THE BIOCHEMICAL COMPOSITION OF BODY FLUIDS THEIR OSMOLALITY AND ULTRAFILTRATES.

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by

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STATISTICAL CALCULATIONS.

The Mean and Standard Deviation (S.D.) on a group of results were calculated by the formulae:-

$$\text{Mean } \bar{x} = \frac{Sx}{n} \qquad \text{S.D.} = \sqrt{\frac{Sx^2 - \frac{(Sx)^2}{n}}{n-1}}$$

where x = individual values and n = number of observations in the group.

Correlation Coefficients (r) between two groups of results were obtained from the formula:-

$$r^2 = \frac{S(x'y')}{Sx^2 - \frac{(Sx)^2}{n}} \times \frac{S(x'y')}{Sy^2 - \frac{(Sy)^2}{n}} \quad \text{A}$$

where $S(x'y') = S(xy) - \frac{SxSy}{n}$

PART 1

STUDIES ON HYPOCHLORAEMIA IN RELATION TO SERUM OSMOLALITY.

INTRODUCTION.

Osmolality and freezing point depression.

Osmotic pressure is one of the colligative properties of a solution i.e. it varies with the total number of particles present in solution but not on their size or shape, and is closely related to the other colligative properties, viz., vapour pressure, freezing point depression and elevation of boiling point. Since the direct measurements of osmotic pressure by the use of apparatus involving semipermeable membranes presents difficulties and inconvenience, in dealing with biological fluids such as serum, urine etc., the osmotic pressure is always arrived at indirectly through the use of one of the above properties, and the freezing point depression is one of the most accurate and easiest method available for the determination.

Beckmann in 1888 first devised a thermometer for the determination of freezing point with some reasonable accuracy. Since then, many improvements and advances have been made in this field. Instruments with high degree of sensitivity are now available for the determination of freezing point depression. Some of these instruments are calibrated directly in units of osmotic pressure i.e. the osmol, so that the concentration of a solution in terms of osmolality can be read off directly in the smaller unit, the milliosmol per kg. water.

Osmotic pressure and distribution of water and solute in body fluid compartments.

The importance of osmotic pressure, in living organisms is hard to over-emphasise. It is of primary importance in the distribution of water and permeable solutes throughout both animals and plants. Plant and animal

tissues are composed of cells containing simple and very complex molecules enclosed in membranes which are essentially semipermeable and these cells are generally surrounded by, or suspended in, solutions. These cell membrane are mostly permeable to water and to some of the solutes. They are more or less impermeable to others and thereby maintain different concentrations of certain substances on the two sides of the membrane even though the osmotic pressure of the external and internal solutions may be same. Water diffuses from the more dilute solution to the more concentrated solution in protoplasm because the membranes of the cell are permeable to it. Salts and other solutes diffuse from the more concentrated to more dilute solutions in protoplasm if they can pass through the membranes. When the blood is diluted by absorption of water, the diffusion pressure of water in the blood is increased, the osmotic pressure is lowered, and more water passes from the blood to the tissues. In such cases the diffusion pressure of water in the blood through the kidneys is also increased, and these organs put out a more dilute urine until the osmotic pressure of the blood and tissues is restored to normal. Similarly a patient may loose much water from his tissues and blood as a result of fever, vomiting, or other clinical disorders, and his blood and tissue fluids become more concentrated than normal. In such circumstances the blood osmotic pressure is high and the kidneys are unable to excrete water and dissolved permeable solutes and the patient suffers from oliguria. The hydrostatic pressure of blood in the kidneys (depending upon the blood pressure) operates with the water diffusion pressure to cause filtration through the kidneys glomerular membranes. For filtration the blood pressure must exceed the opposing osmotic pressure.

The magnitude of osmotic forces which develop when blood plasma, interstitial fluid and cell fluids are separated from pure water by a semipermeable membranes are surprisingly large; a force of the order of 5100 mm. Hg. is required to prevent the transfer of water across the membrane. The magnitude of these forces is responsible for the rapid distribution of water through the capillary walls and cell membranes, and hence this preserves equal osmotic pressure in all the body fluid compartments, except in renal tissue.

Osmolality and blood concentration.

The total osmotic pressure of normal human serum is one of the body constants and it has an average value of 289-290 milliosmols (mOsm.) per kg. water with a standard deviation of 3.5-4.0. Extracellular osmolality is negligibly affected by concentration of extracellular colloids, and it is determined by the amount of crystalloids per unit volume. In normal human serum sodium, chloride and bicarbonate ions make up the bulk of the total ions present and as such, are the largest contributing factors to the total osmotic pressure. Table I shows the calculated contributions of the various ions and molecules to the total serum osmotic pressure. The values have been taken for the mean contributions in millioquivalent per litre (or mg. per 100 ml.) have been assembled from numerous standard sources and most of them have been confirmed by the methods in use in this department. The first step in the calculation is to convert these figures to millioquivalent per kg. water based on the fact that normal serum contains 94% of water (Miller, (1942), Hald (1946)). The osmotic coefficient ϕ, derived from

solutions of known concentration and osmolality is a factor for correction of the deviation from the "ideal" behaviour of a solute.

Osmolality = ϕ n c mOsm. per kg. water.

ϕ = osmotic coefficient

n = number of ions into which the molecule dissociates.

c = concentration in meq. per litre.

This osmotic coefficient for univalent ions is approximately 0.92 (Abelo (1963)). In the last column, the osmotic pressure of each constituent is expressed as a percentage of the total osmotic pressure.

Examination of the data in table I leads to the following conclusions:-

(1) If the serum sodium, chloride, and bicarbonate (which together account for 92.2% of the total osmotic pressure) and the urea and glucose concentrations are all within normal limits, then the serum total osmotic pressure must also be within normal limits.

(2) Conversely, if the observed total osmotic pressure lies outside the limits of normality (282-296 mOsm. per kg. water) there must be major changes in the concentration of one or more of these five constituents; there may also be a major change in the osmotic pressure due to organic anions in cases of ketosis.

(3) Major variations in two or more of the main five constituents may compensate each other osmotically, e.g., chloride ions may be replaced by bicarbonate ions with a resulting osmotic pressure which is within normal limits.

(4) Some of these constituents listed in table I are of no osmotic importance. Any change in serum calcium concentration compatible

Table I. The Contributions of the various Constituents of normal human Serum to the Total Osmotic Pressure of Serum.

Constituent.	Concentrations. (mec. per litre.)	Osmotic Pressures. (mOsm. per Kg. water)	Percentage of the Total Osmotic Pressure.
Sodium	142	139.0	48.3
Potassium	5	4.9	1.7
Calcium (Ca^{++}).	2.5	1.2	0.4
Magnesium	2	1.0	0.3
Chloride	102	99.8	34.7
Bicarbonate	27	26.4	9.2
Protein	16	1.0	0.3
Phosphate	2	1.1	0.4
Sulphate	1	0.5	0.2
Organic anions	3.5	3.4	1.2
	(mg. per 100 ml.)		
Urea	30	5.3	1.8
Glucose	70	4.1	1.4
		Total.....287.7	(99.9)
		Observed Mean....289	

with life, will have a negligible effect on the total osmotic pressure.

(5) The minor constituents of serum which have been omitted from Table I, may be ignored from the osmotic pressure point of view. Thus, a creatinine concentration of 2.0 mg. per 100ml. is equivalent to only 0.19 mOsm. per kg. water.

The sum calculated is usually 5 to 8 mOsm. per kg. water less than the measured serum osmolality in normal subjects which is accounted by these minor constituents of the serum (Holm (1962)). However, the significance of large differences between the measured and calculated values for serum osmolality is still in doubt. Rubin, Braveman, Dexter, Vanamee, and Roberts (1956) compared the measured and the calculated values in 250 patients. Serum osmolality ranged from 220 to 475 mOsm. per kg. water. They reported that 98% of 78 patients with differences in measured and calculated values ranging from 40 to 125 mOsm. per kg. water died within two weeks of the study. The remaining patients were said to have equal, but not necessarily normal, observed and calculated osmolality. The solutes causing the differences in values could not be identified. Diseased states included uraemia, metastatic carcinoma, lympoma, liver cell necrosis, myocardial infarction, and infection. The observation of Rubin et. al. (1956 on the significance of these differences were not confirmed in studies of similar clinical conditions by Edelman, Liebman, O'Meara, and Birkenfeld (1958

Osmotic compensation.

The most interesting situations are however, those in which osmotic "compensation" occurs either by the substitution of one ion for another

(chloride ion replaced by bicarbonate ion) or the replacement of ions (sodium and chloride) by urea. Particular attention has been paid to the role of sodium ions in the regulation of the osmotic pressure. Apart from the fact that it is of the most important quantitative source (48.3%) of the osmotic pressure (Table I, P. 6), it must be balanced by anions, of which chloride and bicarbonate are the most important, in order that electrical neutrality be maintained. Edelman et. al.,(1958) found that the total osmotic pressure was a function of the sodium concentration provided that appropriate corrections were made for abnormal concentrations of urea and glucose: and a similar relationship was found by Olmstead and Roth,(1957) in many of the cases of hyponatraemia which they studied. On the other hand, the serum sodium concentration is relatively constant both in health and disease, and marked changes may occur in the ionic composition of serum while the sodium concentration is still within the limits of normal range, albeit nearer the normal extremes than the normal mean.

Chloride ion is the second largest contributor (34.7%) to the total osmotic pressure and changes in the chloride concentration occur far more frequently, and are of greater importance, than changes in the sodium concentration. The commonest cause of loss of body fluids is vomiting. In those cases where the gastric juice is highly acid the loss of sodium is small relative to the loss of chloride: the patient is losing more hydrogen chloride than sodium chloride. Another point which is frequently overlooked is that the body has no store of chloride, whereas there are quite large stores of sodium in bone and these can be mobilised if necessary.

Outlines of present work.

This investigation began as a study of patients who were admitted to this hospital and who were found to have hypochloraemia (serum chloride less than 90 meq. per litre) on admission. In such cases, the serum sodium concentration is never lowered in proportion. Since the chloride ions contributes such a high percentage of the total osmotic pressure of serum, it is here that the greatest divergencies from the normal osmotic pressure are to be expected, unless the chloride ion is replaced by bicarbonate ion. Any marked hypochloraemia should theoretically lead to a fall in the serum osmolality, but this this is not always so, for more reasons than one. There appears to be no standard pattern of serum chemistry in chloride depletion, and this study is an attempt to find reasons for the differences which are found to occur.

Subjects:-

All the patients had hypochloraemia and many of them were acutely ill on admission. Any scientific study of such cases must inevitably labour under many handicaps. A "base line" is never available and it has been assumed that the patient was in a state of biochemical normality before the cause of chloride depletion began to operate. In nearly all the cases, the hypochloraemia was due to vomiting, less often to continuous gastric suction, and rarely to diarrhoea or the presence of fistulae. Specimens of vomitus were seldom available for examination, and often were of limited value for it is well known that the longer vomiting continues, the less hydrochloric acid is secreted into the juice. Futhermore, it is known that considerable proportion of elderly patients such as comprises the general hospital population, are permanently achlorhydric. Most histories of "vomiting" by patients are notoriously unreliable both in respect of the event and the volume of fluid which is said to have been lost. For this reason alone, the state of the serum biochemistry is of great clinical importance. It also happens quite regularly that vomiting stops for no apparent reason as soon as patient is admitted. From the biochemist's point of view, the primary cause of the chloride depletion is immaterial: since chloride can be neither stored nor metabolised, a depletion must be due to loss of body fluid by one or another route combined with inadequate replacement. Some of these patients were so dehydrated that they required intravenous replacement therapy immediately on admission. The data given in the following tables

refer in every case to the blood chemistry on admission and before any therapy had been started.

There was no diabetic cases in this study so that the effects of hyperglycaemia and of increased concentration of organic anions need not be considered.

MATERIAL AND METHODS.

Determination of Osmolality by the method of Freezing Point Depression.

All the osmolality determinations in the present series were made on a Fiske Osmometer model No. G*. This instrument essentially consists of two parts:-

(1) Equipment to hold, cool, stir and freeze the specimen.

(2) A highly stable thermistor capable of measuring minute temperature differences.

The diagram of the main operational unit is shown in Figure 1. The specimen is placed in a specially designed test tube for the osmometer, which is then mounted in a special bracket in such a way that the temperature probe is located in the centre of the sample. The electrical resistance of the probe varies directly with the temperature of the sample. This resistanc is balanced with a Wheatstone bridge. This electrical resistance of the thermistor is directly proportional to the osmolality. The variable resistan of the Wheatstone bridge can be calibrated directly in milliosmols per Kg. water by using standard solutions, hence the depression of freezing point can read directly in milliosmols per Kg. water.

A thin rod adjacent to the temperature probe vibrates gently, and the amplitude of the vibration is so adjusted that an optimum stirring rate is obtained which reduces the temperature gradients within the sample. This whole assembly consisting of thermistor probe, stirring rod and the sample holder are mounted on a movable arm, supported on a vertical rod, hence it can be raised or lowered in the freezing bath. The sample tube is not immersed in the bath but is suspended just above its surface and a pumping

* Advanced Instruments Inc., Uxbridge, Mass. U.S.A.

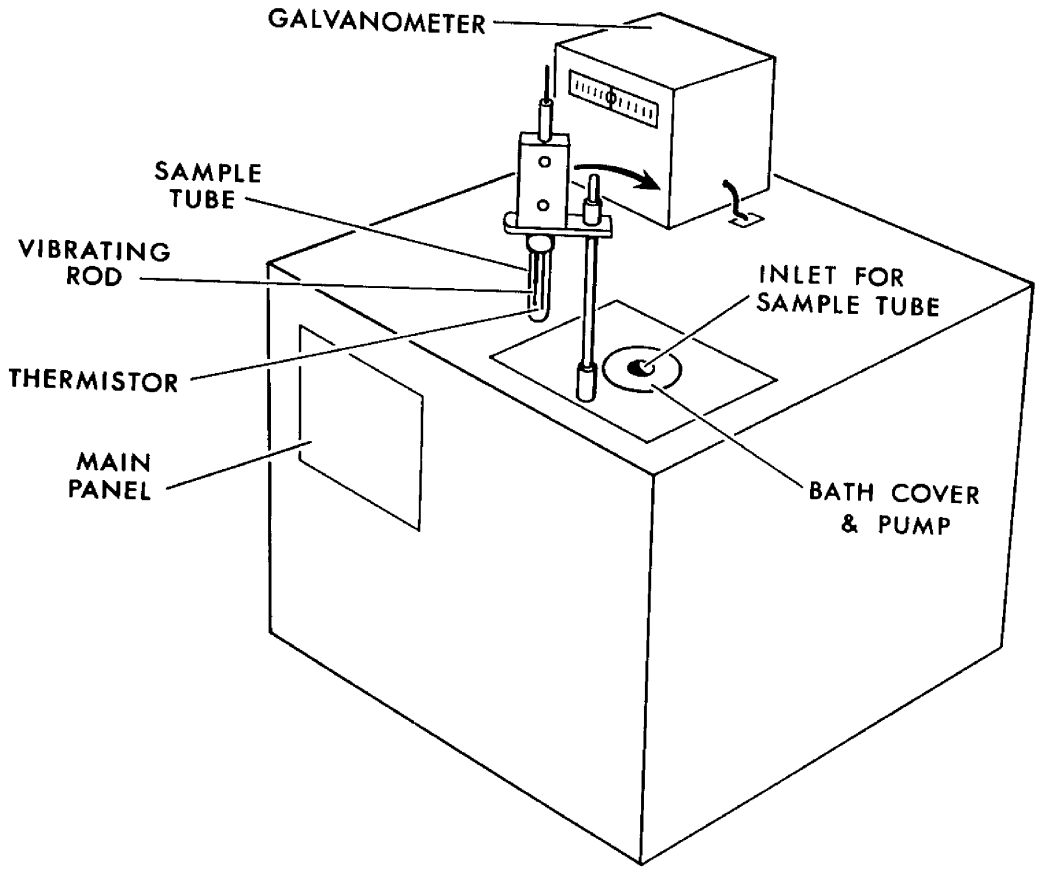

FIGURE 1. OPERATIONAL UNIT OF FISKE OSMOMETER.

mechanism throws jets of liquid of the cooling mixture on the outside of the sample tube to cool it.

The cooling mixture consists of 50% (V/V) ethylene glycol, and the temperature of the bath is controlled within $-6^{\circ} \pm 2^{\circ}C$ thermostatically.

Procedure.

All determinations of serum osmolality were made within 2 - 3 hours after the collection of blood specimens. 2.0 ml. of serum was placed in the osmometer test tube and mounted on the bracket, which was then lowered in the cooling bath. A typical freezing curve during the various procedures of freezing is shown in Figure 2. The operational switch is set on to position I. The galvanometer light is switched on and at the same time the coolant pump starts cooling the sample. Initially the heat is removed very rapidly at a rate of about 1.0 - 2.0 divisions per second on the galvanometer scale, as shown in Figure 2 from 1 to 2. When the galvanometer light reaches 18 divisions to left of the zero the operational switch is moved to the position (2), thereby the cooling pump is switched off and the sample continues to cool but at a slower rate which allows the specimen to attain a uniform temperature throughout. This period of supercooling is shown in Figure 2 from 2 to 3. At the point (3) i.e. 20 divisions to the left of the zero on the galvanometer scale, the operational switch is moved on to the position (3) whereby a larger pulse of current is sent through the coil momentarily, which sets the stirring rod into violent motion and the stirring action ceases after 1 - 2 seconds. This causes large numbers of nuclei to be formed in the specimen on which crystallisation takes place. As the sample freezes the galvanometer light swings rapidly to the right. On

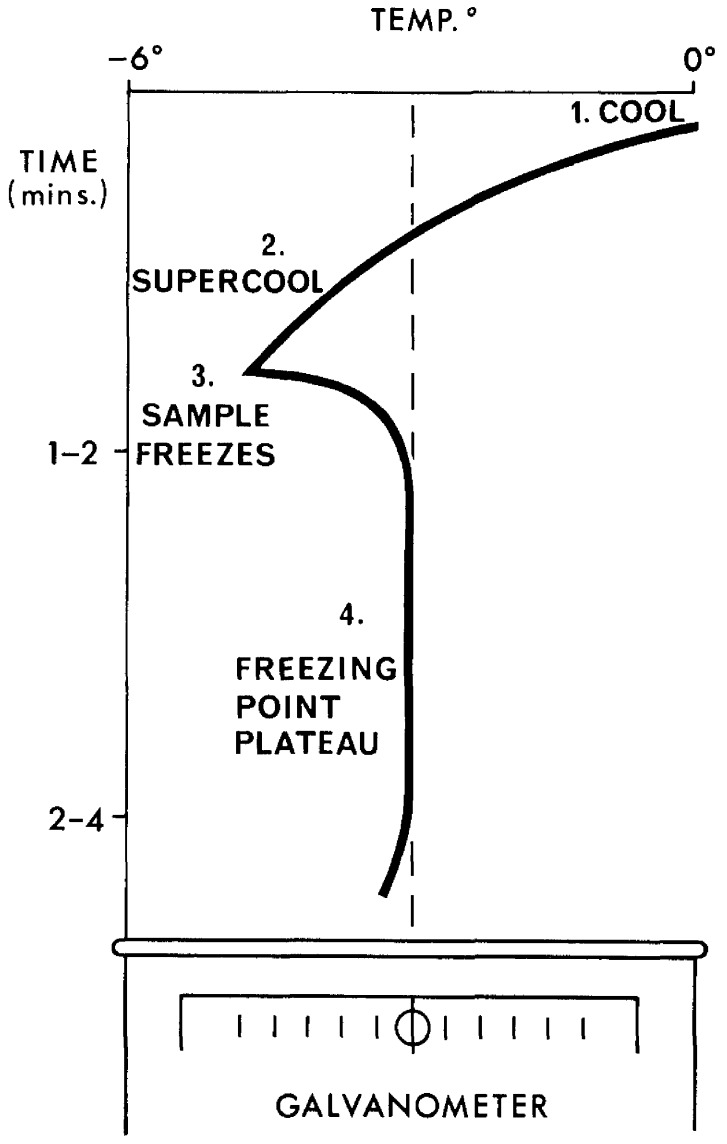

FIGURE 2. FREEZING CURVE.

Spotlight of the galvanometer traces out the pattern shown by solid lines.

1 to 2 - rapid cooling

2 to 3 - supercooling period

3 - buzzer freezes sample

3 to 4 - temperature rises as frozen sample moves to freezing plateau.

4 - freezing plateau

freezing, the sample releases its latent heat of fusion and this change in temperature alters the resistance of the thermistor which, in turn, is recorded by the galvanometer. This is read off directly in milliosmols per kg. water by pressing the sensitivity knob to low position and then turning the milliosmol knob to bring the galvanometer light to zero. Sufficient time 20 - 40 seconds is allowed after the freezing is induced before this reading is taken; during this period of time the sample reaches its plateau temperature.

Calibration of the Instrument.

The instrument can be calibrated for direct reading for a desired range with the use of two standard solutions. As the osmolality in the present study was between 200 and 400 milliosmol per kg. water, the standards chosen were 100 mOsm. per kg. water.and 500 mOsm. per kg. water. The standards consist of aqueous solutions of sodium chloride. Analar grade sodium chloride was recrystallised once from water, dried in an oven at 120°C overnight and then kept in a desiccator over phosphorus pentoxide. In all calculations, due allowances were made for the activity of sodium chloride at various concentrations. The amount of sodium chloride in grams per kg. water required for a desired osmolality was obtained from the international critical tables and for convenience this was further converted into grams per litre of water at 20°C. Hence the amount of sodium chloride required for two standard of osmolality 100 and 500 mOsm. per kg. water was 3.089 and 15.901 grams per litre at 20°C.

Accuracy of the Instrument.

On repeated determination the instrument freezes the sample within 1 mOsm. per kg. water which corresponds to 0.00186°C.

Other Estimations.

During the initial part of the investigation, sodium was estimated by the EEL Flame photometer,* chloride by the method of Schales and Schales, (1941) and bicarbonate by the manometric method of Van Slyke(1924). Later, all electrolytes were determined by the Technicon Autoanalyser.** There was no significant difference in the results obtained from the two methods of analysis provided that the sodium concentration was corrected for the "protein error" i.e., 4 meq. per litre were added to all the estimations done on the flame photometer (EEL).

Urea was estimated throughout by the Technicon Autoanalyser. The following is a summary of the normal mean values and normal ranges for the constituents measured:-

Serum content.	Mean.	Range.
Chloride	102	96 - 106 meq. per litre.
Sodium	142	136 - 148 meq. per litre.
Bicarbonate	27	24 - 31 meq. per litre.
Urea	30	20 - 40 mg. per 100 ml.
Osmolality	289	282 - 296 mOsm. per kg. water.

* Evans Electroselenium Ltd., Halstead, Essex, England.

** Technicon Instruments Ltd., Chertsey, Surrey, England.

RESULTS.

All cases of hypochloraemia were further examined by determining the serum concentrations of sodium and bicarbonate, the blood urea concentration, and the serum osmolality. Although chloride ions account for over one-third of the serum total osmolality, the cases of hypochloraemia could be divided into three groups according to whether the serum osmolality was normal, decreased or increased. These three groups were then examined separately, but before doing so, some justification for grouping the data into separate divisions seems necessary.

