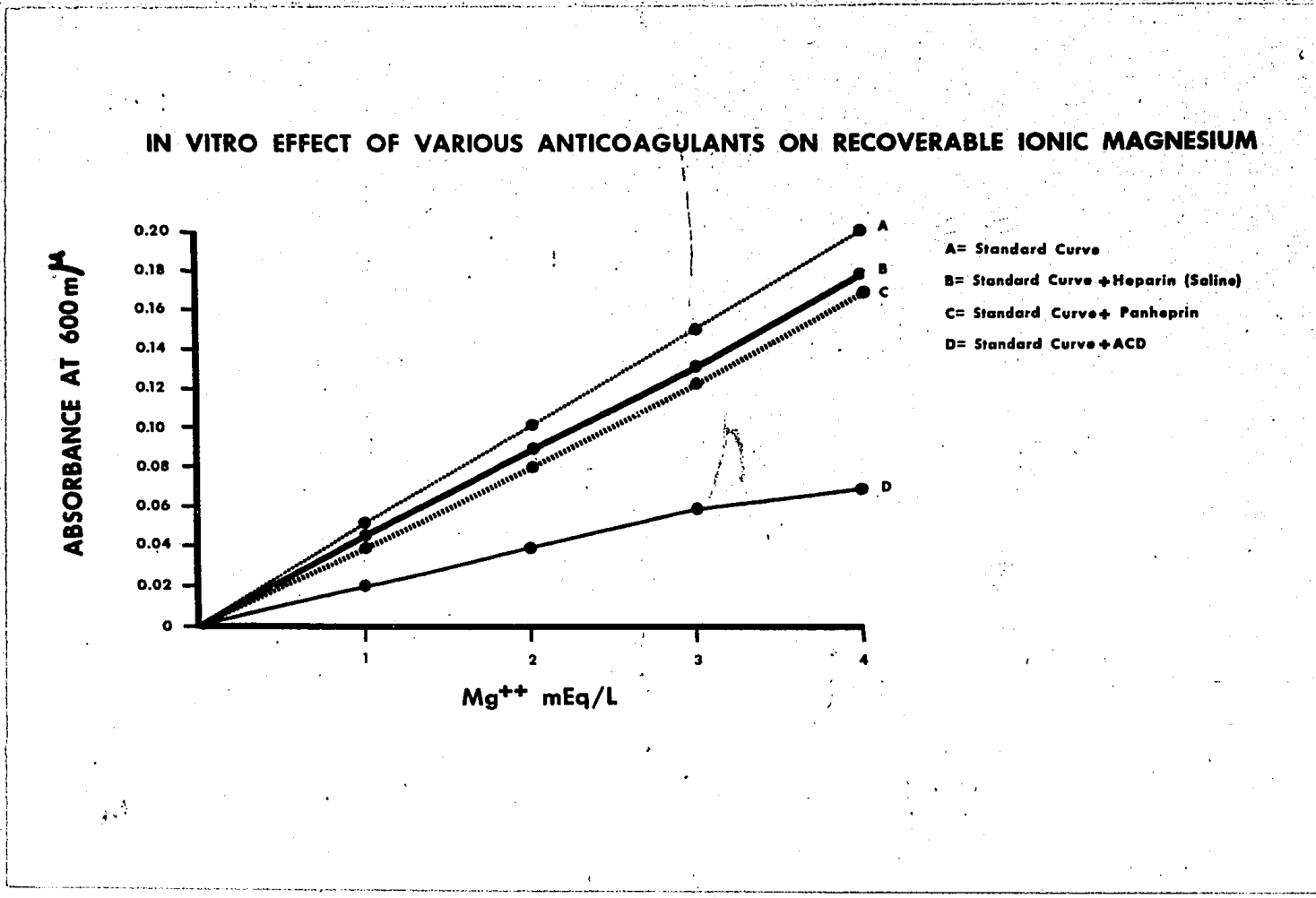

Fig. 3 The in vitro effect of various anticoagulants on the standard Mg^{++} recovery curve. Recovery is measured as absorbance at 600mμ. Little effect is seen on the addition of either heparin-saline or panheprin. In contrast ACD shows marked depression of the curve at all four Mg^{++} concentrations used.

In Figure 4, one can see the effect of the progressive addition of increasing amounts of citrate to a 2 mEq/L (4 mM/L) magnesium standard. The citrate increments are produced by adding a progressively more concentrated solution of sodium citrate to the magnesium standard. The 2.0 mEq/L standard was chosen because it is the closest approximation to the in vivo serum magnesium levels. The progressive disappearance of recoverable magnesium with the increasing concentration of the citrate solution is apparent. Beyond a citrate concentration of 75 mM/L no recoverable ionic magnesium is demonstrated.

- Discussion -

The data obtained from this study clearly indicate a fall in the level of ionized serum magnesium when citrated blood is used for exchange transfusions. This effect is not seen when heparin is used as the anticoagulant. The in vitro studies further support the contention that it is the citrate which is responsible for this effect.

Two factors are responsible for the in vivo reduction in ionized serum magnesium in the infant transfused with citrated blood. These are the citrate binding effect on the ionic magnesium of the recipient and the reduction of magnesium in the donor blood. The latter is due both to citrate chelating and to the dilution effect of the ACD mixture used. (120 ml to a 500 ml bottle of donor blood is not blood but ACD.) While Panheprin actually contains more citrate per ml of anticoagulant solution than ACD, the total amount of citrate per 100 ml of blood is much less because only 28.2 ml of anticoagulant is added to 470 ml of donor blood. This, together with the smaller dilution factor, accounts for the normal ionized serum magnesium levels in the Panheprin donor blood. This is reflected by a slight and transient drop in vivo in the serum magnesium when Panheprin was used, as against the heparin-saline. The