Any mass of data can be split up into small sections but the dividing lines must be meaningful, and unless the sections are quite distinct in at least one important property they may be nothing more than gradual transitions separated by arbitrary boundaries. In the present cases the dividing lines are quite distinct, as one group of patients has succeeded in maintaining one important property of the extracellular fluids within normal limits in spite of forces which would tend to upset that state of normality, i.e. in spite of a decrease in chloride concentration, the osmolality is still maintained within the normal limits, while in the other two groups the serum osmolality is abnormal, being low in one group and high in the other.

Group I. Normal serum osmolality.

In all these cases, the total serum osmotic pressure has been kept within the normal limits, and in spite of grossly altered blood chemistry, at least one physical constant has been maintained.

In all these cases there is bicarbonate retention, serum bicarbonate

is elevated in all with the exception of two cases where it is just on the upper normal limits. The serum sodium concentration is within the normal limits in only four of these cases, and in nearly all of these remaining cases the serum sodium is either at the lower normal limit or below it. The disturbances in the ionic balance due to the hypochloraemia is therefore corrected by an increase in bicarbonate and a decrease in sodium concentrations. However, even the casual inspection of the data makes it clear that in no case is the decrease in chloride exactly balanced by an equivalent increase in bicarbonate, i.e. quantitative replacement of one univalent anion by another type of univalent anion does not occur. This does not mean that such a replacement is a practical impossibility: it is not biologically necessary due to the simultaneous decrease in the concentration of cation (sodium). Again it should be kept in view that there are many other ions involved apart from sodium, chloride, and bicarbonate: and the fact that in almost every case there is nitrogen retention makes it quite certain that significant increases have occurred in the concentration of phosphate, sulphate etc. The point to be emphasised is that ionic balance and the osmotic pressure normality are not achieved by the simple replacement of one chloride ion by one bicarbonate ion.

The interesting thing about these cases is that, although all of them are chloride depleted, and more than half of them are sodium depleted as well, they all have succeeded in maintaining normal serum osmolality. The increases in bicarbonate are only small factors in the preservation of a normal serum osmotic pressure. Since no diabetic cases are included in these, urea is the only possible constituent of serum which can supply

Table II. Hypochloraemia with Normal serum osmotic pressure.

No.	Case.	Osmolality.	Sodium.	Chloride.	Bicarbonate.	Urea.
1.	J.A.	295	140	89	32	84
2.	H.W.	295	135	83	32	111
3.	E.H.	295	130	82	34	106
4.	M.McL.	293	126	87	32	87
5.	C.R.	293	128	64	48	150
6.	M.S.	293	142	85	37	68
7.	E.Ham.	292	130	89	31	118
8.	E.P.	292	140	86	37	55
9.	H.M.	290	135	82	42	19
10.	V.	289	130	88	36	35
11.	C.C.	288	130	82	32	67
12.	J.N.	288	131	84	31	124
13.	R.T.	287	130	79	38	158
14.	A.A.	286	140	82	35	95
15.	M.M.	286	134	88	33	70
16.	H.B.	286	131	87	33	42
17.	J.S.	285	133	81	36	66
18.	E.McG.	285	124	82	32	126
19.	J.V.	285	135	89	36	54
20.	E.W.	285	135	89	32	99
21.	M.J.	285	125	75	44	91
22.	J.G.	285	126	72	32	214
23.	J.H.	283	130	84	37	66
24.	N.McL.	283	129	84	39	84
	Means.	288	132	83	35.5	91

the deficiency in osmotic pressure. The blood urea is elevated in all of these cases, and there is a relationship between the decrease in chloride concentration and the increase in blood urea concentration. This is shown in Figure 3. It is not a very striking one but the correlation coefficient (r) is 0.62. This leads to the possibility that the renal system deliberately retains urea in such cases in order to restore the total osmotic pressure of the body fluids to normality. There is no other relationship between any of the quantities set out in Table II.

There is one very important conclusion to be drawn from the data in Table II. The serum osmolality cannot be used as a general screening test of chemical normality, because in cases where osmotic compensation takes place, the serum chemistry in spite of normal osmotic pressure, may be highly abnormal. (See Table II for examples of this).

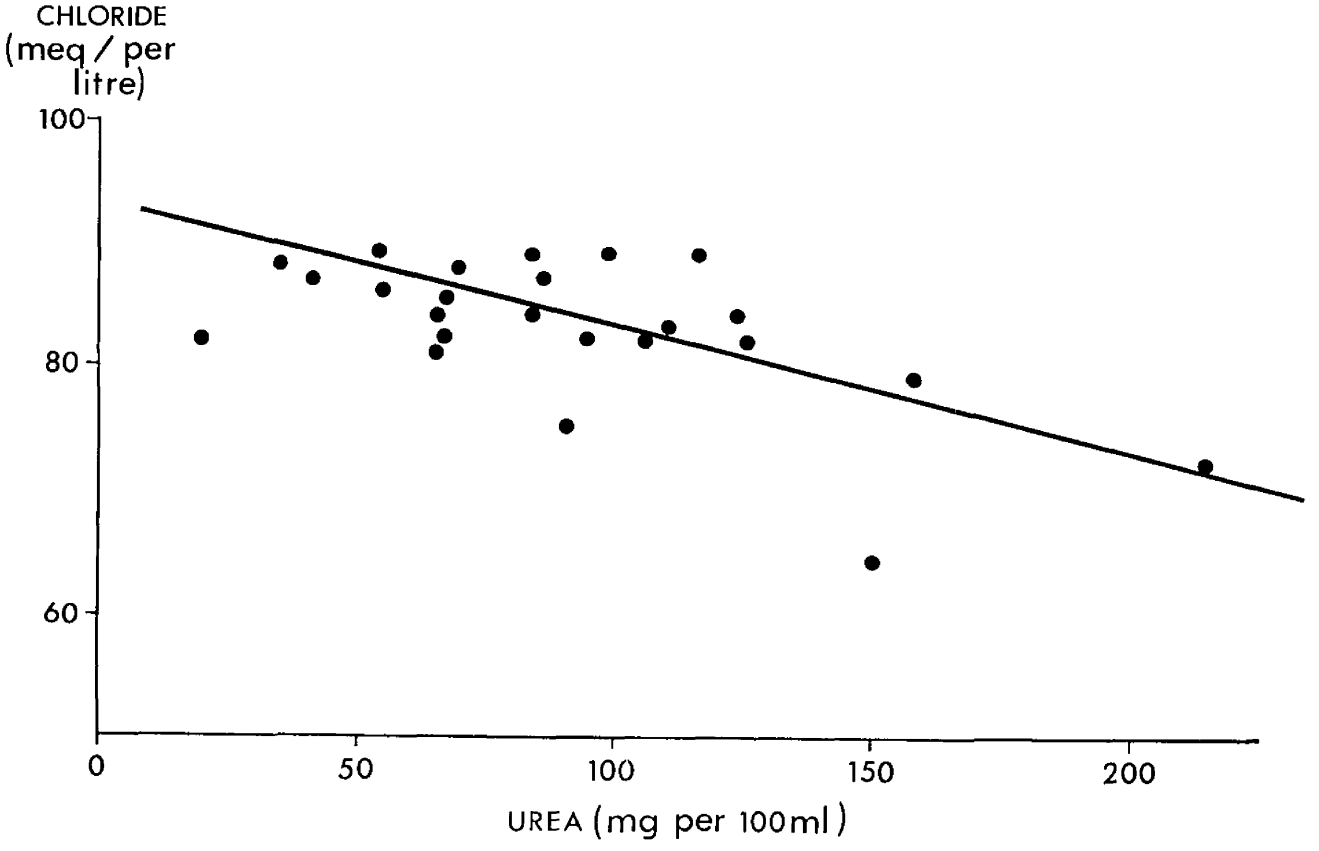

FIGURE 3. RELATION BETWEEN THE CHLORIDE CONCENTRATION AND BLOOD UREA CONCENTRATION IN GROUP I PATIENTS.

Group II Abnormally low serum osmolality.

In the cases of this group, the total osmotic pressure is below the lower limits of normality, and markedly so in the great majority of them. The decrease in serum osmolality in some of these cases, such as those falling in the range 277 - 281 mOsm. per Kg. water was so small, that they came very close to the cases of group I, and must be regarded as borderline cases.

There seems to have been little attempt to replace chloride by bicarbonate. In only 17 of the 63 cases did the patients have an increase in serum bicarbonate and even in 5 of these was the concentration just on the upper normal limit. And furthermore, in 9 of these 63 cases the serum bicarbonate is below the lower limits of normality. In only 6 of these cases there has been an effort to retain bicarbonate to replace the loss of chloride ions and this is rather surprising, since bicarbonate is continuously and plentifully available to the body. Ionic equilibrium has been maintained by reduction of the sodium concentration.

There is no correlation between the serum chloride and the serum bicarbonate, nor has there been much attempt to retain urea in order to restore the serum osmolality to normal. It will be seen from the data in Table III that the blood urea is within the normal limits in 27 of the 63 cases (43%) and in further 11 of the cases (17%) it is only slightly elevated to the range 41 - 46 mg. per 100 ml.

There is no correlation whatever between the serum chloride concentration and the blood urea concentration. As a result of failure to compensate, either by retaining bicarbonate or, more especially, urea,

Table III. Hypochloraemia with low serum osmotic pressure.

No.	Case.	Osmolality.	Sodium.	Chloride.	Bicarbonate.	Urea.
1	C.O.	281	126	87	21	122
2	N.B.	281	134	88	32	58
3	R.L.	280	140	88	29	91
4	R.B.	280	131	89	28	66
5	S.H.	280	139	86	30	38
6	J.D.	280	128	83	27	94
7	R.Mc.	279	124	87	28	41
8	M.S.	279	130	86	32	42
9	E.C.	279	125	84	26	68
*10	C.McF.	278	135	88	29	36
*11	H.L.	278	140	81	33	90
12	I.C.	278	130	87	33	42
13	S.B.	277	135	86	25	52
14	E.B.	277	133	86	30	45
15	E.C.	277	130	89	27	39
16	R.S.	276	127	78	16	65
*17	M.O.	275	134	89	39	37
18	P.Mc.	274	135	87	31	55
*19	C.H.	274	130	87	29	59
20	J.L.	274	133	87	30	31
21	C.K.	273	130	74	39	81
22	D.T.	273	146	88	27	59
*23	E.McW.	273	124	88	23	100
24	T.G.	273	130	84	39	72
25	J.W.	273	126	82	27	108
26	J.S.	273	133	86	38	41
27	J.R.	273	127	86	24	45
28	M.A.	273	127	88	28	63
29	A.D.	272	134	86	30	26
30	S.D.	271	130	87	31	42
31	C.B.	269	125	85	24	46

Cont.-

Table III contd.

No.	Case.	Osmolality.	Sodium.	Chloride.	Bicarbonate.	Urea.
32	Gill.	269	125	78	30	35
33	R.R.	269	134	88	22	68
34	E.M.	268	130	88	30	35
35	G.W.F.	268	126	89	28	44
36	C.I.	267	130	87	29	32
37	A.J.	267	130	81	29	27
38	G.B.	267	129	83	37	38
39	E.Mc.	266	119	87	19	81
40	G.S.	266	129	87	27	58
41	D.H.	264	135	88	32	27
42	C.S.	264	119	87	27	18
43	M.Mc.	263	135	88	29	20
44	R.Mc.	263	135	86	31	29
45	J.A.	262	135	85	29	20
*46	C.McG.	262	125	87	26	20
47	T.B.	262	128	87	32	35
48	D.R.	261	125	89	23	23
49	A.H.	261	134	84	30	19
50	D.L.	261	125	87	26	60
51	E.Cullen	261	109	68	31	85
52	J.Doch.	261	123	84	31	18
53	J.B.	261	125	85	29	16
54	M.T.	260	129	85	24	46
*55	J.R.	259	135	88	27	16
56	E.C.	258	104	64	29	170
57	T.C.	258	130	82	29	22
58	T.D.	256	130	89	25	54
59	E.S.	256	126	65	40	41
60	H.N.	255	120	89	15	68
61	H.Q.	250	109	82	22	28
62	A.C.	243	115	82	25	37
63	B.D.	238	114	79	23	37

* Died

the serum osmolality falls, in some cases to a very low levels.

Group III. Abnormally high serum osmolality.

As would be expected the majority of cases falling into this group were cases of advanced renal disease and since the biochemistry at this stage is so well known, these have not been included in this study. As in Group II, in some of these cases the serum osmolality is only very slightly elevated from the normal range, and hence cases where the osmolality lay in the range 297 to 301 mOsm. per kg. water are regarded as bodorline cases. In this group, the serum total osmotic pressure has increased beyond the upper normal limit in spite of a low serum chloride concentration. Although in a few cases the chloride depletion has been partially compensated by retention of bicarbonate, but there is also considerable urea retention which results in an increased serum osmolality. In about half of these cases, the bicarbonate concentration is at or above the normal limits, but in only two instances (cases J.L. and W.G.) is the increas in bicarbonate chemically equivalent to the decrease in chloride. In three of these cases the urea concentration is slightly raised: in all others it is markedly increased. The total osmotic pressure has been raised to normal and then to a level above normal by urea retention, but there is no correlation between the serum chloride concentration and the height to which the blood urea rises. On the other hand, there is quite a good relationship ($r = 0.63$) between the serum total osmotic pressure and the blood urea concentration, which is shown in figure 4. The increase in urea concentration above the normal limits is related to the increase in serum

Table IV. Hypochloraemia with high serum osmotic pressure.

No.	Case.	Osmolality.	Sodium.	Chloride.	Bicarbonate.	Urea.
1	J.F.	298	138	88	33	90
2	S.R.	299	140	84	35	114
3	M.G.	299	128	86	25	53
4	M.E.	300	132	88	32	107
5	J.G.	301	134	76	32	195
6	W.T.	302	134	88	25	135
7	C.R.	302	139	86	32	91
8	M.McL.	303	134	87	29	43
9	C.W.	306	134	86	23	172
10	D.Mc.	307	140	86	29	117
11	J.Mc.	309	145	82	32	129
12	P.Mc.	310	135	82	29	95
13	C.G.	310	134	74	41	189
14	M.H.	310	134	88	24	93
15	G.F.	311	130	88	26	206
16	H.R.	317	125	75	25	100
17	J.M.	318	104	64	31	231
18	E.Mc.	319	151	80	24	48
19	W.S.	321	146	86	35	159
20	G.F.W.	324	140	84	32	282
21	D.B.	328	140	84	24	257
22	J.L.	336	160	83	49	90
23	W.G.	342	150	83	45	207
24	J.Fr.	348	141	82	22	340
	Mean	313	137	83	31	148

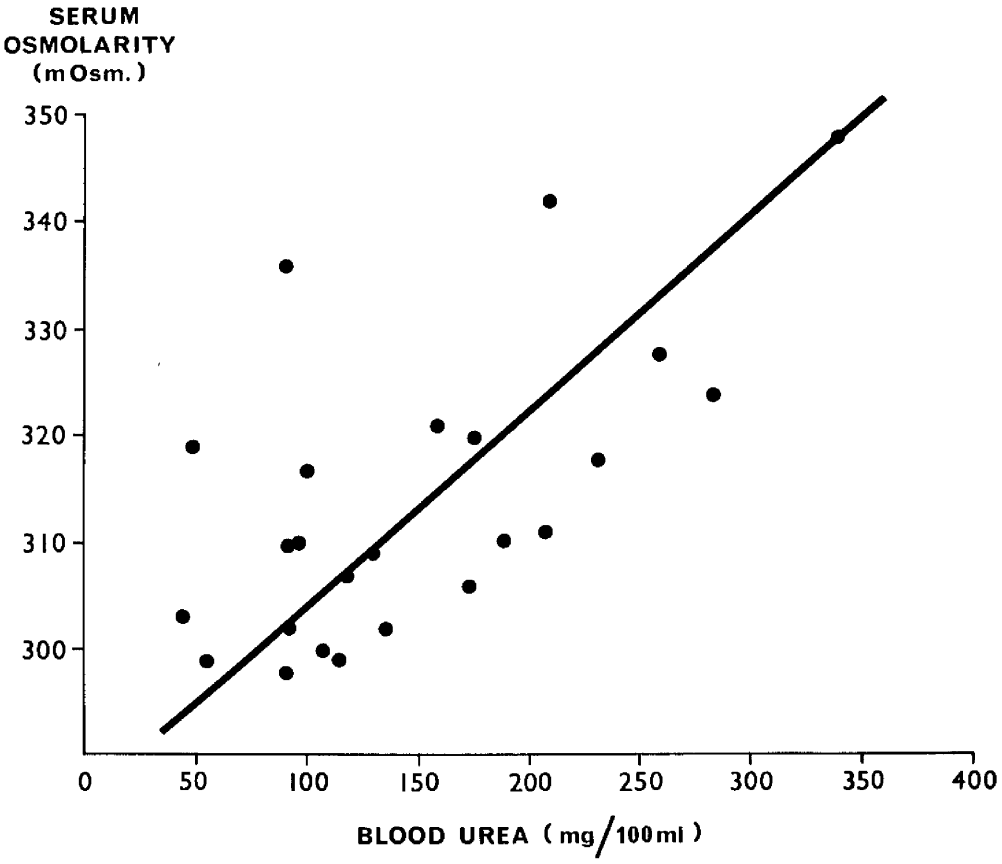

FIGURE 4. RELATION BETWEEN THE SERUM OSMOLALITY AND BLOOD UREA CONCENTRATION IN GROUP III PATIENTS.

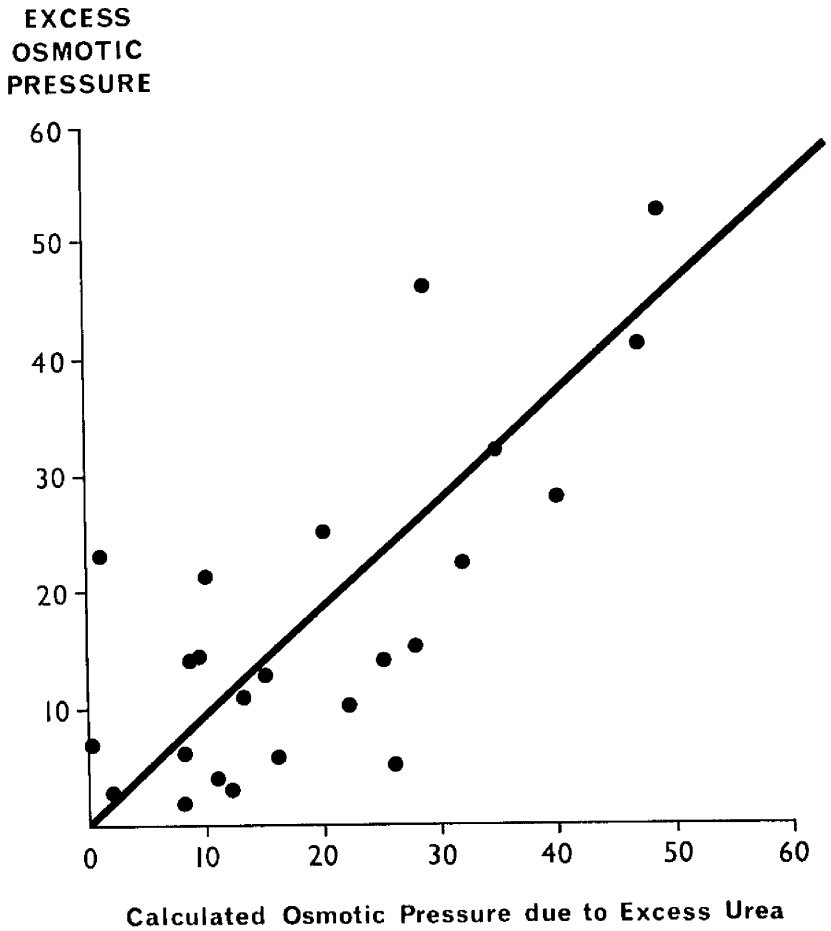

FIGURE 5. RELATION BETWEEN INCREASE IN SERUM OSMOLALITY AND BLOOD UREA CONCENTRATION ABOVE THE NORMAL LIMITS, THE LATTER HAVING BEEN CONVERTED TO mOsm. PER Kg. WATER.

(SEE TEXT P.32)

osmolality as shown in figure 5 where the increase in osmotic pressure above the upper limit of normal is plotted against the increase in blood urea above the upper limit of normal, the latter having been converted to mOsm. per kg. water on the basis of each 60 mg. urea per 100 ml. being equivalent to 10 mOsm. per kg. water. In other words, the increase in serum osmotic pressure is caused by nitrogen retention.

DISCUSSION.

The body has a remarkable ability for maintainting the osmotic pressure of its fluid compartments within narrowly defined limits. A significant depletion of chloride and sodium ions, with subsequent lowering of fluid osmolality, for example, is counterbalanced by the renal excretion of free water to raised fluid ionic concentration and restore its osmolality. Conversely, increased concentration of sodium and chloride ions in body fluids promotes thirst and stimulates oral intake of water, followed by renal excretion of salt and water to restore the fluid volumes.

Various clinical disorders associated with excessive loss of chloride ions, and with renal retention of bicarbonate ions, will evetually precipitate hypochloraemic alkalosis, and due to the depletion of eletrolytes, the serum osmolality will fall, which should stimulate the compensatory mechanism of the kidney to restore the status quo. During the initial one to three days of gastric alkalosis, the renal mechanism for reabsorption of bicarbonate ions will be normal and the patient will excrete an alkaline urine. However, with continued and unreplaced gastric losses of chloride ions, water, and potassium ions, dehydration will follow resulting in a decreased of glomerular filtration rate. With this decreased filtered load the bicarbonate ion reabsorption will occur and the urine become acidic.

It is surprising to note that in great majority of cases studied this compensatory mechanism does not appear to function. Apart from cases listed in group I (which comprises only a small percentage of total cases studied) the majority of the cases in group II and III did not show bicarbonate retention and in 10% of the cases it is well below the normal

limits. There are, of course, other factors which also influence the excretion of bicarbonate ions. Among them, the most important is the maintenance of ionic balance of the serum. Since the ratio of the sodium ions to chloride ions is low in vomitus, sodium ions are not lost in appreciable amounts in the early stages, but if the vomiting is marked and persists for some time without fluid replacement, severe depletion of sodium ions is also likely to develop along with chloride ions. In these circumstances, retention of bicarbonate ions cannot make up the deficiencies of chloride ions, since this would upset the ionic equilibrium. The total osmotic pressure, therefore, should fall. In these cases, the mean serum sodium concentration in the low osmotic pressure group (Group II) is 127 meq. per litre which is appreciably below the lower normal limits. The majority of cases in this group did not show compensatory bicarbonate retention. Two cases in Group III with normal serum sodium had low bicarbonate concentration. However, these cases had uraemia and it is probable, therefore, that there was renal retention of other anions, e.g. phosphate and sulphate which might cause depression of bicarbonate concentration.

It is surprising to find that urea and other end products of nitrogen metabolism are retained in some cases but not in others. There is controversy in the literature as regards the development of uraemia following the excessive loss of gastric secretions. Some workers believe that azotemia does not develop as a result of continuous loss of gastric juice (Grantham and Schloerb, (1963); Kirsner and Knowlton, (1941)). Kirsner and Knowlton (1941) produced severe hypochloraemia in dogs by continuous suction of gastric juice for a long period, and reached a critically low plasma chloride value of 47 meq. per

litre, but they failed to demonstrate any appreciable increase in the blood urea which rose only to 45 mg. per 100 ml. Kirsner, Knowlton, and Palmer (1943) repeated these experiments on human subjects and again could not detect any appreciable change in blood urea. Similar observations were made by Grantham and Schloerb (1963) in dogs and they concluded that severe hypochloraemia is not necessarily accompanied by uraemia. On the other hand, Dragstedt and Ellis (1930) found an elevation of the urea nitrogen from 12 to 154 mg. per 100 ml. and of non-protein nitrogen from 27 to 187 mg. per 100 ml. when the serum chloride was decreased experimentally from 300 to 108 mg. per 100 ml. McCance (1938) found that acute salt deficiency produced by diet and sweating, caused a reduction in volume of body fluids by 28 to 38%; the subjects were in negative nitrogen balance and it was maintained that both in clinical and experimental salt deficiency, the loss of chloride is followed by a marked loss of body water, and explained the nitrogen retention simply on the basis of excessive protein breakdown associated with anhydremia.

It seems that two factors probably play an important part in the development of uraemia in hypochloraemia:-

(1) Dehydration

(2) Abrupt and rapid depletion of chloride ions

In patients who develop acute and severe vomiting an abrupt and rapid depletion of chloride ions occurs, accompanied by marked loss of body water. This happens so quickly that the body has no time to readjust to the new environment, and hence these events probably stimulate some

mechanism of the kidney whereby it retains the end product of nitrogen metabolism and hence attempts to restore osmolality of serum to normal. On the other hand, in patients where the vomiting is not so severe or is only intermittent, or in those who are on gastric suction, the depletion of chloride ions is more gradual and this allows the patient to make fairly satisfactory adjustments to the electrolyte changes by the controlled renal excretion of water. However, if the vomiting persists for sometime without proper replacement of fluid and electrolyte, the patients become grossly dehydrated. In these cases at first there is some shrinking of the extracellular fluid volume, but little change in the acid-base balance, as the excessive loss of chloride ions is compensated by the excretion of alkaline urine in which sodium is in excess of chloride ions. As the vomiting progresses, there is a further reduction in blood volume which diminishes the renal blood flow. The condition is aggravated by renal arteriolar constriction induced by stress, and fall in the blood volume. At this stage, the glomerular filtration rate decreases, reabsorption is more complete, and urine output is decreased with the development of uraemia.

Cases in Group I.

The chloride depletion in this group was due to vomiting with one exception (case No. 18) where it was due to a combination of steroid and diuretic therapy. Only one of these patients subsequently died (case No. 15) and this was a case of severe burns in which toxaemia was the main cause of death. All the remaining cases recovered without incident and replacement therapy was straight forward and effective. Thirteen of the cases had lesions of the upper alimentary tract and most of the remainder

were cardiac cases, except one case of malignant disease (case No. 2).

The only correlation which has been found from the data on this group is that which is shown in Figure 3. The fact that the decrease in osmotic pressure due to low electrolyte concentrations has been accurately counterbalanced by retention of sufficient urea to bring the serum osmolality back to normal, is in itself important. However, it further suggests the possibility that the retention of urea may not be a mere coincidence. There are only two ways in which the serum osmolality can be restored to normal, namely, the excretion of sufficient water (i.e. dilute urine), and the retention of non-electrolytes. No patient in this group was severely dehydrated. Since the urea molecule can easily penetrate the cell membrane, its retention in the body would restore to normal both the extracellular and the intracellular osmotic pressures and this would assist in maintaining the normal state of hydration. Urea is the only substance available for such a compensatory mechanism, and it has the further advantage that it is completely non-toxic in the concentrations which are usually encountered in these conditions.

There can be no doubt that the maintenance of any body constant is beneficial to the patient, and there is no reason why the osmotic pressure should by any exception to this general statement. If this were so, then the nitrogen retention which occurs in these cases is a compensatory mechanism.

Cases in Group II.

A history of vomiting sufficient to account for the chloride depletion was obtained in 43 of the 63 cases, of the remainder, there were six cases

of diarrhoea, two with gallbladder fistulae, and four who were having intensive diuretic therapy. There were seven cases in which the cause of chloride depletion was not immediately apparent, but four of these may have been associated with the use of diuretics.

These patients, generally speaking, were much more seriously ill than the cases in group I, clinical dehydration being a common feature in nearly all of them. The most common diagnosis was malignant disease (21 cases) not infrequently with metastases; 13 cases had congestive cardiac failure, 10 had intestinal obstruction, and the remainder formed a group of miscellaneous conditions. As might be expected 14 of these cases died in the hospital and many others could not have survived long after discharge.

These patients form a group which is quite distinct from the cases in group I. They cannot, in the early development of their biochemical upset, have passed through a stage corresponding to the cases of group I (i.e. nitrogen retention) for this would imply that as the condition worsened, the urea concentration return to normal, or near normal, in over half the cases. Indeed if the data in Table III are examined (with the exception of case 1 and 56 it will be seen that the lower the serum osmolality and the more abnormal the sodium and chloride concentrations, the more likely is the urea concentration to be within normal limits or only slightly elevated.

It is impossible to avoid the conclusion that the osmotic pressure abnormality starts <u>ab initio</u> and that there is little or no attempt at osmotic compensation by retaining either bicarbonate or urea.

Reduction of the serum osmotic pressure due to lowering of the concentrations of sodium and chloride, without a compensatory increase in urea, has been described in many conditions. These have been summarised by Rees, Rosalki, and MacLean (1960). To the conditions mentioned by Rees et. al, may be added some of the less common conditions in which hypochloraemia is encountered e.g. cholecystitis (cases 15 and 53), fracture of femur (case 57), prostatic obstruction (case 60), and alcoholic gastritis (case 50). One is, therefore forced to the conclusion that patients suffering from any type of disease may find their way into this group. Although in most cases, the primary cause of the chloride depletion is readily accounted for by excessive loss of body fluids, in a small minority there is no such loss. In some of these cases the depletion is certainly assisted by the use of diuretics but this does not account for all of them. Schwartz, Bennett, Curelopp and Barttor (1957) described two such cases in which the cause appeared to be an abnormal secretion of antidiuretic hormone: in the cases described by Rees et. al. (1960) the cause was attributed to renal tubular damage.

Cases in Group III.

Chloride depletion was caused by vomiting in 18 of the 24 cases; there was one case of diarrhoea and one case of gallbladder fistula. The most frequent diagnosis was duodenal ulceration (9 cases), while most of the others were cases of malignancy. The mortality rate whilst in hospital (7 out of 24) was higher than the cases in group II. In this group, there was sufficient urea retention to raise the serum osmolality well above the normal upper limit. On the other hand, it is possible that

the cases of this group are simply a biochemical extension of the cases in group I.

There is a quantitative relationship (figure 5, P. 31) between the increase in serum osmolality and the increase in the blood urea concentration, the latter being the cause of the former, and this could well be a continuation of a process which is already evident in the group I cases, viz; urea retention. The passage from one group to the other probably coincides with the onset of renal dysfunction which is a well known hazard of prolonged salt depletion. The combination of hypochloraemic alkalosis and renal insufficiency has been described Steel (1936), Nicol (1940) and Kirsner and Palmer (1942). However, the causes of the kidney dysfunction and the nature of the renal lesions have not been clearly defined. The evidence suggests that the kidney may suffer considerable functional impairment as a result of hypochloraemic alkalosis, and subsequently recover completely. This renal dysfunction which is a reversible process, atleast in the early stages, must be distinguished from renal disease. Whether or not permanent renal damage can result from these conditions cannot be said with certainty.

The serum biochemistry of all the 77 patients who survived (and even of some who did not) was restored to normal by appropriate fluid therapy and, as usual, it was easier to restore the chloride to normality than the urea. It is common knowledge that when the fluid is replaced in such cases, the urea returns to normal, falling rapidly at first, and then more slowly as the normal range is approached. In a series of five patients who recovered from hypochloraemic alkalosis due to pyloric obstuction

Burnett, Barrows, and Commons (1950) found that complete restoration of normal renal function was achieved very slowly over a period of months.

It is a problem to decide which of these three groups represent the "normal response" to chloride depletion: one would naturally favour the group in which such an important body constant as the osmolality is maintained within the normal limits, and this in turn, would mean that urea retention is an active process beneficial to the body. The comparitive ease with which it is removed from the body (once the chloride ions are replaced) supports this view. In that event, it follows that failure to retain urea and to maintain the osmotic pressure of extracellular fluid must be due to a defect in some mechanism presumably in, or related to, the osmoregulating centre.

These changes should correlate with the degree of dehydration but unfortunately, this cannot be adequately expressed quantitatively and is largely a matter of clinical judgement.

PART 2

ULTRAFILTRATION STUDIES ON SERUM CALCIUM, MAGNESIUM AND INORGANIC PHOSPHATE.

INTRODUCTION.

There has long been interest in the precise nature in which calcium exists in the extracellular fluids of the body. It is known that normally there are homeostatic mechanisms which control the calcium concentration of the serum within certain limits. Rona and Takahashi (1911) dialysed serum against water and showed that a part of the calcium was not diffusible through a semipermeable membrane. They assumed that the nondiffusible portion was bound to protein. Since then many studies have been undertaken on the partition of calcium in human serum. Various techniques and improvements of this determination were evolved. Methods involving ultrafiltration have become numerous because of practical and theoretical advantages over biological or dialysis techniques. However, previous workers took no special precaution to ensure constancy of pH during the process of ultrafiltration, as this was considered unimportant at that time. It had been reported by Meysenbug, Pappenheimer, Zuker and Murray (1921), Nauhausen and Pincus (1923), and Greenberg and Gunther (1930) that variations in pH during ultrafiltration had no effect on the results obtained. However, it has since been shown by Seekles (1936) and more recently by Hopkins, Howard and Eisenberg (1952) that the ultrafilterable calcium alters with the pH of serum. The alteration of pH was accomplished by these workers with the addition of acid or alkali. It was found that raising the pH of the serum by adding alkali reduced the calcium concentration of the ultrafiltrate; while acidification increased it. The alteration of pH was later accomplished both by equilibrating the serum in the ultrafiltration apparatus with 100% carbon dioxide and by the addition of acid by Toribara, Terepka and Dewey (1957). These workers could not detect any appreciable difference

in the calcium concentration of the two ultrafiltrates thus obtained. Although the carbon dioxide content of the serum would be different when the pH is altered by these two different methods, it is apparent from the results of Toribara, et al. (1957) that the final pH seems to be the important factor in altering the ultrafilterability of calcium rather than the method of attaining it. Little reliance can, therefore, be placed upon the earlier work which ignored this important factor, and hence obtained results significantly lower than the recent workers for normal human serum.

In Table I is recorded the percentage ultrafilterability of the calcium of normal human serum calculated from the data of earlier authors. It is immediately apparent with the exception of Moysenbug, et al. (1921), Rona and Melli (1925), Kirk and King (1926), and Nicholas (1932) the early workers obtained results lower than those of the four most recent workers. It seems most probable that failure to control the pH during ultrafiltration was predominantly responsible for the consistently low values obtained by the early workers. It is of interest that the results of Moysenbug et al. (1921) and those of Rona and Melli (1925) were obtained by compensation dialysis against distilled water in a closed system where the pH was probably maintained fairly constant throughout the whole procedure. The original Rona and Takahashi investigations of 1911 were carried out essentially by the same method. Kirk and King (1926) and Nicholas (1932) took no special precautions regarding pH control but the apparatus they used was closed during the entire ultrafiltration procedure and positive air or nitrogen pressure was maintained over the serum. The ultrafiltration results obtained under pH control by some of the recent workers are in close agreement with each other.

Table I The Percentage of Ultrafiltorable Calcium in Normal Human Serum as Calculated from the Data of Previous Authors.

Author.	Year.	pH Control.	% ultrafilterable Calcium Range.	Mean	Remarks.
Von Meysenbug et. al.**	(1921)	yes	67-73	70	
Nauheusen and Pincus	(1923)	no	40-50	46	
Roma and Nelli**	(1925)	yes	64-69	66	
Kirk and King	(1926)	no	56-81	69	questionable total serum calcium.
Updegraff et. al.***	(1926)	no	41-54	46	
Hertz	(1929)	no	43-61	49	
Snell	(1930)	no	45-60	51	
Greenberg and Gunther*	(1930)	no	42-68		assuming total serum calcium o 10 mg.%.
McCane and Watchorn*	(1931)	no	44-66	52	
Watchorn and McCane*	(1932)	no	45-59	50	
Nicholas	(1932)	no	62-67	64	questionable total serum calcium.
Herbert*	(1933)	no	48-58	53	
Benjamin and Hess*	(1933)	no	- -	52	included 17 infant Total 21.
Morrison et. al.	(1938)	no	37-48	42	
Anning et. al.	(1948)	no	- -	51	
Hopkins et. al.	(1952)	yes	65-75	68	
Prasad and Flink	(1957)	yes	57-72		
Toribara et. al.	(1957)	yes	61-70	65	
Rose	(1957)	yes	57-63	59	

* Ultrafiltration carried out under negative pressure.
** Compensation dialysis.
*** Combined ultrafiltration and compensation dialysis.

Greenwald (1926) suggested that the diffusible fraction of the serum calcium was partly ionised and partly complexed with a citrate-like substance. Most investigators have supported this view. It is now recognised that calcium exists in the serum in three distinct forms. One of these, calcium bound to protein, comprises the nonfilterable portion of the serum calcium. The other two forms, are ionic calcium and the complexed calcium. The "complexed" calcium refers to that portion of calcium which is ultrafilterable but not ionised. Most of this calcium is complexed to small diffusible ions, such as phosphate, bicarbonate, but mainly citrate. There have been considerable differences of opinion on the proportion of ionised to complexed forms in the diffusible fraction. The only accepted method of determining the ionised calcium of plasma has been the biological method of McLean and Hastings (1934) in which they used the frog heart as an indicator of the calcium ion concentration Using this technique, they found that 4.25 to 5.25 mg. per 100 ml. of the total serum calcium exists in ionic form while the complexed calcium was less than 0.6 mg. per 100 ml. Unfortunately the frog heart technique suffers from many of the usual hazards of a biological method, and may be unsuitable for determination in plasma from patients with certain pathological conditions such as uraemia where phosphate or magnesium ions are present in abnormal amounts and possibly affect the frog heart. However, the above findings of McLean and Hastings (1934) are lower than all the estimates using purely chemical methods.

Raaflaub (1951) developed a chemical method by using murexide for the determination of ionised calcium in cerebrospinal fluid. This method is based upon the fact that if a very small amount of murexide is added to a solution containing calcium ions at approximately neutral pH, then the change

in colour of the dye will be proportional to the calcium ion concentration. Sheet and Seekles (1952) applied the same technique to the determination of ionised calcium in milk ultrafiltrates. Later this method was futher modified by Rose (1957), to permit the measurement of ionised calcium in serum ultrafiltrates under normal conditions of pH and buffering. To do this it was necessary to use the physiological carbonic acid-bicarbonate buffering system and each test had to be read against a blank containing concentrations of sodium, potassium, and chloride ions equal to found in normal serum and bicarbonate in concentration equal to the serum specimen under test. The test is compared with two standards containing two different cacium concentrations: one less and one greater than the ultrafiltrate. Again each standard contains sodium, potassium and chloride in normal concentrations and bicarbonate in concentration equal to that of test serum. Fowler, Fone and Cooke (1961) using this technique could not duplicate most of their readings in a number of experiments. Their impression about the method was that the determination of the ionised cacium fraction by this technique was more difficult, more time consuming, and less reliable than the method of determining calcium in an ultrafiltrate. Since the normal range for ionised calcium is covered by a very small extinction range, an extremely small error in the spectrophotometer readings will represent a significant change in measured calcium ion concentration and consequently also in the value for the complexed fraction. Later it was found by Fanconi and Rose (1958) that in some of their cases the measured ionised calcium in the ultrafiltrate exceeded the total ultrafilterable calcium and they attributed these unreliable results to the

expected error of the method. The mean values obtained for the complexed fraction of calcium are only of the order of 0.25 mg. per 100 ml. (Fanconi and Rose (1958)) and 0.3 mg. per 100 ml. (Rose (1957)), which are negligible compared to the total diffusible fraction, so that the whole of the diffusible fraction can be regarded as the physiologically active fraction. Again in various clinical disorders a knowledge of the concentration of the diffusible fraction gives the same information as the ionic calcium, since the latter is determined in serum ultrafiltrates. Any increase in the diffusible fraction will reflect an increase in ionised fraction and vice versa. However, a significant difference between the two fractions may be encountered in instances such as rapid exchange tranfusions where large amounts of citrate ions are also being tranfused, or in disorders where high values for citrate are found, such as after vitamin D therapy (Harrison (1954)). It is believed that citric acid metabolism is essentially undisturbed in clinical disorders involving calcium metabolism, (Neuman and Neuman (1958).

Hence apart from the above mentioned instance, it can be concluded that the diffusible fraction of calcium represents the ionic calcium.

Ultrafilterable Magnesium.

It was found by Rona and Takahashi (1911) and by Stray and Winternitz (1929) that, like cacium, serum magnesium was not freely diffusible through a semipermeable membrane. The magnesium of the serum also appears to be partly bound to protein, and exists in human serum as ultrafilterable and non-ultrafilterable forms. Of these two divalent ions,

the protein-binding property of calcium has been more intensively studied than magnesium. Magnesium has also an important physiological role in many enzymatic reactions. Recently a clinical syndrome resulting from magnesium deficiency has been described (Flink, McCollister, Presad, Melby and Doe (1957)). Since magnesium and calcium are the major divalent cations in the body, it would appear probable that the magnesium would follow the same pattern as the calcium of the serum, and the characteristics of protein binding for magnesium might be very similar to that of calcium.

Green and Power (1931) reported that in a protein containing solution, the ratio of diffusible to non-diffusible magnesium was approximately equal to the ratio of diffusible to non-diffusible calcium. Kleeman, Epstein, McKay and Taborsky (1958) stated that the dissociation constant may actually be the same and may be expressed as follows:-

$$\frac{(Ca^{++})\ (Protein^{--})}{(Ca\ Proteinate)} = K = \frac{(Mg^{++})\ (Protein^{--})}{(Mg\ Proteinate)}$$

The percentage of total serum magnesium which diffuses through a semipermeable membrane has been measured by several investigators. Controversial values for normal human serum have been reported. The majority of early workers again regarded the control of pH during ultrafiltration as unimportant and obtained results which are lower than the values obtained under conditions of controlled pH by other workers. Average normal values reported for diffusible serum magnesium ranged from 57% (Cope and Wolff (1942)) to 84% (Soffer, Cohn, Grossman, Jacobs and Sobotka (1941)), and results within these limits have also been reported

by Bissell (1945), Watchorn and McCance (1932), and Laviotes and Dine (1942).

However, it was shown later by Hopkins, et. al. (1952) that the ultrafilterable magnesium of the serum altered with pH. The values reported for normal human serum magnesium under controlled pH conditions by the recent workers are consistent. Kleeman, Epstein, McKay and Taborosky (1958) obtained an average value of 74% while Silverman and Gardner (1954) and Prasad, Flink, and McCollister (1961) obtained values up to 72%.

It is generally believed that the protein bound or non-diffusible fraction of magnesium is of little physiological significance in human subjects (Copeland and Sunderman (1952)), and that the homeostatic regulations of magnesium, like calcium, concern only the ionised or the diffusible form. On theoretical grounds it has been concluded by Copeland and Sunderman (1952) that the complexed fraction of magnesium is so small that it can be ignored physiologically. Therefore, no attempts have been made for its actual determination in this study. It is believed that the diffusible fraction of serum magnesium is completely ionised and that all of this fraction is physiologically active (Kleeman, et. al. (1950)).

Ultrafilterable Inorganic Phosphate.

In the past, it has been disputed whether all of the plasma phosphate is filterable through a semipermeable membrane or not. Early micropuncture studies in Amphibia indicated that the concentration of inorganic phosphate in the glomerular filtrate was the same as that in

whole plasma (Walker (1933), and White (1932)). However, when the Donnan membrane effect and the plasma water content were taken into account the concentration of inorganic phosphate should be more than the serum itself. It follows from the concentration of the normal plasma water content of 940 g. per litre (Miller (1942), and Hald (1946)) and the theoretical Donnan ratio for divelent anions, 0.92 to 0.93 (since 80% of the plasma phosphate consists of HPO_4^{--} and only 20% as $H_2PO_4^-$) that the concentration of phosphate in the ultrafiltrate would be 1 / 0.925 x 0.94 or 1.15 times that in whole plasma (Walser (1960)). Hence for complete filterability the ratio of phosphate concentration in ultrafiltrate to that in serum should be 1.15.

Values close to this ratio have been reported by Pincus, Peterson and Kramer (1926) and Grollman (1927) in dogs. Smith, Ollayos and Winkler (1943) investigated 13 human subjects and found a mean value of 1.05 for the ratio in nine cases, while a mean value of 0.99 was obtained in the other four cases. Since the ultrafiltration studies in these four cases were carried out after the production of hyperphosphataemia by intravenous infusion of phosphate, there is a possibility that in these cases a colloidal calcium phosphate complex might have formed which resulted in lowering of this ratio. More recently, Hopkins, Conner, and Howard (1953) ultrafiltered, with pH control, sera obtained from normal subjects and found that the concentration of inorganic phosphate in the ultrafiltrate always exceeded that of original serum.

They obtained a mean value of 1.11 for the above ratio. It was noticed by these workers that the concentration of inorganic phosphate was

far less in the ultrafiltrate obtained from a pooled serum specimen whose calcium concentration was increased beyond 19 mg. per 100 ml. by the addition of calcium chloride *in vitro*. This reduced ultrafilterability of inorganic phosphate was again attributed to the formation of a colloidal complex. This phenomenon could not be demonstrated when the concentration of inorganic phosphate was raised *in vitro* in the presence of normal serum calcium. Wallach, Bellavia, Schorr, and Reizenstein (1964) also found reduced ultrafilterability of inorganic phosphate by producing artificial hypercalcaemia *in vivo* in dogs. This was also observed in a number of cases of hypercalcaemia without any pathology of the parathyroid glands by Hopkins, *et. al.* (1953).

Diffusible Calcium in Hypercalcaemic States.