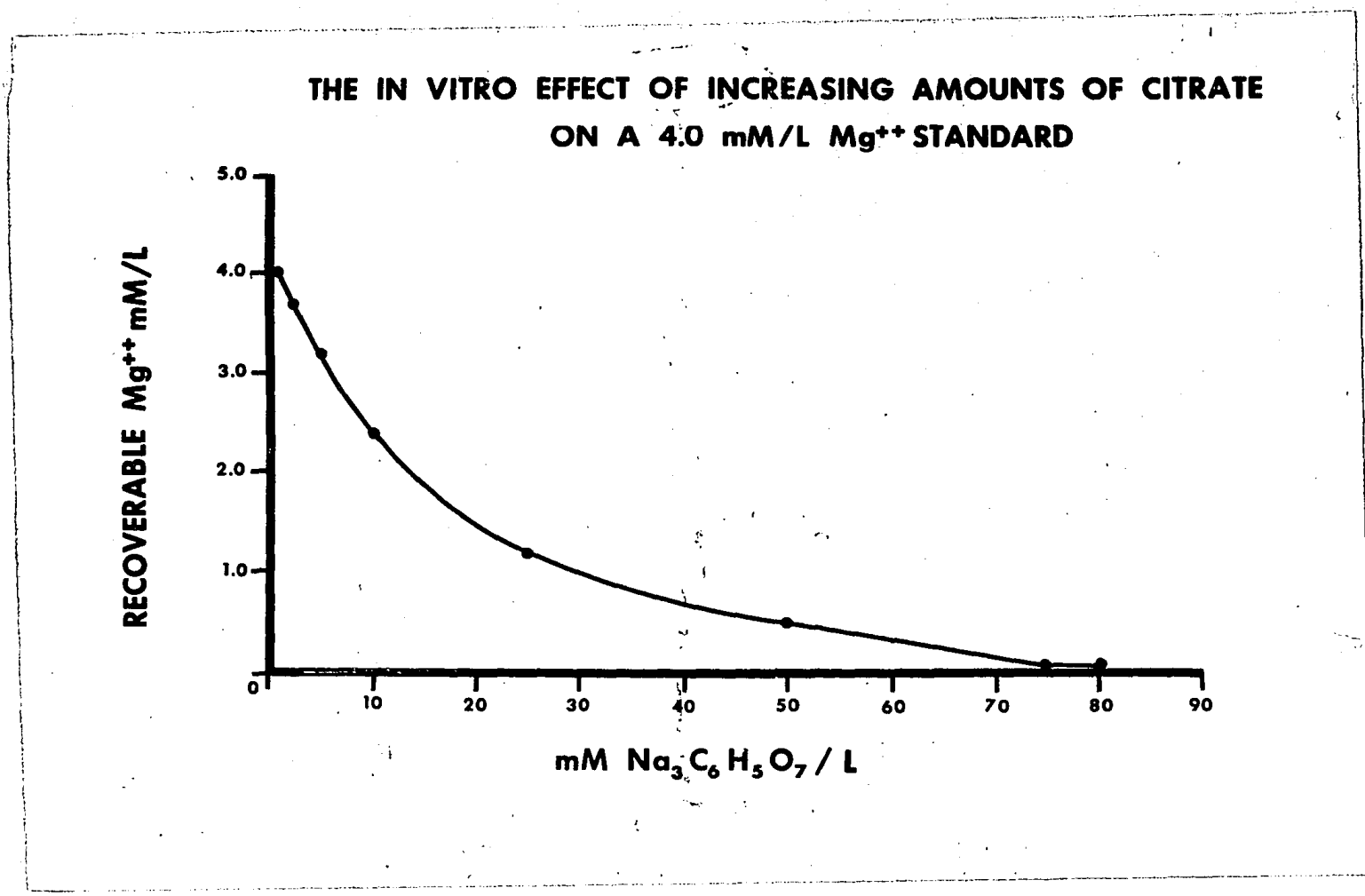

Fig. 4 The in vitro effect of increasing amounts of citrate on a 4.0 mM/L Mg^{++} standard. Note the progressive parabolic fall in recoverability of a 4.0 mM/L (2.0 mEq/L) Mg^{++} standard on the addition of increasing amounts of citrate.

fall was seen in each case but had been corrected by the end of the transfusion. There thus appears to be little serum magnesium reducing effect resulting from the sodium citrate used as a buffer in the Panheprin mixture. By contrast the fall in the ionized serum magnesium seen with citrated blood persists and progressively increases with increasing amounts of blood exchanged. (Figure 3). The depression is increased with repeated citrated exchange transfusions at short intervals, and the serum ionic magnesium may take as long as ten days to return to normal following completion of the exchange. (6)

Symptomatic hypomagnesemia has been described both in human adults (176) and in a newborn infant. (177) Muscular irritability and convulsions are said to be symptoms of hypomagnesemia. Unexplained convulsions occasionally occur during and after exchange transfusions and are generally ascribed to low serum ionic calcium. Because most clinical laboratories measure total rather than ionic calcium, this is difficult to prove, and the possibility that these are due to low ionized magnesium levels, or a combination of both must be considered. It should be kept in mind that citrate will chelate both calcium and magnesium. Since many of the effects of low serum magnesium may be suppressed by the presence of ionic calcium (29), it is possible that clinical effects during exchange transfusions with citrated blood are masked by the regular administration of calcium.

Continuous electrocardiogram monitoring during the course of an exchange transfusion with citrated blood (Section V) has demonstrated a flattening of the T waves when the serum non-protein bound magnesium fell below 0.8 mEq/L. (6) This effect was not seen with heparinized blood where the level of serum ionic magnesium did not change. It is interesting that Hanna and his co-workers (176) reported a similar alteration in the T waves in hypomagnesemic adults which was reversed by the administration of ionic magnesium. Robinson and Barrie (178) have noted similar changes in thirteen of thirty infants monitored continuously during

exchange transfusion. Unfortunately, no accompanying serum magnesium levels were reported. Attention to this possibility might help explain the occasional unexpected sudden deaths which occur during the course of exchange transfusions.

Farquhar and Smith (2) have measured and reported excess citrate levels in citrated exchange transfusion. They also demonstrated that not all of the citrate present is bound to calcium. Bunker, Bendixen and Murphy (174) have demonstrated circulatory depression in man and dogs after citrate infusions. Massive transfusion with citrated blood has been shown experimentally to result in cardiac failure on the basis of both constriction of the pulmonary vascular bed and depression of myocardial activity (184,185). In the studies of Firt and Hejhal (184) this effect was not seen when heparin was used instead of citrate in the transfused blood. Wexler and his coworkers (1) reported severe liver necrosis and death associated with a rising citrate level in an erythroblastotic infant. They suggested that previous liver damage occurring in erythroblastosis where the liver is "stuffed" with hematopoietic tissue might interfere with the metabolism and removal of the infused citrate.

Attention has recently been drawn towards the problem of acidosis in exchange transfusion. Not only is the ACD blood of low pH (pH = 6.6 $\pm$ 0.2), (186,187) but there is evidence of reduced ability, notably in premature infants, to adjust readily to the administration of the blood and to the concomitantly increasing hydrogen ion concentration. Infusion of citrated blood can lead to an initial acidosis, and this will ultimately lead to a metabolic acidosis as citrate is metabolized to bicarbonate. Calladine and his colleagues (180) have suggested that the premature infant may be unable to compensate adequately under these circumstances. They have also demonstrated a reduction in bicarbonate, possibly due to a maturation delay in the enzymes responsible for the metabolism of citrate to bicarbonate. The addition of bicarbonate (187) or

tris-(hydroxymethyl) aminomethane (THAM) (189) to the donor blood has been proposed as a remedy for this situation.

There would, therefore, appear to be considerable merit in any proposal to substitute heparinized for citrated blood in exchange transfusion. There is ample evidence both as to its efficacy and its safety (190-192). In 1958, Valentine (190,191) suggested that the use of heparinized blood for exchange transfusion was a much more rational approach to the problem of citrate intoxication. He suggested that the baby should be kept in a ten ml deficit during the exchange to offset haemodilution and shock. He also advocated the intravenous administration of ten ml of a ten per cent solution of calcium gluconate for every one hundred ml of donor blood to offset the potential hazard of transfusing blood low in calcium. The ACD (120 ml / 380 ml donor blood) lowers the haemoglobin content of the donor blood by simple dilution, causing the erythrocytes to expand by as much as one hundred and twenty per cent. Therefore, if the erythrocyte then returns to normal size after the exchange, the recipient has, in point of fact, received a transfusion of blood of low packed cell volume. This effect is not seen when heparin is used as the anticoagulant because of the small volume of heparin added to the donor blood (2.1 ml heparin; 2110 units; plus 26.1 ml 0.9% Nacl/470 ml donor blood).