(a) Hyperparathyroidism:

Raised values for ultrafiltrate calcium have been found in this condition by Hopkins, *et. al.* (1953) who studied nine cases of hyperparathyroidism and found elevated values for ultrafilterable calcium in eight of them. However, these results when expressed in terms of percentage of the total calcium were within the normal limits. Since then all other workers reporting on ultrafilterable calcium have stated that the highest concentrations of ultrafilterable calcium were encountered in this condition. Prasad and Flink (1958) investigated eight cases of hyperparathyroidism and found raised values of ultrafilterable calcium in all of them and stated that the amount of ultrafilterable calcium depended

mainly upon the total serum calcium. However, their results differed in one respect from that of Hopkins, et. al. (1953) in that they obtained slightly lower values for ultrafilterable calcium both for the normal controls and hyperparathyroid cases. These authors criticised the results of Hopkins, et. al. (1953) who saturated the serum specimens with 5% carbon dioxide in order to standardise the pH prior to ultrafiltration. This, according to Prasad and Flink (1958), would result in lowering the pH far below the physiological range and hence be responsible for greater ultrafilterability of calcium.

Later with the development of a chemical method for the determination of ionised calcium by Rose (1957), the interest shifted to the determination of this purely ionic fraction. In cases of hyperparathyroidism, the ionic calcium, as in normal subjects, also approximates to the total ultrafilterable calcium. Fowler, Fone, and Cooke (1961) stated that the determination of the diffusible fraction would provide the same information as the determination of ionic calcium in these cases, and hence from the purely practical aspect the more time-consumming and less reliable determination of the ionised fraction is unnecessary. Similar observations were made by Hodgkinson (1963). Raised values for ionised calcium have been reported by Fanconi and Rose (1958) and by Lloyd and Rose (1958).

Great reliance is placed upon the serum calcium concentration in the diagnosis of hyperparathyroidism, but when this is not raised the diagnosis is often considered to be untenable, although proven cases with normal concentrations of serum calcium have been reported (Gutman, Tyson,

and Gutman (1936); Hellstrom (1953) and Mather (1953)). The absence of hypercalcaemia may be explained by hypoproteinaemia, renal failure, or temporary remission, but all these factors can sometimes be excluded, as in a case described by Mather, (1953). In such difficult cases, the determination of serum ultrafilterable calcium may be valuable, as according to Lloyd and Rose (1958) the ultrafilterable calcium is always raised with a functioning parathyroid adenoma even if the total serum calcium is within normal limits.

Similar findings have been also described by Fowler, Fone, and Cooke (1961) and by Prasad and Flink (1958), where the determination of diffusible calcium fraction was considered to be valuable in the diagnosis.

(b) Diffusible Calcium in Hypercalcaemia of Causes other than Hyperparathyroidism.

There are a number of other diseases where hypercalcaemia is frequently encountered and with which primary hyperparathyroidism may be confused. These diseases include osteoporosis, hypervitaminosis D, sarcoidosis, myelomatosis with bone involvment, polycystic disease of the kidney, osteomalacia, and the milk alkali syndrome. Ultrafiltration studies on this clinical material have not been extensive, and conflicting reports have been published concerning the significance of ultrafilterable calcium in these types of hypercalcaemia. Hopkins, et. al. (1953) investigated one case of hypercalcaemia due to hypervitamosis D and found decreased values for ultrafilterable calcium in spite of a raised serum calcium concentration, and this was associated with reduced

ultrafilterability of inorganic phosphate as well. Opposed to this, Terepka, et. al. (1958) studied two such cases and found a proportionate rise in the ultrafilterable calcium fraction. Prasad and Flink (1958), investigating cases of hypercalcaemia found decreased values for ultrafilterable calcium in two of four cases of multiple myeloma and in three cases of breast carcinima with secondaries in bone. Hopkins, et. al. (1953) found variable results in multiple myelomatosis; hypercalcaemia was present in six of their eight cases, but in two of these the ultrafilterable calcium was reduced. In these two cases a decreased ultrafilterability of inorganic phosphate was also found.

Diffusible Calcium in Hypocalcaemic States.

(a) In Hypoparathyroidism.

Low valvues for ultrafilterable calcium have been reported by various workers in hypoparathyroidism. Hopkins, et. al. (1953) investigated six cases of post- thyroidectomy hypoparathyroidism and two of the idiopathic type. All of these patients had been on vitamin D and oral calcium therapy, but this was discontinued for several days prior to the experiment. Low values for both serum and ultrafilterable calcium were obtained in all of these cases. In some of them, values as low as half the normal average were found with severe tetany. Decreased values for ultrafilterable calcium were also reported by Terepka, et. al. (1958) in two of their cases. These findings were later confirmed in five cases studied by Prasad and Flink (1958), who stated that although both

serum and ultrafilterable calcium were reduced, the ratio of ultrafilterable to total calcium remained within normal limits. No correlation of the degree of tetany with either the total serum calcium or the ultrafilterable calcium could be found by any of these workers. A decreased value for the ionised calcium was also reported by Fanconi and Rose (1958) in one case of hypoparathyroidism.

(b) Diffusible Calcium in other Hypocalcaemic Conditions.

Hypocalcaemia is often encountered in chronic renal disease with nitrogen retention. It occurs usually in the later stages of this condition and is associated with, and perhaps dependent upon, the increase in serum phosphate which occurs. Tetany is rarely encountered in these cases. However, it can and does occur when marked lowering of serum calcium occurs, with subsequent lowering of diffusible fraction. Absence of tetany in these cases of hypocalcaemia is due to the fact that no significant changes occur in the diffusible fraction of the serum calcium. Fanconi and Rose (1958) investigated four cases of chronic renal failure with nitrogen retention and found that while the total serum calcium was markedly diminished, the ionised fraction was either near the normal limits or only slightly diminished. In a series of seven cases of renal failure Terepka, et. al. (1958) found that the ultrafilterable calcium was within or near the normal range and in one instance was even greater than normal. Consequently the percentage of ultrafilterable calcium in all the cases was abnormally high. Hopkins, et. al. (1953) also studied one case of chronic nephritis and found normal values for the ultrafilterable calcium

in spite of hypocalcaemia and normal total serum proteins with a normal albumin / globulin ratio. Decreased serum albumin concentration is frequently observed in chronic renal failure, and in a small minority of cases a very low serum albumin concentration may be found. It has been reported that in nephrosis, without renal failure, serum calcium values ranging from 6 to 9 mg. per 100 ml. have been observed. This diminution in serum calcium concentration is due entirely to a decrease in the non-diffusible fraction which occurs as a result of a marked reduction in the concentration of serum proteins, particularly serum albumin. In other conditions such as anaemia and cirrhosis of liver, where low serum albumin concentrations are frequently observed, similar results are found. Normal values for ultrafilterable calcium were found in 27 cases of hypoalbuminaemia by Prasad and Flink (1958) and in six cases studied by Hopkins, et. al. (1953). In no case was there any significant alteration in the amount of diffusible calcium and increased neuromuscular excitability was not observed.

Serum and Ultrafilterable Magnesium.

(a) In Hypercalcaemic States.

There is conflicting evidence of the part played by parathyroid glands in magnesium metabolism. Greenberg and MacKay have observed a rise in the plasma magnesium concentration after parathormone injection in dogs. On the other hand Watchorn and McCance (1932) reported a decrease in both total and ultrafilterable magnesium, while Roberts, Murphy, Miller,

and Rosenthal (1954) found no significant change. Hypomagnesaemia has been reported in patients with hyperparathyroidism (Harmon (1956); Agna and Goldsmith (1958)); the same condition has been found to develop after removal of a parathyroid adenoma from patients whose pre- operative serum magnesium concentration were normal (Potts and Roberts (1958); Hanna, North, MacIntyre, and Fraser (1961)). Very little work has been done on the ultrafiltration of magnesium in this condition. Prasad, Flink and McCollister (1961) studied one case of hyperparathyroidism and found raised values for both serum and ultrafilterable magnesium.

Normal values were obtained by Silverman and Gardner (1954) both for total and ultrafilterable magnesium in pre-operative and post-operative hyperparathyroid cases. Variable results are reported by Prasad, et. al. (1961) in cases of multiple myeloma. Hypomagnesaemia with low values for the ultrafilterable magnesium was found in two out of the five cases which they studied. Hypermagnesaemia with high values for ultrafilterable magnesium was found in only one case.

Silverman and Gardner (1954) reported decreased values for ultrafilterable magnesium in two cases of vitamin D intoxication, and Kruger (1932) reported a decrease in magnesium in vitamin D deficiency.

(b) Serum and Ultrafilterable Magnesium in Hypocalcaemic States.

Conflicting findings for the ultrafilterable magnesium have been reported in hypoparathyroidism. Silverman and Gardner (1954) have reported decreased values for both total and ultrafilterable magnesium in the only case they studied. It has also been demonstrated by Roberts, et.al.

(1954) that parathyroidectomy in normal dogs produce little or no effect on the magnesium balance except a minor decrease in the serum concentration. Prasad, et. al. (1961) found normal values for both total serum and ultrafilterable magnesium in one case of hypoparathyroidism.

Variable findings for ultrafilterable magnesium have been reported by Prasad, et. al. (1961) in renal disease. Out of eight cases of nephrosis, both total and ultrafilterable magnesium were found to be decreased in two instances, and the percentage of ultrafilterable magnesium was in three cases associated with the hypoalbuminaemia. Of eleven patients with uraemia five had increased total and two had increased ultrafilterable magnesium. In the majority of cases of hypoalbuminaemia associated with various clinical disorders reported by the above authors, the total serum magnesium was found to be slightly decreased but the ultrafilterable magnesium was within the normal limits and hence resulted in elevated percentage ultrafilterability.

Outlines of Present Work.

In the present study, a new simplified technique for ultrafiltration was designed. Various factors which affect the the ultrafilterability of calcium and magnesium were investigated. The role that ultrafilterable calcium and inorganic phosphate can play in the diagnosis of hyperparathyroidism was also examined. Ultrafilterable calcium, magnesium and inorganic phosphate were determined in the serum of patients with parathyroid disorders and in a large group without any pathology of the parathyroid glands. Particular attention was paid to

cases of hypercalcaemia without parathyroid disorders in the hope of clarifying the ultrafilterable calcium and inorganic phosphate results which are found in this condition.

PART 2

MATERIAL AND METHODS.

Technique of ultrafiltration.

In the absence of any practical method applicable to the direct measurement of ionic calcium, a great deal of effort has gone towards the development of indirect methods of determination in human serum, i.e., measurement of the ultrafilterable calcium, which more or less reflects the ionic calcium. A variety of techniques has been described, but ultrafiltration methods have been most numerous because of the practical and theoretical advantages over the dialysis techniques. In principle, all ultrafiltration techniques utilise a membrane of small pore size and some means of supplying filtration pressure. There are many techniques described in the literature but detailed study showed that these have one or more disadvantages. Generally the techniques require:-

1) A complicated apparatus, not easily constructed.
2) A considerable amount of specimen (serum) is required in order to obtained sufficient ultrafiltrate for analysis.
3) A long time is taken for the ultrafiltration, up to 48 hours.

A new ultrafiltration technique is described here, which is simple to construct and easy to use. Seamless cellophane tubing is used to contain the serum specimen. This is attached to glass tubing supported by a rubber bung, and a positive pressure is applied for the ultrafiltration. The complete apparatus set up is shown in Figure 1 and 2.

A strip of cellophane tubing 6 - 7 inches long is taken and washed in running water for about one hour. After this, the tubing is washed 3 to 4 times in distilled water. One end of the strip is then tightly knotted twice to ensure that no leaks occurred. It was found on

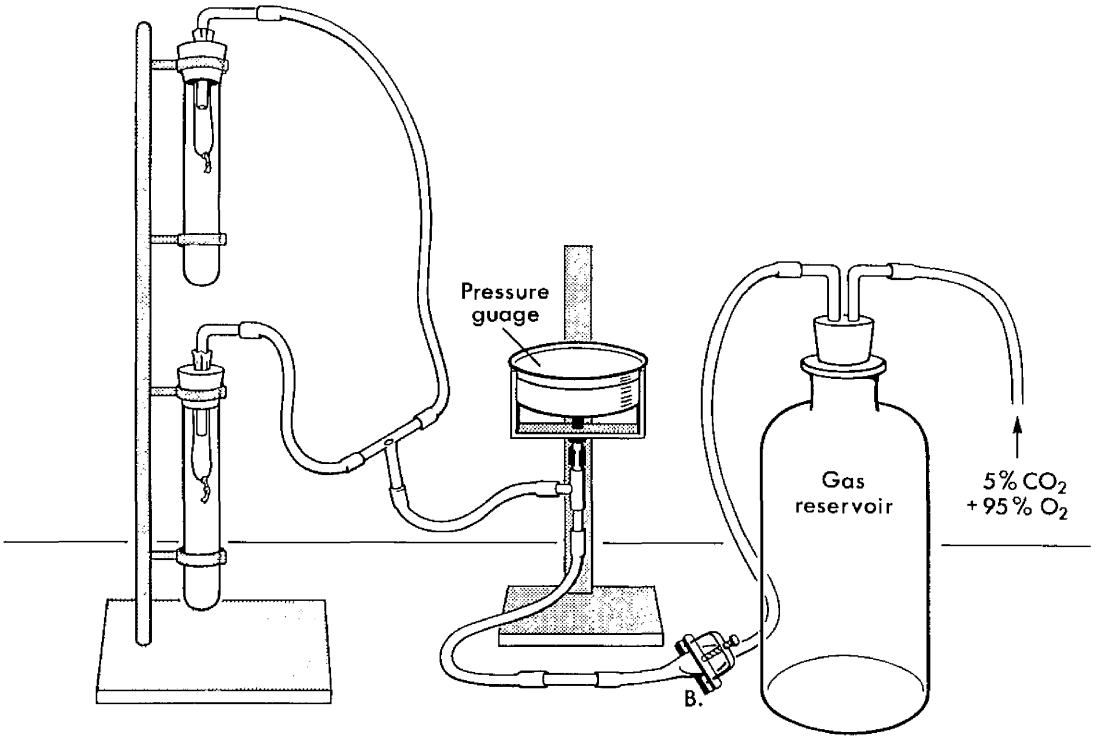

FIGURE 1. THE SET UP OF THE APPARATUS USED FOR ULTRAFILTRATION UNDER 5% CARBON DIOXIDE AND 95% OXYGEN PRESSURE.

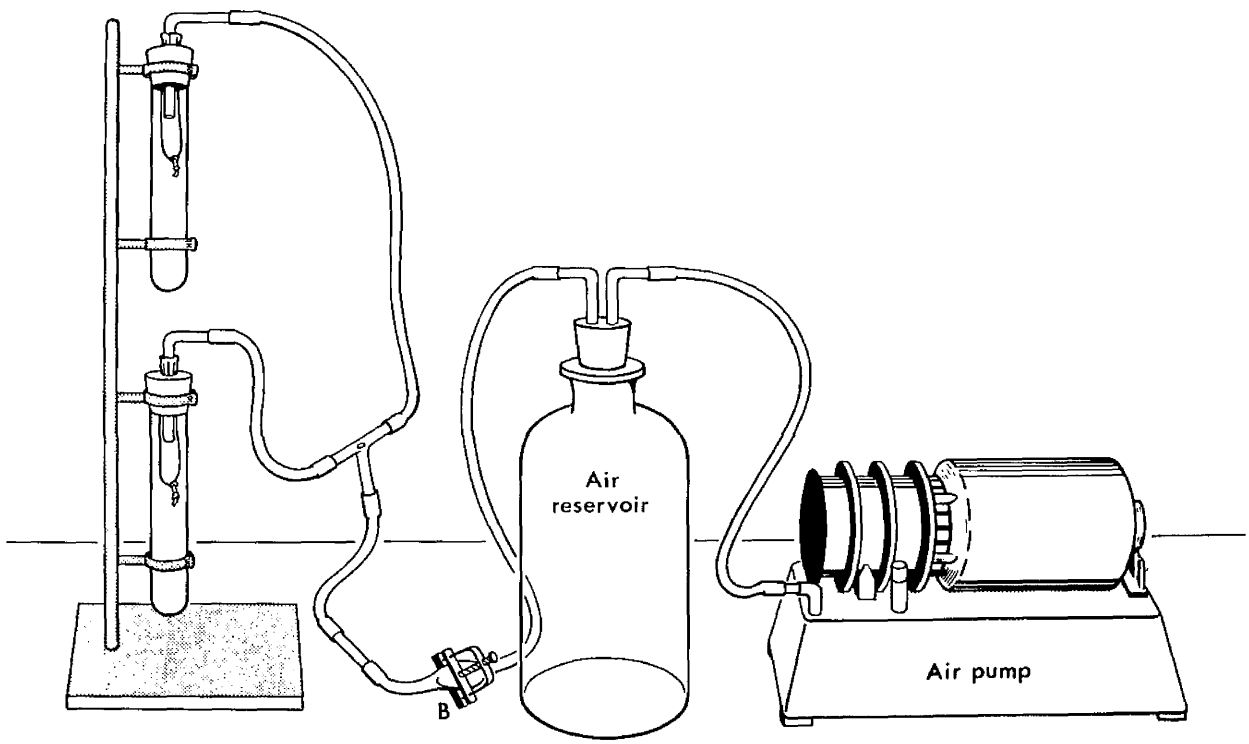

FIGURE 2. THE SET UP OF THE APPARATUS USED FOR ULTRAFILTRATION UNDER AIR PRESSURE.

one or two occasions that the membrane leaked and it is necessary, therefore, to check all ultrafiltrates for protein by addition of ten percent trichloracetic acid. The open end of the cellophane tubing is forced through one of two holes bored in a rubber bung and is pulled through to a reasonable length. A glass bend is then inserted slowly inside the tubing as far as possible without damaging it. The glass bend along with the cellophane tubing is then carefully forced through the bung hole until the other end of the glass bend protrudes through the bung. This gives an air-tight joint between the cellophane and glass tubing. The rubber bung, carrying the cellophane tubing, is clamped firmly and the other end of the glass bend can now be joined by rubber tubing to the device for applying pressure. This consists simply of a litre glass bottle as an air reservoir, fitted with two glass bends through a rubber bung. One end is joined by rubber tubing to the 5% carbon dioxide cylinder, and the other goes to the glass bend holding the cellophane tubing. Between the air reservoir and the cellophane bag a pressure gauge is inserted to regulate the filtration pressure. It was found that the cellophane membrane could withstand a pressure up to 25 lb. per sq. inch before bursting. Experiments were usually performed at a pressure of 16 lb. per sq. inch, this being a pressure which gave rapid ultrafiltration with little danger of bursting the cellophane tubing. As the cellophane tubing has been washed with water, it is necessary to dry it completely before ultrafiltering the serum specimen. After fixing the wet cellophane tubing to the glass bend and rubber bung, a pressure of 16 lb. per sq. inch is applied. The cellophane tubing swells up slightly giving it a sausage-like

shape. This pressure is maintained until the bag is completely dry. After this treatment the cellophane bag does not change on being left, and hence these dried bags can easily be stored for use at any time. The filtration sac is about 12 cm. in length and 1 cm. in diameter.

The serum specimen is introduced slowly through the glass bend with a Pasteur pipette. The specimen tends to accumulate in the glass bend but it can be sucked into the bag by gently pressing and then releasing the cellophane bag. A collecting tube for the ultrafiltrate is clamped to surround the cellophane bag. This is either an ordinary test tube or a graduated tube if the volume of the ultrafiltrate is to be measured. The sac is now connected to the pressure device and the clamp of the 5% carbon dioxide is opened slowly. When the pressure gauge needle approaches the 16 lb. per sq. inch mark the clamp is immediately closed tightly. This pressure is maintained by the reservoir throughout the ultrafiltration period. If the pressure drops due to leakage, it can be readjusted by first closing the stop cock B (Figure 1, P. 64) and then slowly opening the 5% carbon dioxide cylinder clamp until the pressure is again raised to 16 lb. per sq. inch. The advantage of this stop cock is that if the valve on 5% carbon dioxide cylinder is too widely opened the excessive pressure does not reach the membrane and hence the danger of bursting the membrane is eliminated.

When it is desirable to ultrafilter the specimen under air pressure instead of 5% carbon dioxide, the gas cylinder can be replaced by an air pump similar to one which is used with the EEL flame photometer. The advantage of this pump is that the pressure can be adjusted by a valve on

the pump. When this valve is fully closed the pressure cannot go beyond 16 lb. per sq. inch, which virtually eliminates any danger of the cellophane tubing bursting.

Using T-bends and stopcocks a number of specimens can be handled simultaneously. However, frequent adjustments of pressure have to be made, or a much larger reservoir for 5% carbon dioxide has to be used. It was found convenient to handle two specimens at a time.

It takes about 1½ to 2 hours to obtain 1 ml. of ultrafiltrate from 2 ml. of serum.

Estimation of calcium.

Ethylene-diamine-tetra-acetic acid (E.D.T.A.) forms complexes with nearly every metal carrying more than one positive charge. The pH has a marked effect on the stability of various E.D.T.A. metal complexes and this makes the selective titration of these metals in the presence of each other possible.

Murexide (ammonium purpurate) is used as an indicator for calcium titration. It has a violet blue colour in strongly alkaline solutions which turns to salmon pink on addition of calcium due to the formation of calcium-murexide complex. Since this complex is less stable than that between calcium and E.D.T.A., on addition of E.D.T.A. the free calcium is complexed first; then immediately before the equivalence point, the calcium is removed from the calcium-murexide complex with the libration of uncomplexed murexide, causing a colour change. As the change in colour is very slight the end point is detected photoelectrically by the EEL titrator. A curve as in Figure 4 is obtained where the rising part near the end point is a straight line, which is extended to cut the horizontal

branch, the point of intersection being the end point.

Magnesium is the only metal under the chosen experimental conditions which is likely to interfere in the estimation of calcium. This interference is eliminated by carrying out the titration at a high pH where the magnesium-E.D.T.A. complex is not stable. In addition, magnesium hydroxide is insoluble at a pH of 12.

Apparatus:-

The apparatus used was the EEL photo-electric titrator model no. X-135 with a galvanometer which gives direct reading of extinction, when using spectrum filter no. 606 (peak 575-580 mu).

Reagents:-

1. Murexide indicator. (prepared freshly before use)

Approximtely 4 mg. murexide (ammonium purpurate, B.D.H.) were dissolved in 100 ml. of distilled water in a polythene bottle. The extinction of this solution, with water as the blank, is between 70 and 75 divisions on the galvanometer scale.

2. E.D.T.A. (4.80 g. per litre)

The disodium salt of ethylene-diamine6tetra-acetic acid (B.D.H.) was dried at 110°C for four hours and placed in desiccator overnight. 4.80 g. of the salt were dissolved in distilled water and made up to one litre, this solution was stored in a polythene bottle.