Sisson and his coworkers (193) recommended the removal of part of the supernatant citrated plasma after sedimentation of the erythrocytes. This should, they propose, cancel both the dilution effect of the donor blood and the expansion of the erythrocytes. Clinically, the use of heparinized blood is accompanied by much less irritability and restlessness on the part of the infant. Moreover, there is much less variation in the infant's pulse rate.

However, the use of heparinized blood is not without its problems. Compared to citrate, heparin is a poor preservative and blood so anticoagulated must be used within a limit of twenty-four

to thirty-six hours or else discarded. If stored for longer periods of time the blood shows a progressive increase in potassium concentration, resulting from a slow exchange of potassium and sodium ions across the red blood cell membrane. If this hyperkalemic blood were to be transfused into an infant it could ultimately lead to cardiac arrest. Under these conditions the maintenance of stocks of heparinized blood for exchange transfusion would inevitably result in enormous wastage of unused donor blood. It thus becomes necessary to bleed fresh donors as the need arises if heparinized blood is to be used. When readily available and when delay is permitted in its procurement, its use would seem to be justifiable and helpful. This is particularly so in the case of the small premature baby or the infant who is otherwise ill and requires exchange transfusion.

- SECTION VII -

SERUM MAGNESIUM LEVELS IN THE NEWBORN AND OLDER CHILD.

In the past, the reluctance to determine serum magnesium levels in the newborn infant has been due, in part, to the relative unreliability of methods hitherto employed (42) and in part to the need for large amounts of blood, particularly when repeated determinations are proposed.

In 1964 Rice and Lapara (9) described a rapid, accurate ultramicrospectrophotometric method for the determination of magnesium using Mann's Dye. In contrast to the commonly used Titan Yellow Method, the proposed method has the advantage of being unaffected by the presence of calcium gluconate (5), making it particularly useful in following the serum magnesium during the course of exchange transfusions. (6)

It has been well established by other workers that hypomagnesemia in the adult can give rise to certain clinical symptoms. (176) Recent interest in magnesium levels in the newborn infant has been stimulated by the report of Davis and and coworkers, of hypomagnesemia associated with convulsions, and of neonatal respiratory depression associated with hypermagnesemia resulting from magnesium sulfate administration to the mother, as reported by Fishman in 1965. (194) In section five of this thesis, a fall in ionized serum magnesium has been demonstrated during the course of exchange transfusion with citrated blood accompanied in extreme cases by electrocardiographic changes. (6)

The purpose of this section of the study is to carry out serial determinations of serum magnesium levels in the newborn period in an effort to:

(1) Establish normal values in this age group.

(2) Compare the values found in neonates with those of older children.

(3) Compare the values in breast-fed and artificially fed newborn infants.

(4) Compare serum magnesium levels in venous blood to the levels in cord blood and capillary blood.

- Methods And Materials -

Biological Material:

Blood obtained from the Catherine Booth Hospital was analyzed for ionized serum magnesium from a total of fifty-six infants all within the first week of life. For venous blood analysis a No. 21 scalp-vein needle was placed in the antecubital vein and the blood was allowed to drip directly into a dry glass test-tube. For cord blood analysis, the blood was similarily expressed into the tube. For capillary blood determinations a heel prick sample was collected by allowing the blood to drip into the test-tube directly from the warmed heel. The blood was allowed to clot and was then centrifuged at 2,000 r.p.m. for ten minutes. The supernatant serum was aspirated off and frozen until the time of analysis, (usually within twenty-four hours.) Analysis was carried out as previously described in Section IV.

All of the infants studied had serum ionic magnesium determined on the first day of life. In twenty-seven of the fifty-six infants successive determinations were carried out on the third and fifth day as well. Paired simultaneous venous and heel capillary samples were studied in sixteen infants. Cord blood was available for analysis in only eighteen cases. Of the twenty-seven infants in whom determinations were carried out after the third day of life, twenty-two were bottle fed and five were breast fed. The artificial feeding consisted of a standard formula containing a two percent or a four percent fat, evaporated milk. The formula is usually changed from the partly skimmed (2%) to the full (4%) evaporated milk on the fifth day. However,

the mineral composition of the formula remains unchanged.

To compare the findings in the newborn with values for older children, venous blood from sixty-four infants and children ranging from one to sixteen years of age was analyzed. The blood was obtained from normal children undergoing a variety of surgical operations, at the Montreal Children's Hospital.

- Results -

The results obtained in this study are to be discussed under five major headings:

1. Mean Values For The Newborn:

The mean value for the ionized serum magnesium was found to be 1.51 $\pm$ 0.12 mEq/L with a range of 1.20 to 1.80 mEq/L. The bell shaped distribution of the sample population studied is clearly shown in Figure 1. When serial samples are analyzed it is apparent that there are no significant changes with time in the first week of life. This can be appreciated from Figure 2. However, there is a tendency for the cord blood levels to be somewhat higher than the subsequent levels in the infants serum.

2. Cord Blood vs Venous Blood:

The average magnesium level in cord blood was found to be 1.64 $\pm$ 0.12 mEq/L. This value is seen to be somewhat higher than subsequent infant levels, in the first week of life, in thirteen of the fifteen cases. In two cases (No. 10 & No. 12) there is no change at all. It is interesting to note that in no case is the cord level lower than the infant level, as is clearly demonstrated in Figure 3.

3. Venous Blood vs Capillary Blood:

The comparison of serum magnesium levels between simultaneous venous and capillary samples shows a slightly higher

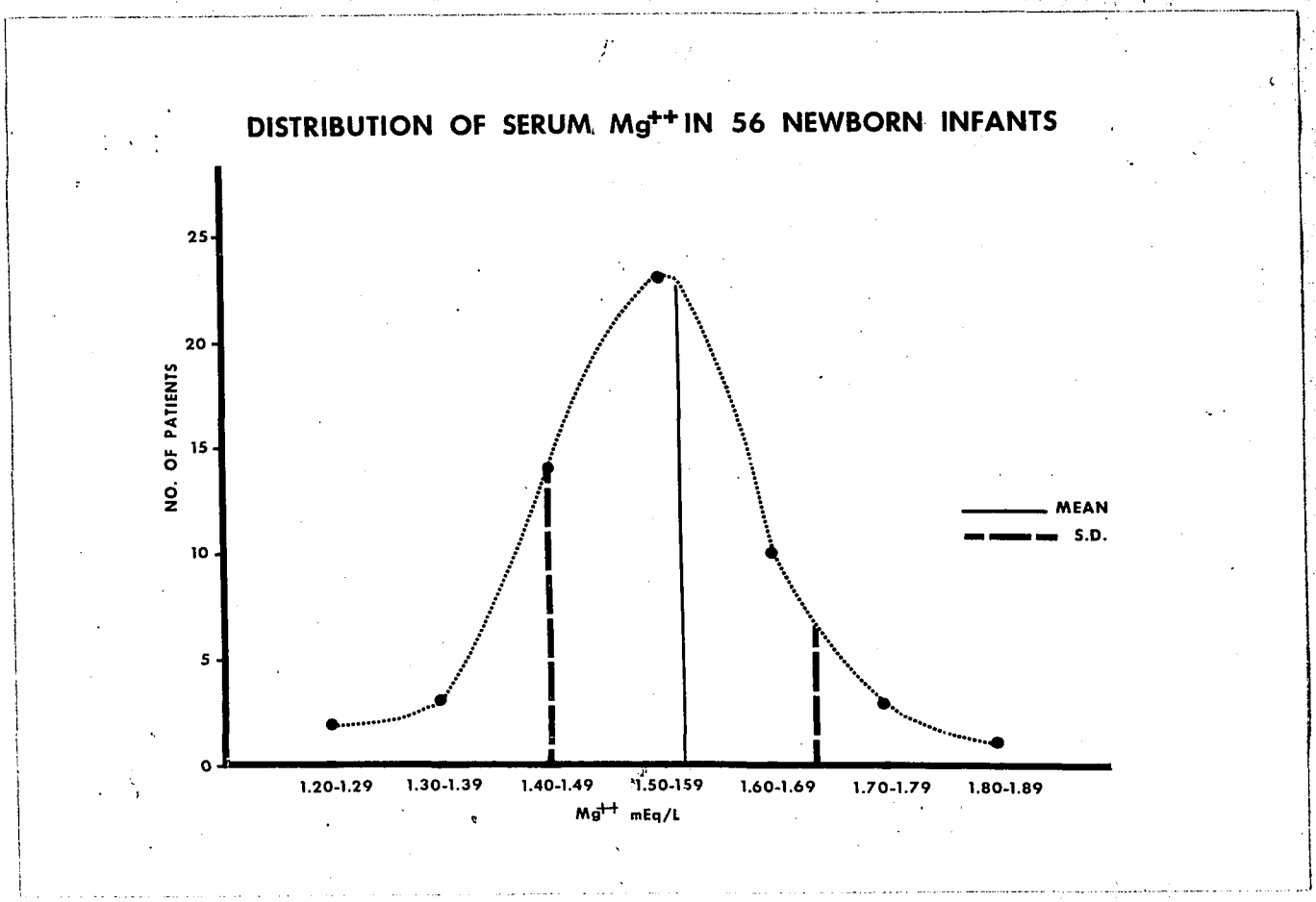

Fig. 1 Serum Magnesium in 56 infants in first week of life. The dashed lines show the limits for the standard deviation.

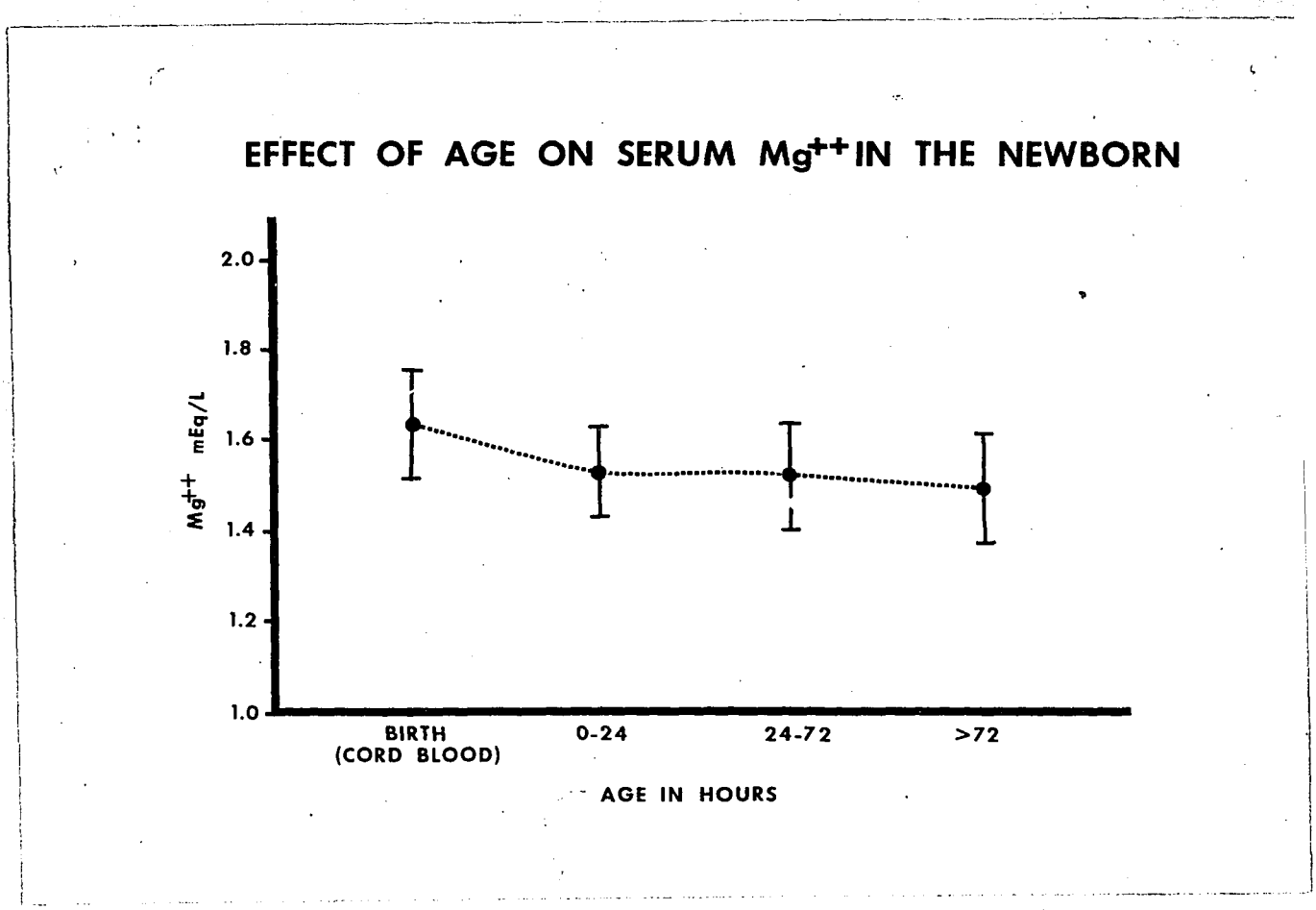

Fig. 2 Serum Magnesium during first week of life. The mean values are joined by the dashed lines. The outside limits indicate the standard deviation in each group.