3. N potassium hydroxide.

56 g. of A.R. grade potassium hydroxide were dissolved in one litre

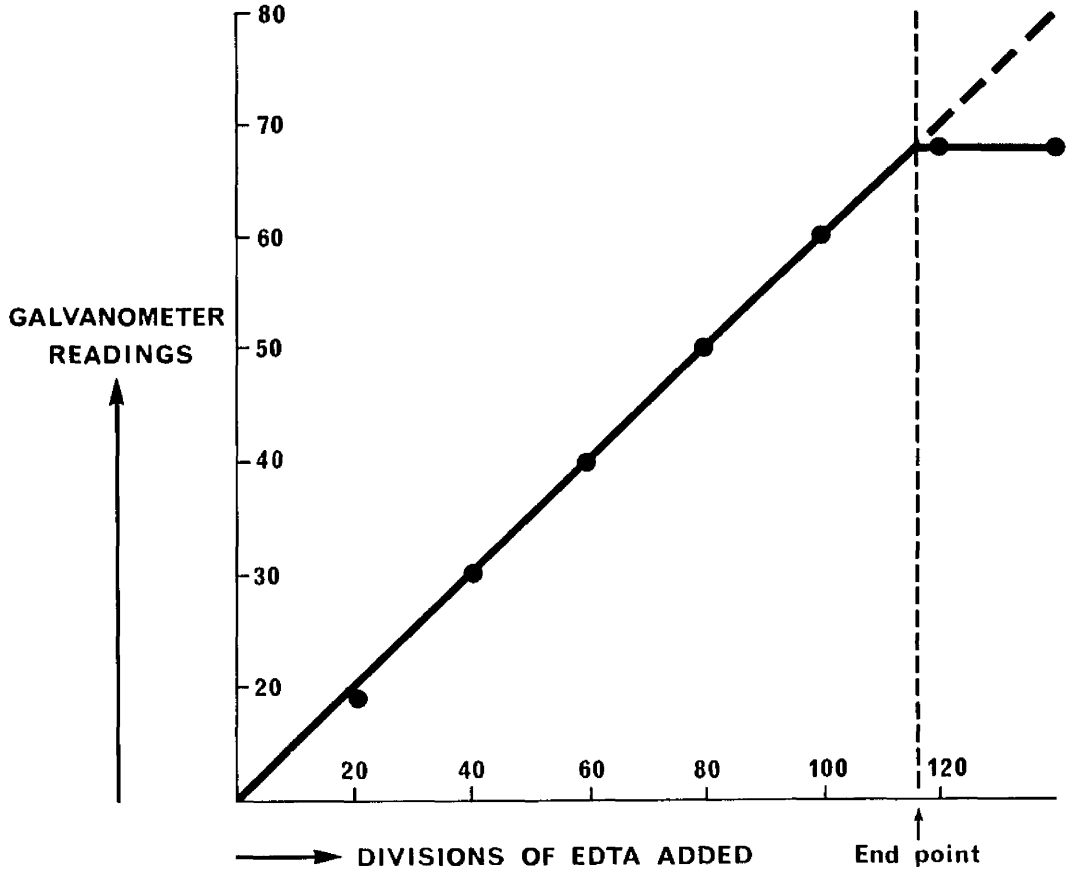

FIGURE 4. CALCIUM TITRATION WITH EGL APPARATUS.

(USING MUREXIDE AS INDICATOR).

of distilled water and adjusted to give a normal solution.

4. Calcium standard. (10 mg. per 100 ml.)

0.250 g. of pure dry calcium carbonate were dissolved in 6 ml. of N hydrochloric acid in a beaker. The beaker was placed on a boiling water bath to drive off excess hydrochloric acid, cooled, and the contents transferred quantitatively to a one litre flask, and made up to one litre with distilled water.

Method:-

2.5 ml. of murexide solution and 0.2 ml. of N potassium hydroxide were placed in a 4 ml. cuvette which was then placed on the titrator platform, the stirrer was switched on, and the sensitivity was adjusted to obtain a galvanometer reading of 70. The microburette tip was then immersed and 0.1 ml. of standard or serum specimen was added. Titration with E.D.T.A. solution was carried out adding 8 microlitres (20 divisions of the micrometer screw gauge) each time and the galvanometer readings were also noted simultaneously, until a peak was reached, indicated by no further movement on the galvanometer scale. The galvanometer readings were plotted against the divisions of E.D.T.A. solution added (Figure 4): the point of intersection of the two straight lines denoting the end point.

Modifications:-

The concentration of murexide solution was reduced to approximately 4 mg. per 100 ml. instead of the original 6 mg. per 100 ml., and the reading corresponding to this solution was usally in the range 70 to 75

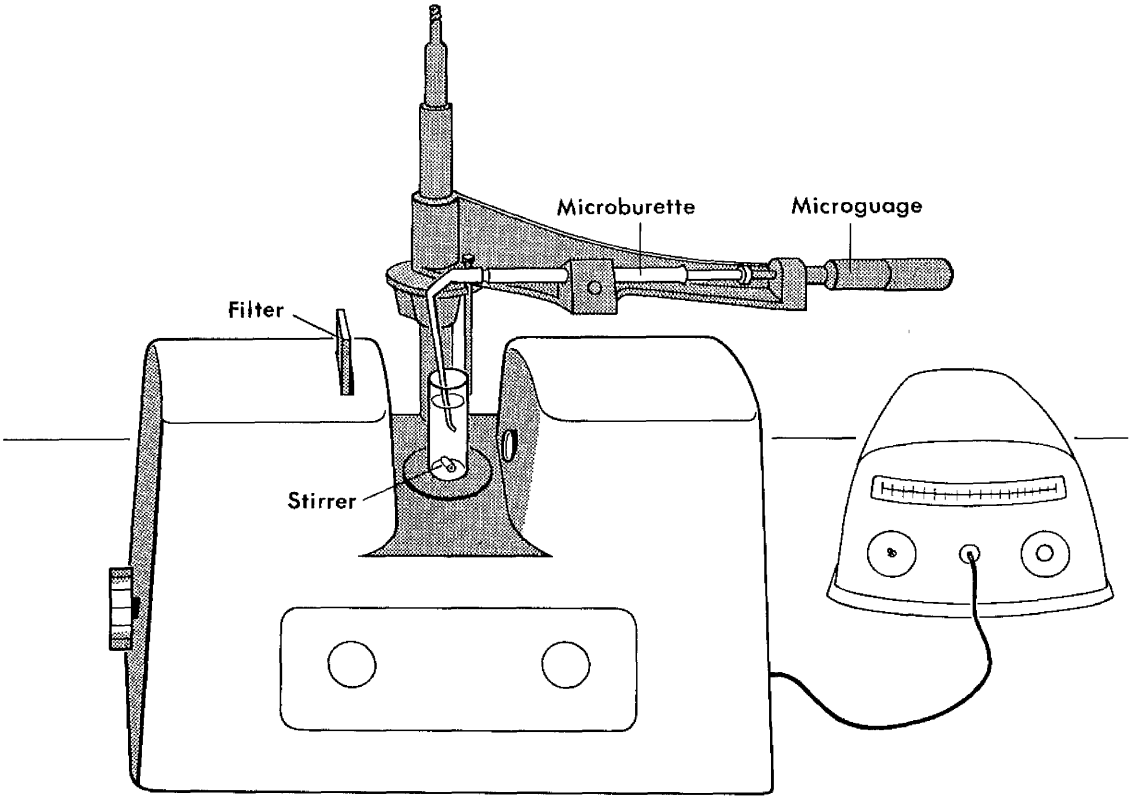

FIGURE 3. DIAGRAM OF E.E.L. PHOTOELECTRIC TITRATOR

on the galvanometer scale.

Estimation of Magnesium.

A new dye, sodium 1-azo-2-hydroxy-3-(2,4-dimethylcarboxanilido)-naphthalene-1-(2-hydroxybenzene-4-sulphonate) was found to be specific and sensitive for the determination of magnesium by Mann and Yoe (1962). In the pH range 8-11 magnesium ions cause a colour change from blue to purple due to the formation of a magnesium dye complex and the absorbance is determined at 500 mμ against a reagent blank. It has been employed by many workers for the direct determination of magnesium in serum, cerebrospinal fluid, and urine. In the present study magnesium was determined by the method of Rice and Lapara (1964) who measured the absorption of unreacted dye at 600 mμ. There is a decrease in the absorption at 600 mμ as the original blue dye becomes purple in the presence of magnesium. The difference in absorbance at 600 mμ between a "reagent" blank and an unknown measures the amount of pink magnesium dye complex formed. This absorption difference is proportional to the magnesium concentration. It is a more sensitive procedure than determining absorbance at 500 mμ against the reagent blank in the direct determination of magnesium as employed by Mann and Yoe (1962). The method was found to be eight times more sensitive than the titan yellow method.

Reagents.

1. Dye solution.

25.0 mg. of the dye, sodium 1-azo-2-hydroxy-3-(2,4-dimethyl-

carboxanilido)-naphthalene-1-(2-hydroxybenzene-4-sulphonate) (obtainable from The La Motte Chemical Products Co., Chestertown, Md., U.S.A.) was dissolved in 200 ml. of absolute ethanol in a 250 ml. volumetric flask, and was diluted to the mark with distilled water and mixed. The reagent is stored in amber bottle and is stable for two months.

2. 0.08 M Sodium borate.

30.51 g. of sodium tetraborate ($Na_2B_4O_7 . 10 H_2O$) was dissolved in 500 ml. of hot water, cooled to room temperature and diluted to one litre with distilled water.

3. Blank diluent.

Mix 44 ml. of distilled water, 8 ml. of sodium borate, and 48 ml. of absolute alcohol.

4. Unknown diluent.

Mix 50 ml. of distilled water, 10 ml. of sodium borate, and 40 ml. of absolute alcohol.

The appropriate volume of "unknown" diluent was prepared freshly each day by mixing accurately 4 volumes of the alcoholic borate solution with one volume of the dye reagent.

5. Magnesium standard solution. (10 meq. per litre.)

0.1233 g. of magnesium sulphate ($MgSO_4 . 7H_2O$) was dissolved in water and diluted to exactly 100 ml. with distilled water.

6. Working standard magnesium solution. (2 meq. per litre.)

A working standard was prepared by diluting accurately the stock standard solution 1:5 with distilled water. The solutions 2,3,4,5, and 6 are stable indefinitely.

Glassware.

All glassware for the magnesium determination was soaked in 50% nitric acid overnight, rinsed with tap water, soaked in glass distilled water overnight and then dried.

Procedure.

By means of a micropipette, 20 microlitres of serum were added to a test tube containing 5 ml. of "unknown" diluent. The test tube was closed with parafilm and the contents mixed thoroughly. Similarly, with every test a serum blank was also put up by adding 20 microlitres of serum to 5 ml. of blank diluent. With each set of unknowns, a "reagent" blank tube and a magnesium standard tube were similarly prepared by adding respectively 20 microlitres of distilled water or standard magnesium solution to 5 ml. of unknown diluent.

After about 5-10 minutes, the absorbance of each unknown was measured against a serum blank at 600 mμ.

The absorbance of the "reagent" blank and standard was measured against distilled water at 600 mμ. The colour was stable for at least 24 hours.

Calculations.

$$\frac{\text{(absorbance "reagent" blank) - (absorbance unknown)}}{\text{(absorbance "reagent" blank) - (absorbance standard)}} \times 2$$

= meq. magnesium per litre.

Other estimations.

Serum inorganic phosphate was determined by the method of

Gomori (1942), and serum proteins by the biuret method as described by Gornall, Bardawill, and David (1949).

Ultrafiltration results may be expressed in two ways: (1) As the actual concentration of calcium, magnesium or inorganic phosphate in mg. per 100 ml. in the ultrafiltrates, or (2) as the percentage of the total calcium etc., in the serum which is ultrafiltrable. The majority of previous authors have emphasised either one or the other way of expressing the results in their writing.

However, both methods of expressing ultrafiltration results are equally meaningful, and in the present study use is made of both ways and emphasis is laid on whichever is the more appropriate.

PART 2

RESULTS.

Many of the early workers determined the ultrafilterable calcium of serum without controlling the carbon dioxide tension. This was considered unimportant at the time, but more recently it has been shown by various workers that the ultrafilterable calcium alters with carbon dioxide tension.

On exposure to air, the plasma or serum loses carbon dioxide and consequently the pH rises. It is therefore necessary to control the pH during ultrafiltration. These pH changes due to the loss of carbon dioxide were prevented by ultrafiltering the serum or plasma in an atmosphere of 5% carbon dioxide (the normal alveolar tension).

Various factors which influence the ultrafilterability of calcium and magnesium were then investigated.

Effect of ultrafiltration with 5% carbon dioxide and 95% oxygen, and with air pressure, on the ultrafilterable fraction of serum calcium.

(1) Twenty five specimens of serum with calcium concentrations varying from 7.3 to 11.1 mg. per 100 ml. were ultrafiltered (1) using a mixture of 5% carbon dioxide and 95% oxygen as the pressure phase, and (2) with air as the pressure phase. The blood specimens were collected without any special precautions for the loss of carbon dioxide, and generally the pH before ultrafiltration was between 7.55 and 7.75. As the 5% carbon dioxide was not being bubbled through the serum specimens it was unlikely that the specimens were saturated with this gas and the pH under these conditions will tend towards the normal range. On the other hand no significant change in pH would be expected when ultrafiltered under air pressure. Calcium was always found in greater concentration in the ultrafiltrates

Table II Comparison of Ultrafilterable Calcium under air and 5% Carbon dioxide pressure.

case	serum total calcium	ultrafiltrate calcium mg. per 100 ml. air pressure	5% CO_2 and 95% O_2 pressure	difference
G.H.	9.9	4.4	5.8	1.4
J.L.	7.3	4.6	5.3	0.7
J.S.	10.1	5.2	5.8	0.6
M.H.	10.2	5.7	6.7	1.0
A.K.	9.7	4.7	6.1	1.4
M.R.	9.8	5.9	6.8	0.9
McK	8.7	4.8	5.5	0.7
H.C.	9.9	6.0	6.5	0.5
J.B.	10.0	4.5	5.5	1.0
V.J.	11.1	5.1	6.1	1.0
M.D.	10.1	5.5	6.5	1.0
J.McM.	7.3	4.4	5.3	0.9
E.B.	7.4	4.7	5.2	0.5
M.R.	9.2	5.9	6.8	0.9
M.McT.	10.1	5.8	7.2	1.4
F.	9.8	5.3	6.4	1.1
J.P.	9.8	5.1	5.6	0.5
S.H.	9.4	4.9	5.5	0.6
J.W.	9.2	5.4	6.6	1.2
M.McM.	9.0	5.1	5.9	0.8
J.C.	9.5	5.2	6.1	0.9
C.	7.4	4.5	5.2	0.7
A.H.	9.8	4.5	5.1	0.6
M.C.	10.3	4.6	5.6	1.0

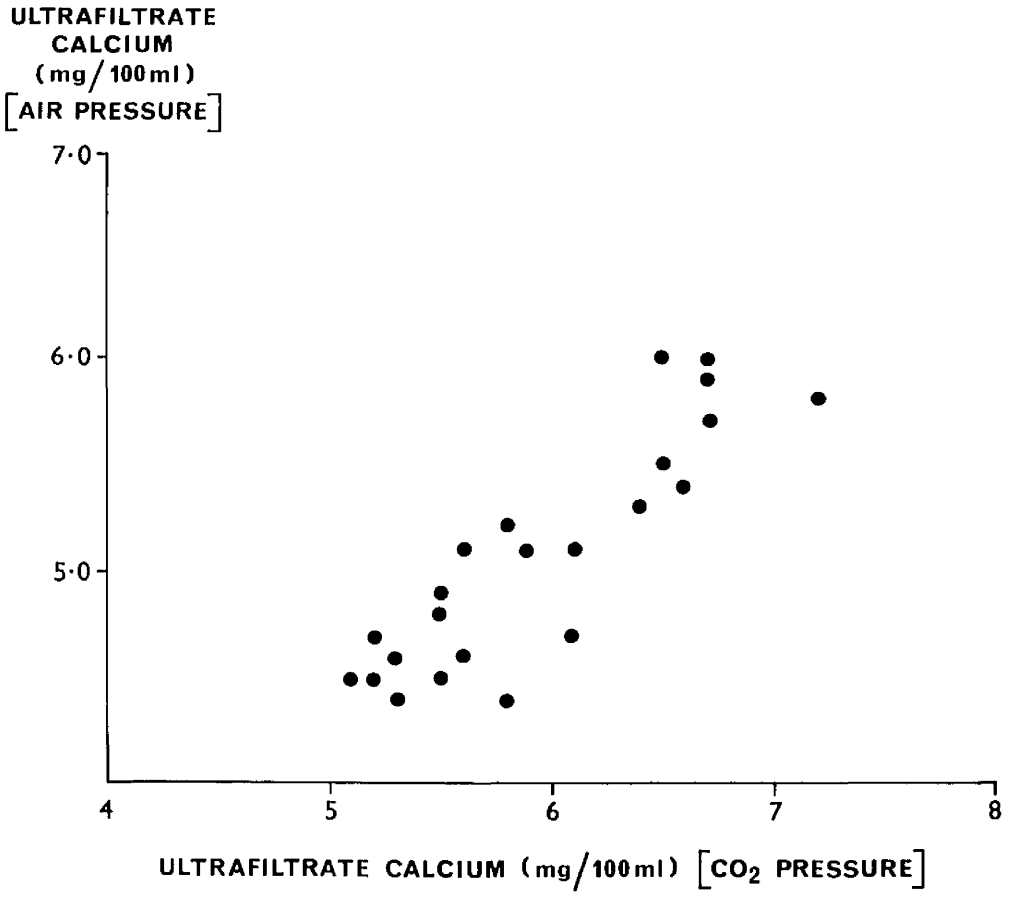

FIGURE 5. RELATION BETWEEN THE ULTRAFILTERABLE CALCIUM FILTERED UNDER 5% CARBON DIOXIDE AND 95% OXYGEN AS PRESSURE PHASE AND AIR AS THE PRESSURE PHASE.

filtered under 5% carbon dioxide and 95% oxygen pressure than in that ultrafiltered under air pressure. Results are presented in Table II. Differences between the two ranged from 0.5 to 1.4 mg. per 100 ml. The values of ultrafilterable calcium under air and 5% carbon dioxide and 95% oxygen were plotted against each other Figure 5. There was a close relationship between the two with a correlation coefficient $r = 0.89$. It appears, therefore, that there is a relationship between pH of the specimen and the ultrafilterable calcium fraction.

(2). The ultrafilterable calcium was then determined at varying serum pH values. The pH changes were produced by the addition of N hydrochloric acid. It was found that 15 microlitres of N hydrochloric acid when added to 2 ml. of serum produced a change of approximately 0.5 pH unit. Four aliquots of the same serum specimen with a normal protein concentration were taken and the pH adjusted to the values shown in Table III.

These specimens were then ultrafiltered under 5% carbon dioxide and 95% oxygen pressure. The results are presented in Table III. The ultrafilterable calcium rose from 5.9 mg. per 100 ml. at pH 7.65 to 7.0 mg. per 100 ml. at pH 6.62. Although the dilution introduced when adjusting the pH was negligible, due allowances were made when calculating the results. The fall in ultrafilterable calcium was proportional to the increase in pH in the region 6.62 to 7.65.

The ultrafilterable magnesium showed a similar slight fall with increasing serum pH while the inorganic phosphate concentration did not change with alteration of pH.

This experiment was repeated over a much narrower range of pH change.

Table III The Effect of pH on Ultrafilterable Calcium, Magnesium and Inorganic Phosphate.

Initial values:

Serum calcium = 9.3 mg. per 100 ml.

Serum magnesium = 2.2 mg. per 100 ml.

Serum inorganic phosphate = 2.8 mg. P per 100 ml.

pH of Serum	Calcium in ultrafiltrate in mg. per 100 ml.	Calcium ultrafilterable %	Magnesium in ultrafiltrate in mg. per 100 ml.	Magnesium ultrafilterable %	Inorganic Phosphate in ultrafiltrate in mg. per 100 ml.	Inorganic Phosphate ultrafilterabl %
7.65	5.9	64	1.6	72	3.2	114
7.29	6.1	65	1.6	72	3.2	114
7.14	6.4	69	1.65	75	3.2	114
6.62	7.0	75	1.8	82	3.2	114

The specimens were adjusted to cover the initial range of pH 7.62 to 7.52. The effect of filtering under pressure with 5% carbon dioxide and 95% oxygen is to lower the pH by approximately 0.3 unit, thereby bringing the pH much nearer the normal physiological range. Results are set out in Table IV. The change in the percentages of ultrafilterable calcium and magnesium over this narrow pH range are too small to be detected.

The effect of varying ultrafiltration pressure on ultrafilterable calcium.

Two millilitres aliquots of the same serum specimen were ultrafiltered at different pressures, varying from 9 to 18 lbs. per sq. inch until one millilitre of ultrafiltrate was obtained. Calcium, magnesium and inorganic phosphate were determined in each. Results are presented in Table V. No change in either ultrafilterable calcium, magnesium, or inorganic phosphate was observed in these ultrafiltrates obtained from different ultrafiltration pressures. This method is not a true reflection of physiological conditions in terms of filtration pressure, as the physiological range of capillary pressure is far lower, i.e., 15 to 25 mm. mercury. These results indicate that the increase in ultrafiltration pressure *in vitro* does not affect the partition of calcium and magnesium. However, increased ultrafiltration pressure greatly reduced the time for adequate ultrafiltration. The time required to obtained an equal amount of ultrafiltrate from the same volume was cut down from 2½ hours at a pressure of 9 lbs. per sq. inch to only one hour at a pressure of 18 lbs. per sq. inch.

Table IV Ultrafiltration of serum over a narrow range of pH.

Original pH of serum = 7.62

Serum calcium = 10.0 mg. per 100 ml.

Serum magnesium = 2.3 mg. per 100 ml.

No.	Initial pH.	Calcium ultrafiltrate mg. per 100 ml.	%	Magnesium ultrafiltrate mg. per 100 ml.	%
1	7.62	5.8	58	1.8	78
2	7.58	5.8	58	1.8	78
3	7.55	5.7	57	1.8	78
4	7.52	5.8	58	1.7	74

Table V Effect of varying pressure on ultrafilterable Calcium, Magnesium and Inorganic phosphate.

1.0 ml. of ultrafiltrate was obtained from 2.0 ml. of serum in each case.

No.	Pressure in lb. per sq. inch	Time in minutes	Ultrafiltrate concentrations in mg. per 100 ml.		
			Calcium	Magnesium	Inorganic phosphate
1	9	180	5.5	2.30	3.4
2	12	140	5.6	2.25	3.3
3	15	90	5.5	2.30	3.4
4	18	60	5.7	2.30	3.5

Effect of storage on Ultrafilterability of Calcium, Magnesium and Inorganic phosphate.

Although all the experiments in this series were carried out immediately after collecting the blood specimens, the effect of storing the serum specimens on ultrafilterable calcium, magnesium and inorganic phosphate was investigated. Ultrafiltration was carried out under 5% carbon dioxide and 95% oxygen pressure, the serum specimens were stored in a refrigerator at 4°C for up to two days. A 2 ml. aliquot of serum was set up for ultrafiltration within half an hour of withdrawing blood, and the rest of the serum was separated from the cells and stored at 4°C. A further 2 ml. of serum was ultrafiltered at the same time the next day and similarly the following day. The ultrafiltrates collected from the 1st. and 2nd. specimens were also stored at 4°C and all the estimations were carried out together on the second day. Results are presented in Table VI. No appreciable difference could be demonstrated in the concentration of calcium, magnesium or inorganic phosphate in the three ultrafiltrates.

Similar observations in respect of calcium were made by Hopkins, et. al. (1952). Toribara, et. al. (1957) and Rose (1957). These observations lead to the conclusion that when ultrafiltration is carried out under 5% carbon dioxide and 95% oxygen there is no necessity for special precautions in the collection of blood specimens, in order to avoid any loss of carbon dioxide.