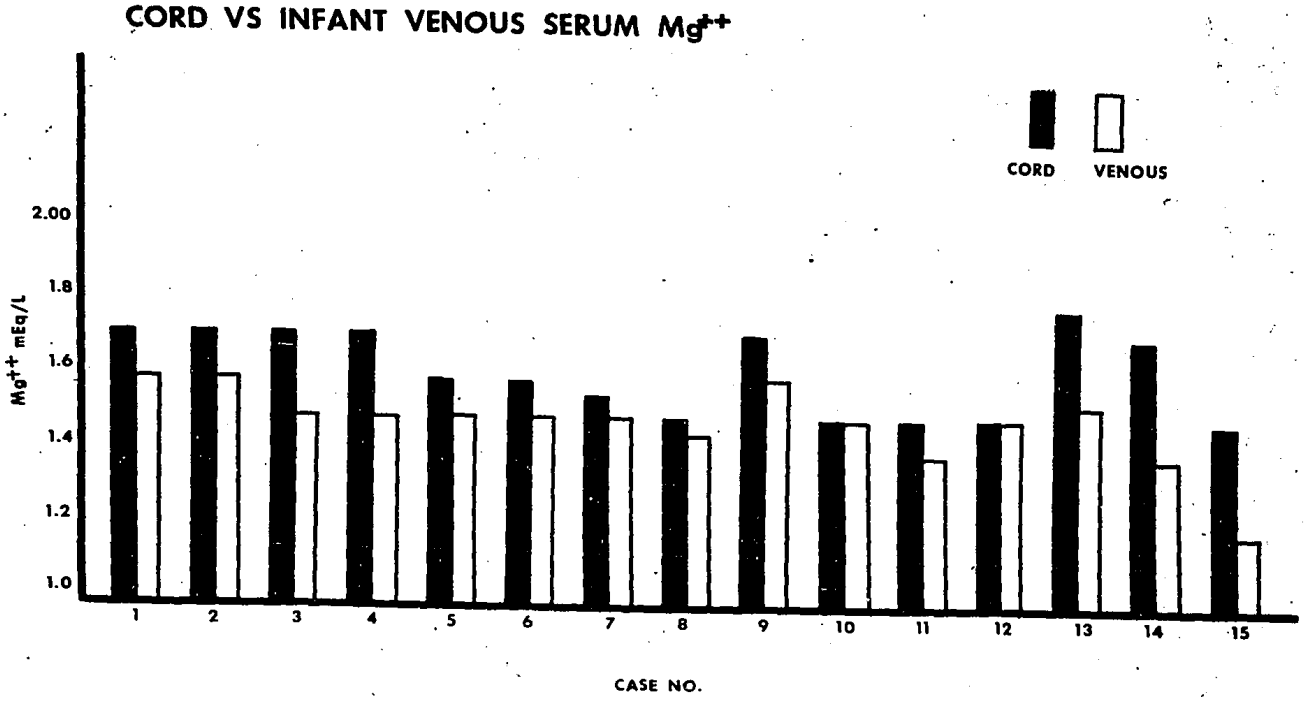

Fig. 3 Serum magnesium levels in cord blood and venous blood for 15 infants within the first week of life.

level of magnesium in the venous blood. This can be appreciated from Table 1. The average venous magnesium level for the group was 1.51 ± 0.11 mEq/L as opposed to 1.45 ± 0.03 mEq/L for the capillary samples. The differences are not significant ($p > 0.05$), and interpretable data can therefore be obtained in either way.

4. Breast Feeding vs Bottle Feeding:

In the infants studied beyond the third day of life, the average serum magnesium levels for the twenty-two artificially fed infants were 1.50 ± 0.11 mEq/L, while the five breast fed infants had an average serum magnesium level of 1.46 ± 0.07 mEq/L. Neither of these values is significantly different from the mean value for the total group. (1.51 ± 0.12 mEq/L)

5. Comparison Of The Newborn With The Older Infant:

The average serum magnesium level, using venous blood, for the sixty-four older infants and children in this group was found to be 1.82 ± 0.14 mEq/L. A bell-shaped curve (Figure 4) similar to that seen in Figure 1, was obtained for this group. However, the average for this group is considerably higher than that found for the newborn infants.

– Table 1 –

PAIRED VENOUS AND CAPILLARY SERUM Mg^{++} LEVELS

PATIENT NO.	VENOUS BLOOD mEq/L Mg^{++}	CAPILLARY BLOOD mEq/L Mg^{++}
1	1.70	1.40
2	1.70	1.50
3	1.50	1.40
4	1.60	1.36
5	1.50	1.36
6	1.40	1.50
7	1.36	1.60
8	1.55	1.40
9	1.65	1.40
10	1.40	1.40
11	1.50	1.40
12	1.54	1.40
13	1.40	1.40
14	1.40	1.40
15	1.40	1.60
16	1.60	1.60
MEAN	1.51	1.45
S.D.	±0.11	±0.03

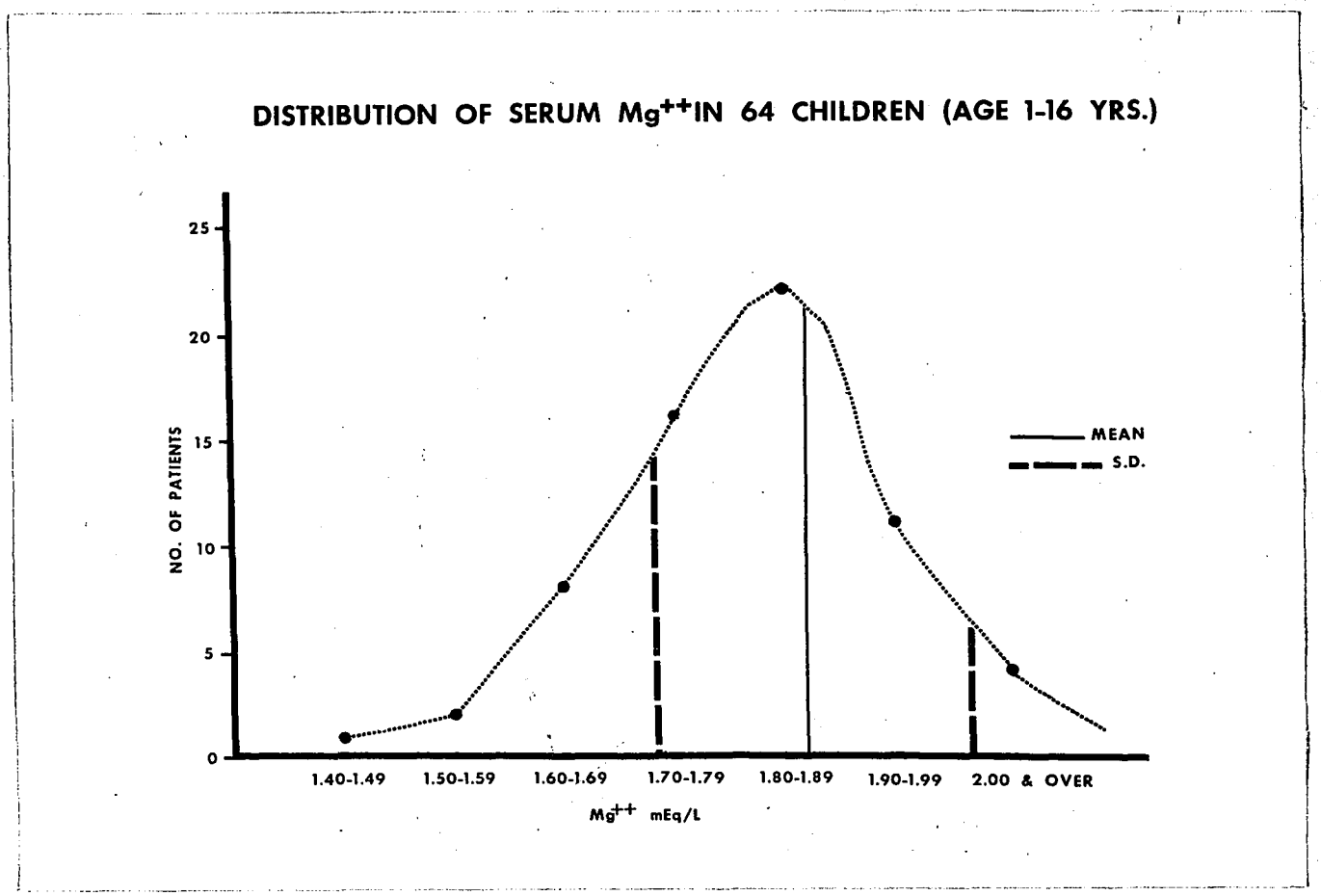

Fig. 4 Serum magnesium in 64 children aged 1–16 years. The dashed lines show the limits for the standard deviation.

- Discussion -

The values reported here for ionized serum magnesium in the first week of life are comparable to, although slightly lower than those previously reported by Anast in 1964, (171) using the Titan Yellow method, and by Orange and Rhein in 1951 (36) who also used the Titan Yellow method. Anast reported a mean value of 1.92 ± 0.27 mg/100 ml (1.57 mEq/L), with a range of 1.36 to 2.90 mg/100 ml (1.11 to 2.38 mEq/L). However, it should be noted that this value was obtained on blood taken by heel prick. The comparable figure in this present series, based on the venous capillary comparison, would be 1.45 mEq/L. From the point of view of clinical applicability, the method proposed in this study is both rapid and truly ultramicro.

Although the theoretical requirement is for 0.04 ml of serum for a duplicate determination, the use of a blank plus the technical problems of pipetting require approximately 0.1 ml of serum. The use of a blank tube in the determination permits an accurate adjustment for the presence of both bilirubin and mild haemolysis. Haemolysis is frequently present in the sera of the newborn. (195) The ability to use very small amounts of serum is a distinct advantage in the neonate with a usually high haematocrit and where repeat determinations may be required.

The absence of interference by the presence of calcium, or gluconate makes this the method of choice in the investigation of ionized serum magnesium during the course of exchange transfusions, and when dealing with convulsive disorders where prior or concurrent calcium therapy is being carried out. In this regard, Anast reported in 1963, (5) that calcium gluconate interfered to such an extent with magnesium determinations, when using the Titan Yellow method, as to render the method unreliable. As seen from Figure 2, there is no significant difference in magnesium levels throughout the first week of life. It is interesting to note, however, that the mean cord magnesium level (1.64 ± 0.12 mEq/L)

is somewhat higher than the ensuing levels in the first week of life. While the difference in the mean values of cord blood magnesium and venous blood magnesium is not very impressive, the individual values show the identical trend in all fifteen cases, in which paired cord and subsequent venous samples were available. (Figure 3)

The comparison of the venous versus the capillary pairs clearly indicates that the latter are quite satisfactory for routine clinical purposes.

The values obtained with this method for older children are within the range usually accepted for adults. (196) There were only a small number of breast-fed infants studied in this series, but no differences were shown between the five breast-fed and the twenty-two bottle-fed infants. This stricking uniformity of values is not in agreement with the suggestions made by other workers that breast-fed infants demonstrate a higher (171,197) or lower (198), magnesium level in the serum. In fact Anast reported that he found increasing levels of serum magnesium in breast-fed infants and decreasing levels in evaporated milk-fed infants. However, he did point out that the differences in some infants was small and suggested that because of the unreliability of the Titan Yellow method, it was probably best to view these differences in formula and breast-fed infants with some reservation until they could be confirmed by other workers. Ideally, such studies should be accompanied by information about the haematocrit levels to allow for variation in hydration of the infants.

Salmi (199) found that serum magnesium levels during the first week of life tended to be higher than the level of the cord blood of the same infant. This tendency of rising levels of serum magnesium during the first week is in keeping with the findings of Anast (171) in breast-fed infants. Perhaps the differences, reported by these workers, in magnesium levels of the breast-fed and formula-fed infants could be explained by

differences in the ratios of dietary phosphorous and magnesium. The magnesium concentration of cow's milk is 0.013% as compared to 0.004% for human milk. The ratio of phosphorous to magnesium in human milk is 4 to 1 and in cow's milk it is 7.6 to 1. (200)

Gardner and his coworkers (201) demonstrated a fall in serum calcium and magnesium and a rise in serum inorganic phosphate in a newborn infant receiving a cow's milk formula. However, not too much credence is given to this report as it has not been confirmed by other investigators.

Since approximately 25% of the serum magnesium is bound to protein, it is possible that variations in protein levels in the two groups may account for the observed differences in serum magnesium. However, since the method used in this present study is measuring ionized magnesium, it is suggested that this is the reason why the present study did not demonstrate differences between breast-fed and formula-fed infants. It should also be pointed out that differences in results by this investigator and others (171,197,198) may be due, in part, to methodology and indicate the importance of each laboratory in establishing its own values.

- SECTION VIII -

ULTRACENTRIFUGAL STUDIES OF PROTEIN-BOUND AND FREE MAGNESIUM IN NORMAL HUMAN SERUM

In 1957 Gerbrandy and his coworkers (202) introduced an in vivo method for determining the protein bound fraction of plasma cations. Their method was based on the principle that a correlation exists between the protein concentration and the concentration of any substance bound to it. Gutman and Gutman (203) used this principle to calculate protein-bound calcium from a large number of individual samples. Chanutin and his colleagues (204) plus Loken (206) made use of this principle in their ultracentrifuge methods.

The cations of plasma, particularly calcium and magnesium, may be differentiated according to their behavior, in the presence of a semipermeable membrane, into a filterable and non-filterable fraction. The non-filterable fraction is generally considered to be identical with the protein-bound cation and the filterable fraction is then referred to as the non-protein bound or "free" cation. The filterable fraction will be referred to by this term throughout the balance of this section.

The filterable fraction is known to contain not only the ionized cation, but also a -generally small- quantity of complex-bound cation (mainly citrate). The filterable complex-bound quantity appears to be negligible in the case of magnesium.

A distinction between the ionized and the complex-bound forms can be made on the basis of the data presented here, and it would appear that for practical purposes, protein bound magnesium may be considered to be non-ionized and the filterable portion to be mostly ionized.

Interest has centered on the biologically active free

fraction and usually only this fraction of magnesium appears to have any clinical significance.

When expressing the free magnesium as a percentage of the total magnesium, one must bear in mind that the total protein concentration, and therefore the total magnesium concentration, can change within a short period of time as a result of changes in posture, hydration or protein metabolism. These changes can occur without any actual change in the free magnesium and magnesium binding capacity of the protein. The fact that changes in the electrolyte binding capacity of proteins might be a characteristic for certain disease states necessitates the calculation of both the protein-bound and the free cation concentration separately.

The present study was undertaken with two goals in mind. The first of these was to attempt to establish a relatively simple, easily standardized procedure which would facilitate the analysis of free magnesium in multiple samples of serum. The second was to demonstrate that the Mann's Dye Method, described in the General Methods (Section IV), of this thesis, is determining free (ionized) magnesium as opposed to total serum magnesium, as usually measured by the atomic absorption or flame emission methods.

- Methods and Materials -

Biological Material

Blood was obtained in the usual manner from ten normal healthy male and female laboratory technicians. In each case the blood was immediately spun at 2,000 r.p.m. for ten minutes and the serum was analyzed for its magnesium and its protein content.

Preparation Of The Sample

The pH of the sera was adjusted to 7.35 $\pm$ 0.15 by the addition of a few drops of 0.1N HCL or 0.1N NaOH.

Methodology

Each serum sample was analyzed for total magnesium content by the standard atomic absorption method of MacDonald and Watson (207) and for free magnesium by the method of Rice and Lapara (9) as described in the General Methods, Section IV of this thesis. The serum was also analyzed for its protein content both by the Standard Biuret Method for total protein and by the Nessler's method for total nitrogen.