Table VI Effect of storing serum on ultrafilterable Calcium, Magnesium and Inorganic phosphate.

All values in mg. per 100 ml.

	Case I			Case II			Case III		
	Ca.	Mg.	PO_4.	Ca.	Mg.	PO_4.	Ca.	Mg.	PO_4.
Serum values	10.1	2.10	2.7	9.3	2.10	3.2	10.3	1.95	3.6
ultrafiltered (immediately)	6.0	1.50	2.9	5.7	1.60	3.5	6.0	1.40	3.9
ultrafiltered (after 24 hours)	6.0	1.50	2.8	5.7	1.50	3.5	6.1	1.40	3.9
ultrafiltered (after 48 hours)	5.9	1.50	2.8	5.7	1.50	3.5	6.1	1.40	3.9

The effect of the extent of Ultrafiltration on ultrafilterable Calcium, Magnesium and Inorganic phosphate.

The following experiment was performed to find how the extent to which the ultrafiltration process is carried out affects the partition of calcium and magnesium. From separate 2 ml. aliquots of the same serum specimen, 0.5, 0.8 and 1.1 ml of ultrafiltrates were obtained and calcium, magnesium and inorganic phosphate were determined on all three ultrafiltrates. No significant difference in the concentration of these constituents could be detected. These observations show that as the volume of ultrafiltrate, and the protein concentration of the residue, increase during ultrafiltration, the calcium-protein relationship remains unchanged.

Another experiment carried out in this connection was as follows:- A 6.0 ml. of serum specimen was ultrafiltered and serial 0.5 ml. sample of ultrafiltrate were collected. Again calcium, magnesium and inorganic phosphate concentrations were determined on each of the six specimens. Results are shown in Table VII. There was no variation in the concentration of these constituents during ultrafiltration due to the increasing protein concentration of the residue.

The effect of increasing the calcium concentration on Ultrafilterable Calcium, Magnesium, and Inorganic phosphate in vitro.

Molean and Hastings (1935) expressed the relationship between the ionised and protein-bound fractions of calcium in normal serum as:-

$$\frac{(Ca^{++})\ (Protein^{---})}{(Ca\ Proteinate)} = K$$

Table VII. Serial ultrafiltration of serum.

Volume of serum taken = 6.0 ml.
Serum calcium = 9.86 mg. per 100 ml.
Serum magnesium = 2.1 mg. per 100 ml.
Serum inorganic phosphate = 3.2 mg. P per 100 ml.

Ultrafiltrates	First 0.50 ml.	Second 0.50 ml.	Third 0.50 ml.	Fourth 0.50 ml.	Fifth 0.50 ml.	Sixth 0.50 ml.
Ultrafiltrate calcium in mg. per 100 ml.	5.50	5.46	5.46	5.50	5.46	5.40
Ultrafiltrate magnesium in mg. per 100 ml.	1.55	1.55	1.55	1.50	1.50	1.55
Ultrafiltrate inorganic phosphorus in mg. P per 100 ml.	3.4	3.4	3.5	3.4	3.5	3.5

It follows from this equation that extra calcium added to serum should also obey this relationship and distribute itself accordingly. To check this, the calcium concentration of a serum specimen was increased *in vitro* from an original concentration of 10.1 mg. per 100 ml. to 14.9 and 19.6 mg. per 100 ml. by the addition of calcium in the form of a concentrated solution of calcium chloride. These three specimens after standing for one hour were then ultrafiltered using 5% carbon dioxide and 95% oxygen under similar conditions. Calcium, magnesium and inorganic phosphate were determined in the ultrafiltrates and the results are presented in Table VIII. 6.4 mg. per 100 ml. (63%) of the total calcium was ultrafilterable before any addition of calcium. According to the mass action effect, the 63% of the added calcium should remain in the ionic state. Calculated on this basis, the calcium concentration in the ultrafiltrates from the second and third specimens should be 9.5 and 12.4 mg. per 100 ml. respectively. However, this was not found to be the case; the calcium concentrations of these ultrafiltrates were 8.8 and 11.3 mg. per 100 ml. which showed a gradual decrease in the ultrafilterability of calcium from 63% in the original serum to 59% and to 57% in the second and third specimens.

The other interesting feature which may be noted was in the behaviour of inorganic phosphate under these conditions. The concentration of inorganic phosphate in the ultrafiltrate from the untreated serum was 5.1 mg. per 100 ml. against 4.1 mg. per 100 ml. in the serum itself (124% ultrafilterable). However, when the serum calcium concentration was raised to 14.9 mg. per 100 ml. there occurred a sharp drop in its concentration in the ultrafiltrate to 3.7 mg. per 100 ml. only 90% being ultrafilterable,

Table VIII The Effect of Increased Serum Calcium on Ultrafilterable Calcium, Inorganic Phosphate and Magnesium.

	Calcium mg. per 100 ml.		Inorganic Phosphate mg. per 100 ml.			Magnesium mg. per 100 ml.		
Serum	ultrafiltrate	% ultra-filterable	Serum	ultra filtrate	% ultra-filterable	Serum	ultra filtrate	% ultra-filterable
10.1	6.4	63	4.1	5.1	124	2.10	1.60	76
15.0	8.8	59	4.1	3.7	90	2.10	1.70	81
19.6	11.3	58	4.1	2.6	63	2.10	1.75	83

and when the serum calcium concentration was raised to 19.6 mg. per 100 ml. there occurred a still further drop in its concentration in the ultrafiltrate to 2.6 mg. per 100 ml. only 63% being ultrafilterable.

This decreased ultrafilterability of inorganic phosphate with the increasing serum calcium concentration suggested the formation of a complex bound to proteins, or an insoluble complex precipitating from the serum. The latter possibility was excluded as follows:-

Calcium and inorganic phosphate concentrations were determined in a serum specimen. Calcium was then added to raise the serum calcium concentration above 15.0 mg. per 100 ml. and the specimen was then centrifuged at high speed. No sediment could be observed after centrifugation, and no difference could be detected in calcium and phosphate concentrations. It would seem most likely, therefore, that the complex was bound to the proteins.

It may be noted that concentration of magnesium increased slightly in the ultrafiltrates from original 1.60 mg. per 100 ml. to 1.70 and 1.75 mg. per 100 ml. when the serum calcium was increased. This probably indicated that a gradual decrease in the protein binding of magnesium occurred to accommodate the increased amount of calcium.

Ultrafilterable Calcium, Magnesium, and Inorganic Phosphate in the Normal and in Pathological Conditions.

Group I. Normal Controls.

Sera from twenty normal healthy donors were subjected to ultrafiltration using 5% carbon dioxide and oxygen. The data on calcium, magnesium and inorganic phosphate is summarised in Table IX, the values being presented both in actual concentrations in the ultrafiltrates and as a percentage of total serum concentration. The serum calcium concentration ranged from 9.8 to 10.6 mg. per 100 ml. with a mean value of 10.2 mg. per 100 ml. and S.D. ±0.20. The ultrafilterable calcium ranged from 5.5 to 6.3 mg. per 100 ml. with a mean value of 5.9 mg. per 100 ml. and S.D. ±0.25. When expressed as percentage of the total serum calcium concentration, the range was 55 to 61%, mean 58%. The protein bound calcium fraction varied from 4.0 to 4.6 mg. per 100 ml.

The serum inorganic phosphate varied from 2.5 to 4.2 mg. per 100 ml. and the ultrafiltrate concentration from 2.8 to 4.9 mg. per 100 ml. It was always found in greater concentration in the ultrafiltrate than the serum itself and represented 105 to 120% ultrafilterability with a mean value of 110% for 20 specimens.

The serum magnesium concentration ranged from 1.8 to 2.4 mg. per 100 ml. with a mean of 2.0 mg. per 100 ml. and S.D. ±0.18. The ultrafilterable concentration ranged from 1.3 to 1.7 mg. per 100 ml. with a mean 1.5 mg. per 100 ml. and S.D. ±0.11. When expressed as a percentage of total serum magnesium it ranged from 70 to 79% with a mean of 74%.

Table IX Ultrafiltration of Serum from Normal Persons.

	CALCIUM		MAGNESIUM		INORGANIC PHOSPHATE		PROTEIN
No.	mg. per 100 ml.	% ultra filterable	mg. per 100 ml.	% ultra-filterable	mg. per 100 ml.	% ultra filterable	Alb./Glob. g. per 100 ml.
1	9.8/5.5*	56	2.0/1.5*	75	4.1/4.6*	112	4.6/2.4
2	9.9/5.8	58	1.9/1.4	74	3.9/4.3	110	5.1/2.4
3	10.0/5.6	56	1.9/1.5	79	3.7/3.9	106	5.2/2.8
4	10.1/5.5	55	1.9/1.4	74	4.2/4.6	110	5.3/2.3
5	10.1/5.8	57	2.3/1.6	70	2.5/2.8	112	5.5/1.9
6	10.1/5.6	55	2.0/1.4	70	4.0/4.2	105	6.1/2.4
7	10.1/6.1	60	2.1/1.6	76	3.0/3.3	110	5.3/2.1
8	10.1/6.1	60	2.1/1.5	71	3.7/4.0	108	5.6/2.3
9	10.2/5.6	55	1.9/1.4	74	4.2/4.9	116	5.3/2.2
10	10.2/5.9	58	1.8/1.4	78	4.1/4.4	107	5.3/2.3
11	10.2/6.1	60	2.0/1.5	75	3.7/3.9	106	6.1/1.8
12	10.2/5.8	57	2.2/1.6	73	3.8/4.0	105	4.8/2.8
13	10.2/6.1	60	1.8/1.4	78	3.4/3.6	106	5.1/2.1
14	10.2/5.8	57	2.1/1.6	76	2.8/3.1	111	4.8/2.6
15	10.3/6.3	61	1.9/1.4	74	4.0/4.8	120	5.5/1.9
16	10.3/5.9	57	1.8/1.3	72	3.4/4.0	117	4.4/2.3
17	10.4/5.9	57	1.8/1.4	78	3.3/3.6	109	5.3/2.4
18	10.4/6.2	59	2.4/1.7	71	3.2/3.4	106	4.6/2.3
19	10.6/6.0	57	2.0/1.5	75	4.0/4.2	105	5.7/1.7
20	10.6/6.3	59	2.3/1.7	74	3.3/3.6	109	5.5/2.0
Means	10.2/5.9	58	2.0/1.5	74	3.6/3.9	109	5.3/2.2

*Results expressed as total serum concentration/ultrafiltrate concentration.

Group II. Patients with primary Hyperparathyroidism.

Pre-operative sera were obtained for ultrafiltration from 12 patients with hyperparathyroidism. The experiments were repeated in 11 of these cases three days after the parathyroid adenomas were surgically removed. The results of ultrafiltrations along with other biochemical data are presented in tables X, XI, and XII. Primary hyperparathyroidism was diagnosed in all of the 12 patients, and in 11 of these cases the condition was shown to be due to a single adenoma at histology. There was evidence of carcinomatous changes of the parathyroid tumor in the case of J.R. who later died. Severe bone involvement was demonstrated radiologically and was accompanied by raised serum alkaline phosphatase activity in three of these patients (J.R., S.L., and M.T.). There was some X-ray evidence of bone involvement in the case of H.F. althought the serum alkaline phosphatase activity was essentially normal.

Renal stones were demonstrated in six of these cases (W.R., F.C.,T.A., H.C.,C.Mc., and S.B.). There was no evidence of either bone disease or renal stones in case of J.W. and the diagnosis was made primarily on the serum and ultrafilterable calcium estimations.

Serum and ultrafilterable calcium.

The serum and ultrafilterable calcium were determined repeatedly before operation and the highest calcium results obtained are recorded in Table X. The total serum calcium concentration ranged from 10.0 to 17.0 mg. per 100 ml. with a mean value of 12.4 mg. per 100 ml. The ultrafiltrate calcium ranged from 6.9 to 10.9 mg. per 100 ml. with a mean of 8.0 mg. per 100 ml.; when expressed as percentage ultrafilterable calcium the range was from 61 to 69%.

Table X Ultrafiltration of Serum from Patients with Hyperparathyroidism.

CALCIUM

	Pre-operative			Post-operative		
Case.	mg./100 ml.	% ultra-filterable	serum alb./glob.**	mg./100 ml.	% ultra-filterable	alb./glob.**
J.R.	17.0/10.9*	64	4.9/2.9	8.5/6.6*	77	3.5/2.2
S.L.	14.2/9.6	67	4.7/1.6	9.5/5.9	62	4.6/1.6
H.F.	12.9/8.2	64	4.2/1.8	9.3/4.9	53	4.0/1.9
J.W.	12.8/7.8	61	4.5/2.0	9.2/5.6	61	4.6/1.8
W.R.	12.3/7.9	64	5.2/1.7	9.8/5.7	58	4.9/1.7
F.G.	12.2/7.6	62	5.3/2.0	8.4/4.5	53	5.1/2.0
H.C.	12.1/7.9	65	5.5/2.2	9.9/5.9	59	5.0/1.8
C.Mc.	12.0/7.7	64	5.3/2.1	7.8/4.4	57	4.8/2.0
S.C.	11.6/7.4	64	4.8/1.9	8.2/4.8	58	4.7/1.9
S.B.	11.3/7.0	62	4.7/2.1	8.6/5.3	61	4.6/1.9
T.A.	10.5/7.0	67	5.0/3.0	6.9/4.0	61	5.0/2.9
M.T.	10.0/6.9	69	3.8/2.0	- -	-	- -
Means	12.4/8.0	64	4.8/2.1	8.7/5.2	60	4.6/2.0

*Results expressed as total serum/ultrafiltrate calcium.

**Values in g. per 100 ml.

The ultrafilterable calcium concentration in each case was well above the upper normal limit of 6.3 mg. per 100 ml., and again in terms of percentage ultrafilterable calcium, it was above the normal limits in each case except J.W. where it was just on the border-line, i.e. 61%. These later findings are not in agreement with the observations of Hopkins, et. al. (1953) and Terepka, et. al. (1958). These workers could not demonstrate any increase in the ultrafilterable calcium in terms of percentage of total serum calcium in this condition.

It is also interesting to note that, in two patients (T.A. and M.T.) with normal serum calcium concentration of 10.5 and 10.0 mg. per 100 ml., the concentration of calcium in the ultrafiltrates was elevated to 7.0 and 6.9 mg. per 100 ml., or 67 and 69% ultrafilterability. Case M.T. had a slightly low serum albumin concentration due presumably to renal involvement as her blood urea was 169 mg. per 100 ml.

Post-operative results.

The tests were repeated three days after the removal of the adenoma in 11 of the 12 cases, except case H.G. where the serum was analysed seven days post operatively. After the surgical removal of the parathyroid adenomas, it was found that both total and ultrafilterable calcium fell (Table X). The decrease in ultrafilterable calcium was not in direct proportion to the total calcium. In case J.R., the percentage ultrafilterable calcium showed an increase to 77% post-operatively, although both total and ultrafilterable calcium fell. A slightly low ultrafilterable calcium (53%) was found in the cases of H.F. and F.C., and results in all

other cases were within normal range.

Case S.L. showed hypocalcaemia post-operatively and was followed up until recovery (Figure 6). The total and ultrafilterable calcium fell gradually, but the percentage ultrafilterable calcium remained elevated. She had persistent tetany and was given repeated calcium transfusions which produced only temporary benefits. Eventually she responded to a high dosage of vitamin D_2 her serum and ultrafilterable calcium gradually increased and she recovered completely after about four months.

Serum and ultrafilterable Inorganic phosphate.

The majority of the patients had pre-operative serum phosphate concentrations at, or below, the lower limits of normal range, except in one case (M.T.) who showed a slight elevation to 5.4 mg. per 100 ml. probably due to renal involvement. The mean serum phosphate concentration for the 12 patients was 2.6 mg. per 100 ml. (Table XI).

Ultrafilterable inorganic phosphate ranged between 100 and 115% with a mean of 108%, this being within the normal limits. In none of these cases was the inorganic phosphate found in less concentration than in the original serum. Hence the possibility of the existence of any calcium-phosphate-protein complex, as was observed by Hopkins, et. al. (1953) in one of their hyperparathyroid cases, was ruled out.

A very slight increase in the serum inorganic phosphate was observed three days after the surgical removal of the tumour. It was slightly decreased in the case of J.R. In case H.C., where the specimen was obtained seven days post-operatively, there was an appreciable increase in

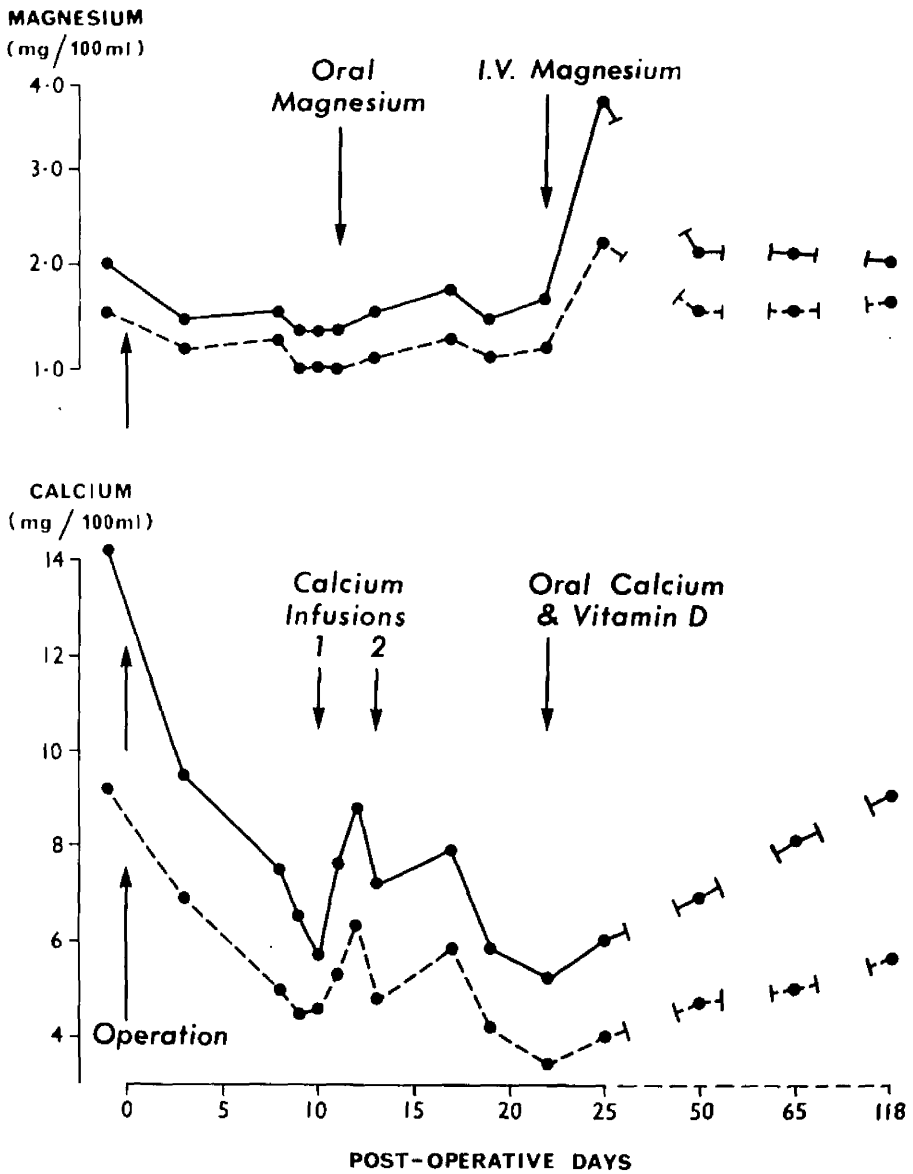

FIGURE 6. SERUM AND ULTRAFILTRATE CALCIUM AND MAGNESIUM

SERUM AND ULTRAFILTRATE CALCIUM AND MAGNESIUM

CONCENTRATIONS AFTER REMOVAL OF A PARATHYROID TUMOUR FROM A PATIENT WITH BONE INVOLVEMENT. (PATIENT S.L.).

Table XI Ultrafiltration of Serum from Patients with Hyperparathyroidism.

INORGANIC PHOSPHATE

Case.	Pre-Operative. mg. per 100 ml.	Pre-Operative. % ultrafilterable	Post-Operative. mg. per 100 ml.	Post-Operative. % ultrafilterable
J.R.	1.9/2.0*	105	1.7/1.8*	106
S.L.	2.2/2.2	100	2.4/2.7	112
H.F.	1.5/1.7	113	2.4/2.6	108
J.W.	2.7/2.9	108	3.0/3.2	106
W.R.	2.4/2.7	112	2.4/2.6	108
F.C.	2.5/2.6	104	2.9/3.1	107
H.C.	2.3/2.4	104	4.3/4.6	107
C.Mc.	2.0/2.3	115	2.4/2.7	112
S.C.	2.7/2.7	100	2.9/3.2	110
S.B.	2.3/2.3	100	3.1/3.4	109
T.A.	3.2/3.8	118	3.4/3.8	111
M.T.	5.4/5.9	108	- -	-
Means	2.6/2.8	107	2.8/3.1	108

*Results expressed as total serum/ultrafilterable inorganic phosphate.

serum inorganic phosphate. The ultrafilterability pattern remained similar to the pre-operative results.

Serum and ultrafilterable Magnesium.

The pre-operative serum magnesium concentrations were normal in these cases and ranged from 1.8 to 2.4 mg. per 100 ml. The ultrafilterable magnesium was also within normal limits with the exception of two cases (T.A. and S.C.) where it was decreased slightly. When expressed in percentage of the total magnesium all the values fell within the normal range except in the case of S.C. where this value was also decreased.

No significant change could be demonstrated in the concentration of either serum or ultrafiltrate magnesium after the surgical removal of parathyroid tumour, except in the case of S.L. where a drop occurred in both serum and ultrafilterable magnesium (Table XII). She was followed up till recovery and results are shown in Figure 6. This magnesium deficiency was associated with mental confusion, but she responded well to magnesium supplements.

Apart from this case (S.L.) the results indicate that a parathyroid adenoma has no effect on the serum or ultrafiltorable magnesium.

Group III. Cases of Multiple Myeloma.

The sera of eight patients with myelomatosis was ultrafiltered under 5% carbon dioxide and 95% oxygen, and the analysis of the ultrafiltrates are presented in Table XIII. Hypercalcaemia (serum calcium greater than 10.8 mg. per 100 ml.) was present in four cases.

Table XII Ultrafiltration of serum from patients with hyperparathyroidism.

MAGNESIUM

Case	Pre-Operative mg. per 100 ml.	Pre-Operative % ultrafilterable	Post-Operative mg. per 100 ml.	Post-Operative % ultrafilterable
J.R.	2.1/1.7*	81	2.2/1.6*	73
S.L.	2.1/1.6	76	1.5/1.1	73
E.F.	2.3/1.6	70	2.2/1.7	77
J.W.	1.9/1.5	79	1.8/1.4	77
W.R.	2.0/1.6	80	2.1/1.6	76
F.C.	2.2/1.5	70	2.0/1.6	80
H.C.	2.2/1.6	72	2.4/1.3	54
C.Mc.	2.1/1.6	76	1.9/1.5	79
S.C.	1.8/1.1	61	2.0/1.5	75
S.B.	2.2/1.6	72	2.0/1.6	80
T.A.	1.8/1.25	75	2.1/1.7	81
M.T.	2.4/1.8	75	- -	-
Means	2.1/1.5	74	2.0/1.5	75

*Results expressed as total serum/ultrafilterable magnesium.