Ultracentrifugation

After the initial analysis the serum was placed in a No. 40 head in a Spinco Model L-2 ultracentrifuge and was spun at 40,000 r.p.m. (105,000 x g) for twelve hours (overnight) at 5°C. This procedure yields a very sharp boundary between the protein-free supernate and the protein-containing infranate. The supernate was then aspirated and analyzed for its nitrogen and magnesium content, as indicated in the section entitled Methodology.

Standard solutions containing 1,2,3, and 4 mEq/L of Mg^{++} were also spun, under the same conditions as the serum, to determine if magnesium and its salts could be sedimented by the high gravitational fields used in this study.

- Results -

The results of this study are to be presented under three major headings:

1. In Vitro Studies Of The Effect Of Ultracentrifugation On Serum Magnesium

As shown in Table #1, there is a striking difference in the serum magnesium levels as determined by the Atomic Absorption Method and the Mann's Dye Method. It is suggested that the former method is measuring total serum magnesium whereas

the latter method is measuring only the free magnesium. The values obtained for the serum magnesium with the atomic absorption method range from 2.15 to 2.38 mEq/L with a mean of 2.27 ± 0.08 mEq/L. On the other hand, the values obtained with the Mann's Dye Method range from 1.60 to 1.90 mEq/L with a mean of 1.76 ± 0.1 mEq/L. The average difference between the values obtained by these two methods is 0.53 mEq/L. The differences are, therefore, highly significant ($P < 0.001$). The average serum protein content was 6.8 gm/100 ml with a range of 6.2 to 7.4 gm/100 ml. The average N.P.N. content of the sera studied was 22.3 mg/100 ml with a range of 20 to 25 mg/L. (See table below).

The data presented in Table 1, also shows the effect of a high centrifugal force (105,000 x g) on the serum magnesium and the serum protein. As can be seen, there is no detectable serum protein in the supernate after the serum has been spun for twelve hours at 40,000 r.p.m. However, there is no detectable change in the N.P.N. levels when the pre and post-spin values are compared. This is clearly shown in the table below:

Sample No.	Nonprotein Nitrogen (mg/100 ml) Pre-Spin	Post-Spin
1	23	24
2	22	21
3	20	21
4	20	20
5	23	22
6	22	20
7	21	20
8	24	25
9	25	27
10	23	25
Average	22.3	22.5

This may be interpreted to indicate that the gravitational field used did not sediment any of the smaller molecules such as amino acids.

SAMPLE NO.	SERUM PROTEIN (gm/100ml.)		MAGNESIUM CONCENTRATION (m Eq/L)			
			ATOMIC ABSORPTION		MANN'S DYE	
	PRE-SPIN.	POST-SPIN.	PRE-SPIN	POST-SPIN	PRE-SPIN	POST-SPIN.
1	6.6	Nil	2.19	1.66	1.64	1.65
2	7.0	"	2.28	1.70	1.71	1.72
3	6.2	"	2.16	1.64	1.62	1.61
4	6.3	"	2.15	1.61	1.60	1.60
5	6.7	"	2.28	1.74	1.72	1.73
6	7.1	"	2.35	1.86	1.86	1.86
7	6.9	"	2.30	1.82	1.80	1.81
8	7.3	"	2.36	1.87	1.85	1.86
9	7.4	"	2.38	1.93	1.90	1.91
10	6.7	"	2.29	1.79	1.75	1.77
AVERAGE	6.8		2.27 ± 0.08	1.76 ± 0.1	1.74 ± 0.1	1.75 ± 0.1

Table 1 Concentration of free and total magnesium in the sera of ten normal adults - before and after ultracentrifugation -

There is no significant difference between the serum magnesium values obtained with the atomic absorption and the Mann's Dye methods, (Table 1) after centrifugation.

Table 2 shows, however, that there is a striking difference between the pre-spin and post-spin values for serum magnesium when the serum is analyzed by the atomic absorption technique. The range of the serum magnesium for the pre-spin values is 2.15 to 2.38 mEq/L with an average of 2.27 $\pm$ 0.08 mEq/L, and the range for the post-spin values is 1.61 to 1.93 mEq/L, with an average of 1.76 $\pm$ 0.1 mEq/L. The average difference here is calculated to be 0.51 mEq/L. The percent difference between the pre and post-spin magnesium values range from 74.56 to 81.09, indicating that an average of 77.4 percent of the serum magnesium is free or non-protein bound.

In Vitro Studies Of Non-Protein Bound Magnesium And Protein-bound Magnesium.

When the initial serum magnesium levels obtained with the Atomic Absorption and the Mann's Dye methods are compared, it can readily be seen that there is a striking difference between them (Table 3). When the magnesium levels obtained by the Mann's Dye method are expressed as a percent of the values obtained by the Atomic Absorption method, it can be seen that from 74.41 to 79.19% of the serum magnesium is free. Table 4, is included to show the percentage of the total serum magnesium that is free and protein bound. On the average 76.7 percent of the serum magnesium is free and 23.3 percent is bound. It should be pointed out that the free fraction contains magnesium that is chelated to citrate plus the truly ionized fraction.

In Vitro Studies Of The Effect Of Ultracentrifugation On An Aqueous Solution Of Magnesium Sulfate.

Magnesium sulfate solutions (1 to 4 mEq/L) were centrifuged under exactly the same conditions as the sera to

SAMPLE NO.	MAGNESIUM CONCENTRATION (m Eq/L) PRE-SPIN.	POST-SPIN	$\frac{\text{POST-SPIN}}{\text{PRE-SPIN}} \times 100$
1	2.19	1.66	75.79
2	2.28	1.70	74.56
3	2.16	1.64	75.92
4	2.15	1.61	74.88
5	2.28	1.74	76.32
6	2.35	1.86	79.14
7	2.30	1.82	78.91
8	2.36	1.87	79.23
9	2.38	1.93	81.09
10	2.29	1.79	78.12
AVERAGE	2.27 ± 0.08	1.76 ± 0.1	77.4

Table 2 Free magnesium as a percent of total magnesium as determined by atomic absorption.

SAMPLE NO.	MAGNESIUM CONCENTRATION (m Eq/L)		$\frac{\text{MANN'S DYE}}{\text{ATOMIC ABSORPTION}} \times 100$
	ATOMIC ABSORPTION	MANN'S DYE	
1	2.19	1.64	74.89
2	2.28	1.71	75.00
3	2.16	1.62	75.00
4	2.15	1.60	74.41
5	2.28	1.72	75.43
6	2.35	1.86	79.19
7	2.30	1.80	78.26
8	2.36	1.85	78.39
9	2.38	1.90	79.83
10	2.29	1.75	76.40
AVERAGE	2.27 ± 0.08	1.74 ± 0.1	76.7

Table 3 Free magnesium as a percent of the total magnesium in the sera of ten normal adults - before ultracentrifugation -

SAMPLE NO.	MAGNESIUM CONCENTRATION	
	% BOUND	% FREE
1	25.11	74.89
2	25.00	75.00
3	25.00	75.00
4	25.59	74.41
5	24.57	75.43
6	20.81	79.19
7	21.74	78.26
8	21.28	78.39
9	20.17	79.83
10	23.60	76.40
AVERAGE	23.3	76.7

Table 4 Fraction of serum magnesium that is protein bound and free.

determine if the centrifugal force used was capable of sedimenting salts of magnesium. The concentration of magnesium in the aliquots removed before and after centrifugation was identical. Therefore, it is evident from Table 5, that no sedimentation of magnesium occurred.