Hyperproteinaemia was present in six of the cases, including all the cases with hypercalcaemia. The percentage of ultrafilterable calcium was extremely variable, being normal in two cases, raised in three cases, and lowered in three cases. There is no relationship of any kind between the ultrafilterable calcium, whether expressed as a concentration or as a percentage of the total, and the serum protein, serum albumin, or serum globulin.

The most striking results were the decreases in the ultrafilterability of inorganic phosphate in the cases E.O. and J.M. in which the percentage of inorganic phosphate in the filtrates was only about half of the expected values. These happen to be the two cases with the highest serum calcium concentrations, and with the lowest percentages of ultrafilterable calcium. This again suggests the possible occurrence of a calcium-phosphate-protein complex (see P. 92).

The serum magnesium concentration is sub-normal in five of the eight cases. The percentage which was ultrafilterable was normal except in one case (J.J.) where the value was 92% : three days later, the percentage rose to 100%.

Group IV. Hypercalcaemia due to miscellaneous conditions.

Sera from six patients with hypercalcaemia due to causes other than hyperparathyroidism and multiple myeloma were examined; the clinical conditions in these cases were as follows:-

J.R. had steatorrhoea for many years while M.K. developed hypoparathyroidism after total thyroidectomy: both had been on high doses

Table XIII Ultrafiltration of Serum from Patients with Multiple Myeloma.

Case.	CALCIUM mg. per 100 ml.	CALCIUM % ultra-filterable	MAGNESIUM mg. per 100 ml.	MAGNESIUM % ultra-filterable	INORGANIC PHOSPHATE mg. per 100 ml.	INORGANIC PHOSPHATE % ultra-filterable	PROTEIN Total/Alb./Glob. g. per 100 ml.
C.K.	9.2/5.6*	61	1.8/1.4*	77	4.6/4.6*	100	7.1/5.1/2.0
W.M.	9.6/7.3	76	2.3/1.8	78	3.4/3.7	108	12.2/3.0/9.2
A.K.	10.1/5.9	58	2.3/1.6	70	3.8/4.0	105	7.6/4.7/2.9
J.J.	10.4/7.1	68	1.4/1.3	92	3.4/3.7	108	10.9/2.3/8.6
C.F.	11.3/5.6	50	1.7/1.2	70	2.6/2.8	107	11.4/4.0/7.4
M.M.	13.5/8.8	65	1.5/1.0	66	3.7/4.2	113	10.8/3.8/7.0
E.O.	14.3/6.2	43	1.5/1.1	73	3.0/1.7	57	9.5/3.9/5.6
J.M.	15.0/7.2	48	1.5/1.1	73	3.9/2.0	51	13.4/3.9/9.5

*Results expressed as total serum concentration/ultrafiltrate concentration.

of vitamin D along with calcium supplements. The hypercalcaemia can be regarded due to hypervitaminosis D in these cases. M.W. has severe osteoporosis secondary to rheumatoid arthritis. E.E. had acute paralysis with sensory loss and later developed urinary retention and was immobilised for some time. R.W. was initially admitted for jaundice but on laparotomy was found to had carcinoma of gallbladder with secondaries. He died later and at autopsy was shown to have normal parathyroids. No cause for the hypercalcaemia could be ascertained in the case of J.S. An exploration of the neck was carried out without any positive findings. His complaints included tiredness of two years duration and aching discomfort in both legs, below the knees.

The results of ultrafiltration are presented in Table XIV. In spite of the elevated serum calcium, the ultrafilterable calcium was decreased to 5.0 and 5.3 mg. per 100 ml. in cases J.R. and J.S. and this was below the lower normal limit of 5.5 mg. per 100 ml. and represented only 43 and 44% ultrafilterability. In the cases of M.W. and M.K., although the ultrafilterable calcium concentration was within normal limits, when expressed as a percentage of the total calcium it was decreased to 45 and 42%. The ultrafilterable calcium was slightly elevated to 6.8 and 7.0 mg. per 100 ml. in E.E. and R.W. but again the percentage ultrafilterability was decreased.

The most interesting feature of these cases was the decreased ultrafilterability of the inorganic phosphate. The concentration of inorganic phosphate in the ultrafiltrates was always less than that found in the serum. The serum inorganic phosphate was elevated in two instances

Table XIV. Ultrafiltration of Serum from Patients with Hypercalcaemia due to Miscellaneous Causes.

	CALCIUM		MAGNESIUM		INORGANIC PHOSPHATE		PROTEIN
Case.	mg. per 100 ml.	% ultra-filterable	mg. per 100 ml.	% ultra-filterable	mg. per 100 ml.	% ultra-filterable	Alb./Glob. g. per 100 ml.
J.R.	11.6/5.0*	43	2.2/1.3*	59	4.8/3.7*	77	4.9/2.4
M.W.	12.8/5.8	45	2.3/1.8	78	5.2/4.0	77	4.8/2.1
E.E.	12.9/6.8	52	2.4/1.7	73	3.7/2.7	73	5.1/2.8
R.W.	13.0/7.0	54	2.1/1.5	72	2.4/1.5	62	3.4/1.9
M.K.	13.2/5.5	42	2.0/1.5	75	3.7/2.7	73	4.9/2.2
J.S.	13.2/5.3	44	2.8/2.1	75	3.6/2.5	66	5.1/2.3

*Results expressed as total serum concentration/ultrafiltrate concentration.

and within the normal range in the others, ranging from 2.4 to 5.2 mg. per 100 ml. The ultrafiltrate phosphate ranged from 1.5 to 4.0 mg. per 100 ml. and the percentage ultrafilterable ranged from 62 to 77% compared with 105 to 120% in normals. This decreased ultrafilterability of calcium and inorganic phosphate again suggested the existence of calcium-phosphate-protein complex. These results are similar to those observed *in vitro* by increase of serum calcium (see Table VII).

The serum magnesium concentration was within the normal limits in all with the exception of J.S., where this was also slightly raised. The percentage ultrafilterable magnesium was also normal except in J.R. where there was a decrease to 59%. J.S. had increased total and ultrafiltrate magnesium but the percentage ultrafilterable was in the normal limits.

The effect of acutely produced Hypercalcaemia by the infusion of Calcium gluconate on ultrafilterble Calcium, Magnesium, and Inorganic phosphate.

Since the clinical conditions so far studied represent, for the most part, chronic alterations in calcium and perhaps protein metabolism, it therefore seemed of interest to compared them with an *in vivo* hypercalcaemia induced by the administration of calcium. One thousand and ten mg. of calcium (14 mg. per kg. body weight) in the form of calcium gluconate in normal saline was infused intravenously in a normal human subject over a period of four hours.

A control specimen before infusion, a specimen taken 30 minutes after commencing infusion, and four subsequent hourly specimens were taken.

A futher specimen was obtained approximately 24 hours after infusion. The results of ultrafiltration are presented in Table XV. The total serum calcium was increased from 10.5 to a peak of 13.1 mg. per 100 ml. during the infusion, and began to fall after the infusion was stopped. The serum calcium had returned to the control value by the following morning. It will be seen that in spite of the increase in total serum calcium, a slight gradual decrease in the ultrafilterable calcium occurred. It decreased from the control concentration of 6.0 mg. per 100 ml. to 5.1 mg. per 100 ml. when the serum calcium concentration had reached the peak of 13.1 mg. per 100 ml., which represented a decrease in percentage ultrafiltorability of calcium from 57to 39%. After the infusion of calcium was stopped, the serum total calcium concentration began to fall and the ultrafilterable calcium gradually increased and by the following morning both the total and ultrafiltorable calcium were near to the control values. It would appear from this that all of the added calcium was being bound to the serum proteins, although there was, if anything, a slight fall in the protein concentration from initial value of 7.1 g. per 100 ml. to 6.5 g. per 100 ml. when the serum total calcium reached the peak of 13.1 mg. per 100 ml. The serum protein concentration was back to the control value by the following morning.

A gradual and persistent rise in serum inorganic phosphate was also observed. It increased from the control concentration of 3.0 mg. per 100 ml. to a peak of 5.9 mg. per 100 ml., this occurred one hour after the highest recorded calcium value, and then it gradually decreased. However, it was still slightly elevated above the control value the following

morning. The concentration of inorganic phosphate in the ultrafiltrates also increased, in the normal percentage ultrafilterable pattern, with the increasing serum concentration, until the serum calcium reached a value of 11.5 mg. per 100 ml. However, the percentage ultrafilterable inorganic phosphate dropped dramatically after this, and remained low in all the subsequent specimens, and was just within the normal limit on the following morning. These results again suggested the existence of a calcium-phosphate-protein complex. There was no evidence of this complex being formed when the serum calcium concentration was below 12.0 mg. per 100 ml.

Serum magnesium gradually decreased from the control concentration of 2.0 mg. per 100 ml. to 1.7 mg. per 100 ml., when the serum calcium had attained the maximal value of 13.1 mg. per 100 ml., and then gradually increased with the decreasing serum calcium after the stoppage of calcium infusion. It was again back to the control value by the following morning. The ultrafilterable magnesium also decreased from 1.6 mg. per 100 ml. to 1.05 mg. per 100 ml. when the serum calcium had reached the peak value. Again, the decrease in ultrafilterable magnesium was not in proportion to the decrease in the total serum magnesium and hence there was a fall in percentage ultrafilterability of magnesium from 80 to 62%. The decrease in ultrafilterable magnesium continued in spite of increasing serum magnesium and decreasing serum calcium after the stoppage of calcium infusion. While the serum magnesium increased from 1.7 to 1.8 mg. per 100 ml. in the two subsequent specimens the ultrafiltrate magnesium dropped from 1.05 to 1.0 mg. per 100 ml., and hence a further drop to 56% in percentage ultrafilterable magnesium occurred. However, the

Table XV The effect of intravenous calcium gluconate (14 mg. Calcium per Kg. body weight) on ultrafilterable Calcium, Magnesium and Inorganic phosphate.

No.	Time	CALCIUM mg./100 ml.	CALCIUM % ultra-filterable	MAGNESIUM mg./100 ml.	MAGNESIUM % ultra-filterable	INORGANIC PHOSPHATE mg./100 ml.	INORGANIC PHOSPHATE % ultra-filterable	PROTEIN Alb./Glob. g./100 ml.
1	control	10.5/6.0*	57	2.0/1.6*	80	3.0/3.4*	113	5.2/1.9
				INFUSSION STARTED				
2	0.5 hr	10.5/6.0	57	2.0/1.6	80	3.5/3.6	103	- -
3	1.5 hr	11.3/5.8	51	1.9/1.2	63	4.3/4.4	102	- -
4	2.5 hr	11.5/5.3	46	1.8/1.1	61	5.0/5.1	102	- -
5	3.5 hr	13.1/5.1	39	1.7/1.05	62	5.8/5.2	89	4.7/1.8
				INFUSSION STOPPED				
6	5.5 hr	12.8/5.2	41	1.8/1.0	56	5.9/5.4	91	- -
7	22.5 hr	10.6/5.9	56	2.0/1.7	85	3.6/3.6	100	5.1/1.9

*Results expressed as total serum concentration/ultrafiltrate concentration.

ultrafilterable magnesium was found slightly raised above the control value on the following morning. In spite of the decreasing total serum magnesium the protein bound fraction rose from 0.4 mg. per 100 ml. to 0.65 mg. per 100 ml. (when calcium had reached the peak value).

Group V. Patients with Hypoparathyroidism.

The sera from patients with hypoparathyroidism were subjected to ultrafiltration. These patients included both idiopathic and post-thyroidectomy cases of hypoparathyroidism. The patients were on oral calcium supplements and vitamin D_2 and this therapy was discontinued several days prior to the initial ultrafiltration studies. The results in Table XVI show that the total serum calcium concentrations ranged from 5.6 to 8.6 mg. per 100 ml. while the ultrafilterable calcium varied from 3.7 to 5.7 mg. per 100 ml. Although the total serum calcium was reduced in every instance, the ultrafilterable calcium was within normal limits in two cases (E.G. and A.H.), and was increased in one case (E.G.).

Serum inorganic phosphate was elevated in all the cases studied except case W.G. where it was within the normal limits. The pattern of ultrafilterability was essentially normal.

Hypomagnesaemia was present in only one case (E.G.) and the concentration of magnesium in the ultrafiltrate was also found to be decreased in this case. Apart from one case (E.G.) the serum proteins were within the normal limits. Tetany was observed in three of these cases (E.M., E.G., and J.F.).

Patient E.M. was studied in more detail during a course of therapy for idiopathic hypoparathyroidism. Treatment with 3.7 mg. of

Table XVI Ultrafiltration Data on Serum from Patients with Hypoparathyroidism.

	CALCIUM		MAGNESIUM		INORGANIC PHOSPHATE		PROTEIN
Case.	mg. per 100 ml.	% ultra-filterable	mg. per 100 ml.	% ultra-filterable	mg. per 100 ml.	% ultra-filterable	g. per 100 ml.
E.M.	5.6/3.7*	66	1.8/1.3*	81	6.2/6.5*	106	5.0/2.5
J.F.	6.4/4.0	62	1.8/1.4	77	5.8/6.2	107	5.8/2.8
E.G.	6.8/5.7	83	1.3/1.2	92	6.0/6.4	106	2.9/1.1
W.G.	7.1/4.1	57	1.9/1.4	74	3.4/3.9	114	4.3/1.8
H.K.	7.2/4.1	57	2.0/1.5	75	5.2/5.6	107	4.9/2.3
E.B.	7.4/4.0	54	2.2/1.6	73	6.4/5.7	104	4.5/2.4
H.O.	7.6/4.7	61	1.8/1.4	77	4.5/4.7	104	5.3/1.9
E.L.	8.2/5.2	61	2.0/1.3	65	5.3/5.5	103	5.6/2.1
A.H.	8.6/5.7	66	2.0/1.4	70	5.0/5.3	106	5.3/1.9

*Results expressed as total serum concentration/ultrafiltrate concentration.

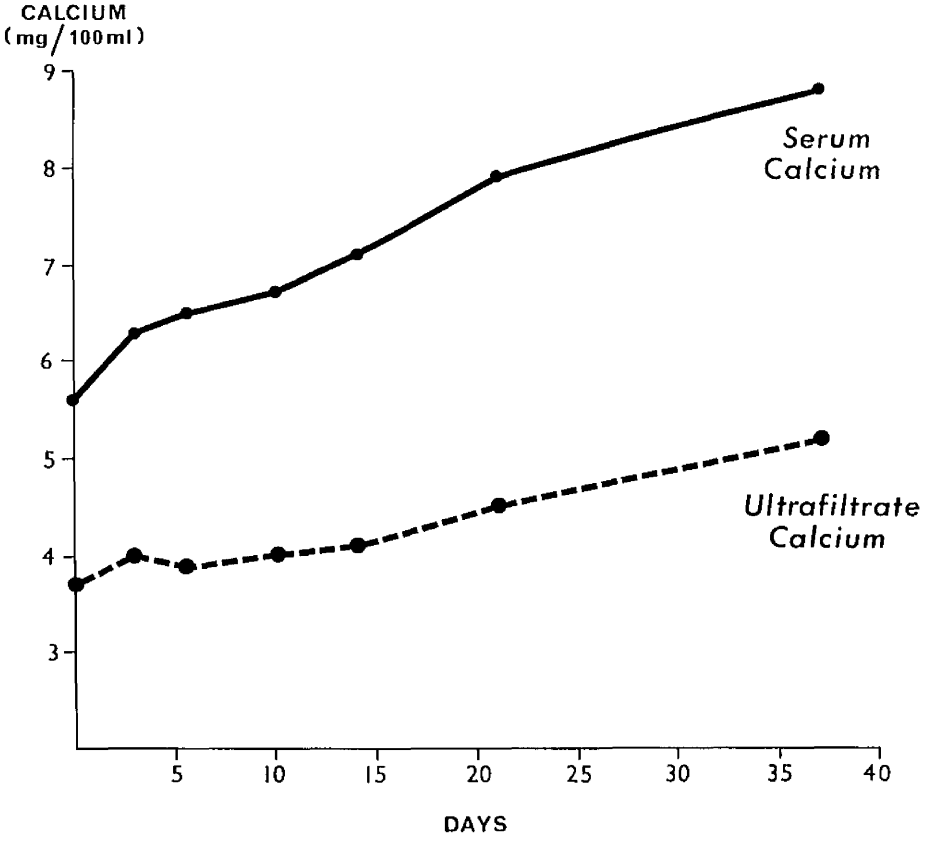

FIGURE 7. SERUM AND ULTRAFILTERABLE CALCIUM AFTER ORAL CALCIUM AND VITAMIN D THERAPY IN A PATIENT WITH IDIOPATHIC HYPOPARATHYROIDISM. (PATIENT E.M.)

A.T. 10 and 6.0 grams of calcium gluconate per day was maintained for two days. Thereafter he was put on 150,000 units of vitamin D per day and the A.T. 10 was discontinued. The follow-up results are shown in Figure 7. After two weeks of this therapy, the ultrafilterable calcium had increased by only 0.4 mg. per 100 ml. while the total calcium had increased by 1.5 mg. per 100 ml. The last estimations, carried out after four weeks of therapy, showed an increase of 1.5 mg. per 100 ml. in the ultrafiterable calcium with an increase of 3.2 mg. per 100 ml. in the total serum calcium, so that the percentage of ultrafilterability fell from 66% to 56% after two weeks, and rose again slightly to 59% after four weeks of treatment.

Group VI. Patients with "Hypoalbuminaemia"

As the bulk of the non-filterable calcium is bound to serum albumin, any decrease in its concentration should affect the bound calcium fraction. Sera from 14 patients with hypoalbuminaemia due to various causes, were ultrafiltered and the results are shown in Table XVII. None of these patients had any history of renal insufficiency, and the blood urea concentration was within the normal limits in every instance.

Serum albumin concentration varied from 2.3 to 2.9 g. per 100 ml., the serum globulins were within the normal limits in eight of these patients and slightly decreased in another four. Two cases with hypoalbuminaemia from patients with cirrhosis of the liver had increased serum globulins. Hypocalcaemia was present in all of these cases, with serum calcium concentrations ranging from 7.0 to 8.9 mg. per 100 ml.

Table XVII Ultrafiltration of Serum from Patients with Hypoalbuminaemia.

	CALCIUM		MAGNESIUM		INORGANIC PHOSPHATE		PROTEIN
Case.	mg. per 100 ml.	% ultra-filterable	mg. per 100 ml.	% ultra-filterable	mg. per 100 ml.	% ultra-filterable	Alb./Glob. g. per 100 ml.
O.B.	7.0/5.7*	81	0.8/0.7*	88	2.5/2.7*	108	2.9/2.2
E.G.	7.0/6.3	90	1.5/1.3	87	1.7/1.9	111	2.9/1.1
J.M.	7.3/5.3	73	1.6/1.4	87	5.3/5.6	105	2.8/0.7
J.H.	7.7/5.9	76	1.6/1.4	87	5.5/5.5	100	2.6/2.0
J.C.	8.1/5.9	72	1.8/1.5	83	2.4/2.7	112	2.3/3.0
J.F.	8.2/6.2	75	2.0/1.6	80	4.0/4.6	115	2.6/1.4
M.W.	8.3/6.0	72	2.0/1.5	76	4.6/4.8	104	2.6/2.7
I.C.	8.3/6.1	73	1.8/1.5	83	3.1/ -	-	2.7/2.5
I.G.	8.6/6.7	78	1.6/1.4	87	5.0/5.2	104	2.6/1.8
M.H.	8.7/5.5	65	1.7/1.5	88	2.4/2.7	112	2.6/4.3
E.V.	8.8/6.5	73	2.0/1.3	65	5.0/5.3	106	2.8/2.5
H.B.	8.8/6.0	68	2.0/1.5	75	3.8/4.8	126	2.8/2.0
I.G.	8.9/6.9	77	1.9/1.6	84	2.5/1.7	108	2.4/1.4
S.T.	8.8/6.2	70	1.7/1.5	88	3.2/3.5	109	2.6/5.0

*Results expressed as total serum concentration/ultrafiltrate concentration.

On the other hand ultrafilterable calcium was within normal limits in 11 of the 14 cases and was slightly elevated in the others. However, when expressed in terms of the percentage ultrafilterable calcium, it was always markedly elevated varying from 65 to 90%. It would appear, therefore, that the decrease in serum calcium associated with hypoalbuminaemia is due to decrease in the bound fraction, and not to the free or ionised fraction.

There was no direct correlation either between total proteins and total serum calcium (correlation coefficient (r) = 0.41) or between albumin and the ultrafilterable calcium (r = 0.25).

The decrease in serum calcium was associated with elevated inorganic phosphate in five of the 14 cases. One case had a decreased inorganic phosphate concentration and the remainder had normal values. The percentage ultrafilterable inorganic phosphate was within the normal range in all cases.

The hypocalcaemia was also associated with hypomagnesaemia in seven out of 14 cases; it was very severe in the case of O.B. (with a serum calcium concentration of 7.0 mg. per 100 ml.) where the serum magnesium concentration was only 0.8 mg. per 100 ml. In other seven cases, serum magnesium concentration was within the normal limits. The ultrafilterable magnesium was within normal limits except case O.B. (who had very low total serum magnesium as well), and the decrease in magnesium concentration, where it occurred, was again probably entirely in the protein bound fraction. When expressed as a percentage of the total, it was elevated

in 11 out of 14 cases and was decreased in one case; the other two were within the normal range.

Group VII. Patients with Chronic Renal Disease.

In Table XVIII are collected together a group of patients with hypocalcaemia secondary to renal disease. Serum calcium concentration varied from 5.3 to 8.6 mg. per 100 ml. and the ultrafilterable calcium from 4.2 to 6.1 mg. per 100 ml. It is immediately apparent that the percentage of ultrafilterable calcium in all the cases is abnormally high, regardless of the concentration of total calcium, and as a consequence, the concentration of calcium in the serum ultrafiltrates was within the normal limits in four of these cases. It was slightly decreased in the rest of the cases, but was well above the values expected on the basis of the McLean and Hastings (1935a) equation. In two of the cases the protein-bound fraction was reduced to a value as low as 0.7 mg. per 100 ml. while the ultrafilterable fraction approached normal values.

The serum total protein concentrations were reduced in only half of these cases, and in all 14 cases the serum globulin concentrations were within normal limits. The reduction in the total protein concentration wherever it occurred, was in the concentration of albumin which is also responsible for the binding of calcium. Since the serum protein concentrations were within normal limits in half of these cases, and some of these were also associated with very low serum calcium values, some other factors are also concerned in the maintenance

Table XVIII Ultrafiltration Results in Patients with Chronic Renal Disease.