- Discussion -

Most of the earlier studies dealing with cation binding by proteins have dealt mainly with calcium and have used some type of ultrafiltration technique. Theoretically the ultrafiltrate and the supernate of ultracentrifuged serum should show identical cation and serum protein concentrations.

By means of ultracentrifugation or ultrafiltration, a protein-free fluid may be obtained which is identical to the protein-free phase of native serum. In the case of ultracentrifugation, free magnesium remains evenly distributed throughout the fluid phase while protein-bound magnesium is sedimented at 105,000 x g. (Table 1)

In 1935 McLean and Hastings (208) demonstrated that most of the calcium which passed through a semi-permeable membrane was in the ionized form. Their data was derived from experiments using the classical frog heart method as an indicator of ionized calcium levels. This method is based on the observation that the frog heart is sensitive to changes in ionized calcium concentrations and that solutions containing equal concentrations of this ion induced equal responses in the heart. Complexed calcium ions and non-ionized calcium fail to show any effect upon the frog heart.

Serum protein, being amphoteric, is dissociated in the anion form at the physiological pH of 7.35. McLean and Hastings (208) demonstrated that in the calcium proteinate complex, the protein moiety acted as a divalent anion, and was therefore able to chelate one calcium ion, as shown below.

determine if the centrifugal force used was capable of sedimenting salts of magnesium. The concentration of magnesium in the aliquots removed before and after centrifugation was identical. Therefore, it is evident from Table 5, that no sedimentation of magnesium occurred.

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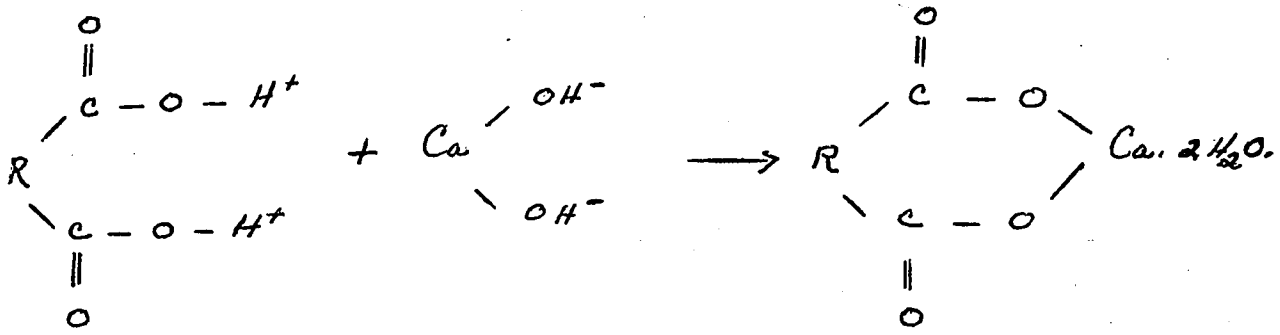
SAMPLE NO.	MAGNESIUM CONCENTRATION (m Eq/L)	
	PRE-SPIN.	POST-SPIN.
1	1.01	1.00
2	2.00	2.00
3	3.02	3.00
4	4.00	4.00

Table 5 Effect of ultracentrifugation on an aqueous solution of magnesium sulfate.



Since calcium and magnesium both occur in the Group II A of the Periodic Table it would seem likely that the serum magnesium should follow the same pattern as the serum calcium. Therefore, the ratio of the diffusible to the non-diffusible magnesium should be approximately equal to the ratio of diffusible to non-diffusible calcium.

The ratio of free magnesium to protein-bound magnesium is dependant on the concentration of protein in the serum. Therefore the percentage of bound magnesium would be expected to decrease with increasing centrifugation time. As the protein-bound magnesium is concentrated in the bottom of the centrifuge tube there is a tendency for this bound magnesium to dissociate from the protein in accordance with the Law of Mass Action. This, in theory, should be equally applicable to the ultrafiltration techniques.

Average normal values reported for diffusible magnesium range from 57% to 84% (85). A considerably narrower range was obtained by the method used in this study. The range of values obtained was 74.4% to 79.8% which is well within the previously mentioned range.

The concentration of free magnesium is relatively constant in the serum from the ten adults whose magnesium metabolism was believed to be normal. Their average serum ionic magnesium was approximately 76% of the total. The balance, approximately 23%, is the protein-bound fraction. Therefore, most of the freely circulating magnesium must be classified as diffusible or

non-protein bound. Since the total protein concentration is known, the amount of magnesium bound per gram of protein may be calculated. In calculating the protein binding of magnesium using the above method, no correction for plasma water or the Donnan Factor need be applied.

When citrate is added to a magnesium solution in vitro, the diffusible magnesium would be expected to increase, but the magnesium ion concentration would decrease. The chelated magnesium citrate is considered to be freely diffusible and is therefore determined as part of the free magnesium by other methods. However, the method used throughout this study is apparently unable to determine chelated magnesium as was demonstrated in Sections V and VI of this thesis.

This study has demonstrated that a metal-indicating dye such as Mann's Dye can be used to determine free serum magnesium concentration. The biological importance of this method lies in the fact that it presumably determines the physiologically effective concentration of serum magnesium. When combined with the determination of total magnesium as described herein, this method provides an estimate of the amount of magnesium present in the complexed form.

The reproducibility of values obtained for magnesium partition by means of the ultracentrifuge permits an accurate distinction between protein-bound and non-protein-bound or free magnesium.

- SUMMARY -

Evidence has been presented that the use of citrated blood for exchange transfusions results in a fall in the level of ionized serum magnesium, the degree of which increases when repeated exchanges are carried out at short intervals. The role of citrate as the responsible agent for this reduction has been confirmed by the in vitro effect of added citrate in depressing the recovery curve of ionized magnesium. This reduction of serum ionic magnesium is also due to a quantitative dilution of the donor blood with the ACD mixture.

Although no clinical signs could be attributed specifically to this depression in serum magnesium levels, electrocardiographic changes were noted when the serum ionized magnesium level was below 0.8 mEq/L.

Evidence has also been presented to indicate that when either of the two heparin preparations were used as the anticoagulant instead of citrate, neither significant magnesium binding nor dilution occurred, and the ionized serum magnesium levels remained essentially unchanged.

In spite of the advantage of heparin over citrate as an anticoagulant for the blood used in exchange transfusions, its disadvantages as a preservative make the routine use of heparinized blood difficult. However, consideration should be given to the use of heparin in the small premature or sick "high-risk" infant requiring replacement transfusion.

Data on ionized serum magnesium levels in 56 newborns and 64 older children, using a rapid ultramicro method, have been presented. No appreciable changes could be found in the first week of life, though cord blood levels tended to be somewhat higher than the subsequent serum levels. No differences were noted with breast as compared to bottle feeding. The lack of any significant differences between venous and capillary blood samples, allows the confident use of capillary blood for this determination in the newborn period.

Data on ionized serum magnesium and protein bound magnesium in ten normal healthy adults has been presented, using the Atomic Absorption Method of MacDonald and Watson, and the ultramicrospectrophotometric method of Rice and Lapara. The evidence presented indicates that from twenty to twenty-five percent of the serum magnesium is protein bound. The data presented also indicates that the method of Rice and Lapara is actually measuring ionized or non-protein bound serum magnesium as opposed to total serum magnesium.

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