Case.	CALCIUM mg./100 ml.	CALCIUM % ultra-filterable	MAGNESIUM mg./100 ml.	MAGNESIUM % ultra-filterable	INORGANIC PHOSPHATE mg./100 ml.	INORGANIC PHOSPHATE % ultra-filterable	PROTEIN Alb./Glob. g./100 ml.	Blood Urea mg./100 ml.
A.S.	5.3/4.6*	86	1.6/1.3*	84	13.6/14.6*	107	4.8/1.7	295
V.W.	5.3/4.4	82	1.4/1.3	92	14.0/14.6	104	4.5/2.4	420
E.G.	5.4/4.7	88	1.6/1.5	94	5.4/5.7	106	2.8/1.9	429
J.H.	5.5/4.2	75	1.8/1.5	83	15.2/16.2	107	3.2/1.8	378
C.G.	5.8/4.8	82	1.4/1.3	87	15.5/16.8	108	4.2/1.9	432
W.R.	6.0/4.3	72	1.5/1.3	86	9.5/9.5	100	4.6/1.6	224
E.C.	6.7/4.4	66	1.9/1.5	79	13.4/14.1	105	3.4/2.1	480
H.M.	6.8/5.0	73	2.0/1.4	73	9.2/9.6	104	3.3/1.6	352
J.F.	6.9/4.8	70	1.8/1.6	87	15.2/16.6	109	3.5/2.0	320
E.M.	7.5/4.7	63	1.8/1.4	78	13.0/13.1	101	5.0/1.9	283
M.A.	7.8/5.5	70	2.3/1.7	75	14.4/15.3	106	4.2/1.8	330
P.D.	7.9/5.5	70	2.5/1.8	70	12.0/12.0	100	4.2/2.1	262
J.R.	8.5/5.8	68	1.6/1.2	73	7.6/8.1	106	3.6/2.1	238
M.D.	8.6/6.1	71	2.8/2.1	74	8.2/9.1	110	3.3/1.4	224

*Results expressed as total serum concentration/ultrafiltrate concentration.

of ultrafilterable calcium, e.g. pH. Acidosis, as judged from the low serum bicarbonate concentration and low blood pH (Table XIX.), was present in each case except in cases M.M. and M.D. where the pH was within the normal limits. It is known that low pH favours less binding of calcium to protein, therefore in these cases the accompanying acidosis probably plays an important part in the maintenance of ultrafilterable calcium. There is a relationship between pH and ultrafilterable calcium (correlation coefficient, r = 0.66) and also between bicarbonate concentration and ultrafilterable calcium (r = 0.78).

Other pertinent blood chemistry determinations in these patients are also listed in Table XVIII. The blood urea and serum inorganic phosphate concentrations were elevated in the subjects to varying degrees. The total or ultrafilterable calcium concentrations did not correlate with blood urea or serum inorganic phosphate. In all of these cases the concentration of ultrafilterable inorganic phosphate always exceeded that of serum concentration thus representing normal pattern of ultrafilterability of this ion.

Variable magnesium results were obtained. In 6 out of 14 cases the serum magnesium was decreased but in only one instance (J.R.) was ultrafilterable magnesium also decreased, so that the percentage ultrafilterability was elevated in every case except J.R. where it was within the normal limits. Increased values for serum magnesium were obtained in only two cases (P.D. and M.D.) and in the case of M.D. ultrafilterable magnesium was also increased. In the rest of the cases both serum and ultrafilterable magnesium were within normal limits.

Table XXIX. Total and Ultrafilterable Calcium with Blood pH and Bicarbonate concentration in Chronic Renal Disease.

Case.	Total Serum Calcium mg. per 100 ml.	Ultrafiltrate Calcium mg. per 100 ml.	Blood pH.	Serum Bicarbonate meq. per litre.
A.S	5.3	4.6	7.15	12.0
V.W	5.3	4.4	7.17	11.0
E.G.	5.4	4.7	7.10	13.0
J.H.	5.5	4.2	7.22	16.0
C.C.	5.8	4.8	7.15	10.5
W.R.	6.0	4.3	7.14	13.0
E.M.	6.7	4.4	7.30	15.0
M.M.	6.8	5.0	7.38	19.0
J.F.	6.9	4.8	7.29	16.5
E.M.	7.5	4.7	7.32	15.5
M.A.	7.8	5.5	7.30	22.0
P.D.	7.9	5.5	7.32	20.0
J.R.	8.5	5.8	7.35	21.0
M.D.	8.6	6.1	7.39	20.5

Tetany has been described in cases of chronic renal failure with nitrogen retention, but none of the patients in the present exhibited any symptoms of latent tetany at any stage. The implication of these findings will be discussed.

PART 2

DISCUSSION

Methodology.

The ultrafiltration apparatus used in the present study is very simple and yet has many advantages over the most commonly used methods of Toribara, et. al. (1957) and Rose (1957). Samples as small as 1.5 ml. gave a sufficient volume of 0.7 ml. ultrafiltrate in 1½ to 2 hours compared with the large volumes of specimen, 16 to 17 ml., and longer time required, up to 6 to 9 hours, in other methods.

Another major factor, the maintenance of the pH during the ultrafiltration procedure, was reasonably achieved by maintaining a carbon dioxide tension approximately equal to that of alveolar air over the specimen. The control of pH during the ultrafiltration is essential, as is evident from the data in (Tables II and III, P. 79 and 82) and that of Toribara, et. al. (1957). However, when only minor changes were produced in pH within the physiological range, no difference in the diffusible fraction of serum calcium could be observed, which indicated that minor changes in blood pH, such as occur in an individual from day today, probably will not affect the partition of calcium. Similar observations were made by Hopkins, et. al. (1952) and Toribara, et. al. (1957). When blood is exposed to air it loses carbon dioxide and the pH rises, but if the specimen, after exposing to air, is kept under an atmosphere of gas with a carbon dioxide tension similar to that of normal alveolar air, the carbon dioxide will dissolve and the pH of the specimen will return to approximately normal physiological values and hence the partition of calcium will not be affected. This was shown when two specimens, one collected under oil and the second exposed to air, were

utrafiltered under these conditions. No difference could be demonstrated in the calcium concentration of ultrafiltrates thus obtained. This confirmed the findings of Hopkins, et. al. (1952) and Toribara, et. al. (1957) that when ultrafiltration is carried out under an atmosphere of 5% carbon dioxide there was no need for any special precaution to be taken in the collection of blood specimen, i.e. collecting under oil.

Ultrafiltration and Calcium-Protein relationship.

In the present study the relationship between calcium, magnesium and inorganic phosphate were further studied by ultrafiltration of these substances through a cellophane membrane under 16 lbs. per sq. inch pressure at physiological pH. As with all such techniques, as ultrafiltrate is formed, the protein and calcium concentrations in the residue rise, and water content falls. However, it is evident from the data in Table VII (P. 89) that the concentration of calcium in serial ultrafiltrates obtained from a fixed volume of serum specimen did not differ significantly. This fact strengthens the belief that the calcium-protein relationship remains unchanged during the ultrafiltration procedure, and also refutes the frequently voiced criticism (Bennett and Kirby (1965)), that since ultrafiltration is a dynamic process and concentrates the serum proteins, the concentration of calcium in the ultrafiltrates may not reflect the original conditions in the serum. This criticism can also be shown to be invalid on theoretical grounds. According to McLean and Hastings (1935a) the relationship between calcium and serum proteins may be expressed by the simple equation:-

$$Ca^{++} + Protein^{--} \rightleftharpoons Ca\text{-proteinate}$$

assuming that one protein molecule binds only one calcium ion.

The dissociation constant of the calcium complex may be expressed as:-

$$\frac{(Ca^{++})\ (Protein^{--})}{(Ca\text{-proteinate})} = K$$

where the quantities in the brackets are activities. According to this equation the ratio of bound to unbound calcium is a function of the calcium ion activity in the solution. In serum of total ionic strength 0.15, the protein concentration is about 0.001 M and can be considered to contribute a negligible amount to the total ionic strength. Thus relatively large alterations in protein concentrations must be produced before any change in the ionic strength occurs. Chen and Neuman (1955) showed that diffusible forms of calcium in 0.15 M sodium chloride move through a cellophane membrane at the same rate as water. Similar behaviour during ultrafiltration of serum would then cause no change in the concentration of any diffusible ions in the free water of the bulk solution, an increase in the concentration of both forms of protein, but only a negligible change in ionic strength. Since the largest factor governing the activity coefficient is ionic strength, the activity coefficient of all constituents in the remaining serum would remain essentially constant during the ultrafiltration process. Therefore, with no change in activity coefficients, calcium ion concentration and the ratio of the two forms of protein do not change.

The above discussion would also apply equally to any reaction

involving a fixed number of one or more calcium ions with a single protein molecule. Thus:-

$$nCa^{++} + Protein^{--} \rightleftharpoons Ca_n\text{-proteinate}$$

$$K = (Ca^{++})^n \frac{(Protein^{--})}{(Ca_n\text{-proteinate})}$$

The ultrafiltration procedure would not change the calcium ion concentration and the protein / Ca_n-proteinate ratio would remain constant although the concentration of protein is continuously increasing.

Large amounts of serum may be ultrafiltered without any appreciable change in the composition of the ultrafiltrate, and the reduction in the volume of the serum accompanying the usual ultrafiltration would not be expected to effect the results obtained.

Normal Values, Ultrafiltrate Analyses:

The mean value for ultrafilterable calcium in the normal subjects was found to be 5.9 mg. per 100 ml. which gave a mean value of 58% for the percentage ultrafilterable calcium. McLean and Hastings (1934), by their frog heart technique, concluded that about 1.3 mM per litre (5.3 mg. per 100 ml.) of calcium exists in the ionic form in normal serum, and Rose (1957), by determining the total diffusible and ionic calcium, reported that 0.3 mg. per 100 ml. of calcium exist as unionised citrate etc. The sum of these two values, 5.6 mg. per 100 ml. is very

close to the mean value of 5.9 mg. per 100 ml. found in the present experimently determined range for normal ultrafilterable calcium. These values are in close agreement with the results obtained by some of the most recent authors using different techniques under pH control (Prasad and Flink (1957), Rose (1957), and Fowler, Fone, and Cooke (1961)). Hence it is apparent that as long as control of the pH is maintained during the ultrafiltration procedure, comparable values for ultrafilterable calcium should be obtained regardless of the technique used.

A mean of 1.5 mg. per 100 ml. of the total serum magnesium was found in the ultrafiltrates of normal subjects which represented 74% ultrafilterability. This value was in close agreement with the results obtained by Kleeman, et. al. (1958) and that of Prasad, et. al. (1961). These workers also maintained a constant control of pH during the ultrafiltration procedure.

It has been shown (P. 52) that the inorganic phosphate is completely filterable when the theoretical consideration of Donnan membrane effect and the serum water content are taken into account. However, Manery (1954) has pointed out that the observed Donnan ratio for various ions in serum are all less than this theoretical value. Greenberg and Gunther (1930) reported a value of 1.05 in the presence of 7% proteins. The deviation is very small in case of the monovalent cations, sodium and potassium (Manery, 1954), but greater in case of the anions, chloride, sulphate, and phosphate (Swan, Feinstein and Madisso (1956)). In the present series of normal subjects a mean value of 3.9 mg. per 100 ml. which represented 109% ultrafilterability of inorganic phosphate was obtained and this

approaches the values calculated on theoratical grounds. Hopkins, et. al. (1953) and Smith, et. al. (1943) reported similar results. The present results confirmed the findings of Hopkins, et. al. (1953) that the inorganic phosphate is completely ultrafilterable in the sera of normal subjects. In calculating the protein binding of calcium and magnesium no corrections either for plasma water or the Donnan factor were applied, as, according to Walser (1960), in the cases of divalent cations which are partly bound to protein these two corrections are opposite in direction and of approximately equal magnitude and hence cancel each other. However, in the case of anions these corrections are in the same direction and have to be applied.

Serum Calcium in Hyperparathyroidism.

Twelve proven cases have been investigated. Parathyroid adenoma was removed at operation in 11 of these cases. The twelfth patient had secondary involvement of the kidneys with high blood urea and was not operated upon. The diagnosis was based upon the characteristic radiological signs of hyperparathyroidism in bone or on kidney stone formation. The serum calcium was raised in all except in three of the patients (S.B., T.A., and M.T.) where the total serum calcium never exceeded 11.3, 10.5, and 10.0 mg. per 100 ml. respectively. Diagnosis in two of these (S.B. and T.A.) was particularly difficult, since the serum inorganic phosphate was also normal, the bones were normal radiologically, and the serum alkaline phoshpatase activity was within normal limits.

Ultrafilterable and protein bound Calcium.

(a). Before operation.

Inspection of the present data (Table X, P. 96) shows that whereas the serum ultrafilterable calcium is always elevated in patients with active parathyroid adenoma, the protein bound fraction (in actual concentration) is normal or only slightly elevated, thus giving increased values for the percentage ultrafilterable calcium. There has been controversy over these findings of increased diffusible calcium in hyperparathyroidism. It is apparent from McLean and Hastings equation (P. 125) that if the serum protein remain constant, an increase in the concentration of the calcium ions must be accompanied by a proportionate increase in the protein bound fraction. Later these workers (1935b) found that following administration of parathyroid extract to man and animals, the redistribution of calcium in serum followed the above equation. Hopkins, et. al. (1953) from the determination of ultrafilterable calcium in these disease states, supported the findings of McLean and Hastings, and stated that the presence of an elevated ultrafilterable calcium concentration was of no importance in establishing the diagnosis of hyperparathyroidism. Ludewig, Chanutin, and Masket (1942) concluded that the equation was unreliable, and Sartori (1955) found that it did not apply to hypocalcaemic conditions in man. However, most of the recent authors agree that the diffusible portion of the serum calcium is raised in this condition. It is also accepted that ultrafilterable calcium will be found to be raised in cases of primary hyperparathyroidism even though the serum total calcium may be normal (Fanconi and Rose (1958),

Lloyd and Rose (1958), Fowler, Fone, and Cooke (1961), and Hodgkinson (1963)); in such cases this may be of even greater diagnostic importance.

(b). After operation.

After the surgical removal of adenomas the relative fall in ultrafilterable fraction was greater than that of the protein-bound fraction. The operation was always followed by a fall in the ultrafilterable calcium to normal or subnormal concentrations, but the fall in the protein-bound fraction was only slight and was negligible in three of the cases (H.F., W.R., and H.C.). Case J.R. was an exception, where the fall in the protein-bound fraction was appreciable. However, a simultaneous fall in the serum albumin concentration was also observed and this factor was probably responsible for low protein-bound fraction in this particular case. Since the post-operative serum protein concentrations were within the normal limits in rest of the cases, the calcium results are unexpected on the basis of McLean and Hastings equation. Similar observations were also made by Lloyd and Rose (1958). These authors measured ionised calcium in 17 patients with hyperparathyroidism before and after removal of adenomas; the ionic calcium concentration fell in all cases studied but the protein-bound fraction remained essentially unchanged. Hellstrom (1953) also reported three cases with primary hyperparathyroidism, in whom the concentrations of serum calcium were within normal limits, but the values for the diffusible calcium fraction were above normal. After surgical removal of the adenomas in his cases also, the concentration of serum diffusible calcium fell, while the protein-bound fraction remained

virtually the same in one case and was increased in the other two. However, Hellstrom did not estimate the serum protein, nor did he describe the method for the determination of diffusible calcium. In one of the cases of hyperparathyroidism reported by Hopkins, et. al. (1953) the surgical removal of the adenoma was followed by a slight rise in the protein-bound fraction of the serum calcium, although no change in the serum protein concentration occurred. There can be two possible explanations for this normal protein-bound calcium concentration in the presence of increased ultrafilterable calcium, either by reduction in the concentration of serum protein or by a decrease in the capacity of the serum protein to bind calcium. Since serum protein concentrations were within the normal limits in the present series, the parathyroid adenoma was accompanied by a decrease in the ability of the serum proteins to bind calcium. This phenomenon seems to be abolished in the post-operative state, without any significant change in the serum protein concentration.

Mechanism of action of Parathyroid Hormone.

It is generally accepted that the parathyroid glands are initially concerned with the maintenance of a normal serum calcium concentration. Although the precise physiological mechanism involved is still incompletely understood, it is now believed that the glands are concerned with the equilibrium between calcium in bone and extracellular fluids (Copp, 1957). The parathyroid hormone regulates the ionic calcium homeostasis in extracellular fluid by a direct action on bone tissue. Secondly, it decreases renal tubular reabsorption of inorganic phosphate from the

glomerular filtrate; excessive amounts of hormone therefore cause excessive loss of phosphate in the urine and a fall in its concentration in the plasma. It also produces other physiological effects which are not obviously related to its calcaemic action.

Parathyroid hormone and binding of Calcium to Protein.

Since the concentration of ionic calcium in the tissue fluids is controlled by the secretion of parathyroid glands it is very likely to have some effect on the protein binding of serum calcium. It is known that artificial elevation of serum calcium suppresses the parathyroids (Howard, Hopkins, and Connor (1953); Nordin and Fraser (1954); and Kyle, Schaff, and Canary (1958)) and parathyroid size varies inversely with the level of serum calcium (Ham, Littner, Drake, Robertson, and Tisdale (1940), and Steenbock and Bellin (1953)). Patt and Luckhard (1942) demonstrated that an increase in parathyroid hormone secretion occurred in response to the perfusion of parathyroid glands with fluids of low calcium concentrations. Talmage, Elliott, and Enders's (1957) osteoclast count, which is believed to measure parathyroid activity, rises in response to rapid removal of calcium from the extracellular fluid of intact rat. However, in the presence of functioning parathyroid adenoma, there is an excessive secretion of the hormone with an increase in concentration of total and ionic serum calcium, while there is little or no effect on the protein-bound fraction. Therefore, it seems possible that excessive parathyroid hormone has modified the serum proteins, in such a way, which causes a change in the chelating ability of the atoms situated near the

carboxylic acid groups in the molecule (Lloyd and Rose (1958)). It is more likely that the parathyroid hormone itself has reduced the capacity of proteins to bind calcium by blocking the binding sites of calcium in the protein molecule. It seems unlikely that formation of new proteins with different properties could have occurred after the removal of the adenoma.

Intravenous Calcium infusion.

This hypothesis was further tested by the intravenous infusion of calcium in a normal human subject. It was anticipated that increase in serum calcium will suppress the parathyroid secretion which should result in a decrease in the concentration of diffusible fraction and an increase in the protein-bound fraction. The precise ionic calcium concentration which stimulate and suppress the parathyroid are not known, but calcium infusion suggest that parathyroid suppression occurs when total serum calcium rises above 11.0 mg. per 100 ml. and the stimulation probably occurs when the concentration of calcium falls below about 9.0 mg. per 100 ml. (Nordin (1961)).

It is evident from the data in Table XV (P. 110) that during the calcium infusion period the total serum calcium rose from 10.5 mg. per 100 ml. to a peak level of 13.1 mg. per 100 ml. after about four hours, and the diffusible fraction decreased from 6.0 mg. per 100 ml. to 5.1 mg. per 100 ml. and the protein-bound fraction rose from 4.5 mg. per 100 ml. to 8.0 mg. per 100 ml. Immediately after stopping the calcium infusion the total serum calcium began to fall and with this the ultrafilterable

fraction began to increase again, and by next morning both the total and ultrafilterable calcium were near to the control values. Since the parathyroid hormone promotes the excretion of phosphate, its suppression by calcium infusion might be expected to raise the serum phosphate concentration. This was found to be the case, and indicated that efficient suppression of hormone occurred: this effect on serum inorganic phosphate became evident before there was any change in the ultrafilterable calcium fraction. This experiment demonstrated that parathyroid hormone has some definite effect on the protein binding of calcium. It seems that after the suppression of glands , which result in less secretion of parathyroid hormone, the additional binding sites which presumably had been under the control of parathyroid hormone were made available to the additional calcium. However, after the serum calcium had reached a concentration over 11.5 mg. per 100 ml. there also occurred a reduction in the ultrafilterability of inorganic phosphate, which remained apparent in the subsequent specimens until the calcium concentration was again below 11.5 mg. per 100 ml. This reduced ultrafilterability of both calcium and inorganic phosphate suggested the formation of a complex between these two which is bound to the protein. This decrease ultrafilterability of both calcium and inorganic phosphate were also observed *in vitro* by simply raising the serum calcium concentration. It seems likely that with the depression of parathyroid activity the conditions *in vivo* becomes similar to that *in vitro*. The nature and mechanism of the formation of this complex is unknown. The effect of intravenous calcium on the serum ultrafilterable calcium in normal human subject was investigated by Terepka, *et. al.* (1958).

These workers found that hypercalcaemia produced by intravenous infusion of calcium was accompanied by no alteration in the percentage of calcium that was ultrafilterable, i.e. both total and ultrafilterable calcium increased proportionately. This may be due to the faulty control of pH during the process of ultrafiltration. However, Wallach, et. al. (1964) has shown in dogs that the artificial production of hypercalcaemia does results in a slight fall in the ultrafilterable fraction of calcium and maintained that despite hypercalcaemia, sufficient plasma protein binding sites were available to accomodate the additional calcium. They also observed a simultaneous increase in serum inorganic phosphate and a significant decrease in its ultrafilterability.

Similar conclusions were reached that the parathyroid hormone reduces the binding capacity of serum proteins, by the administration of parathyroid extract to normal human subjects (Lloyd and Rose (1958)).

Ultrafilterable Calcium in Hypercalcaemia of Other Causes.

There are various conditions where hypercalcaemia is encountered without any disorder of parathyroid and a diagnosis of primary hyperparathyroidism may sometimes be confused with these diseases. By clinical and laboratory observations it is usually possible to differentiate these conditions from hyperparathyroidism. However, when the latter condition is complicated by secondary renal involvement, the diagnosis may become difficult. In such cases the hypercalcaemia present will suppress the parathyroid glands with less secretion of parathormone and this should result in decreased ultrafilterability of calcium and increased

protein binding. Hence, results similar to the artificial hypercalcaemia produced *in vivo* or *in vitro* should also be obtained in these conditions.

Five patients without any disorders of the parathyroid glands were studied. It is immediately apparent from the results of ultrafiltration (Table XIV, P. 106) that the hypercalcaemia is associated in each case with decreased percentage ultrafilterability. In only one instance (R.W.) does this value approach the normal. In this case a decreased concentration of serum albumin was also found which may be responsible.

J.R. was a case of steatorrhoea on long standing calcium supplements and high dosage of vitamin D. Both total and ultrafilterable calcium were within normal limits after the withdrawal of therapy. One case (M.K.) of post thyroidectomy hypoparathyroidism was included, who was also on the same treatment and results similar to above were obtained. Similar observations were also made by Hopkins, *et. al.* (1953) in one of their two cases of hypervitaminosis D. As hypercalcaemia was not apparent in their second case, no such results were obtained. Terepka, *et. al.* (1958) in two of their cases of vitamin D intoxication could not detect any difference from normal in the distribution of protein-bound and ultrafilterable calcium fractions, but again this was probably due to their faulty control of pH during the ultrafiltration. As these workers did not estimate the inorganic phosphate, which is not completely filterable in these conditions, no conclusions can be drawn from their results.

Case E.E. had acute paralysis of the lower limbs with sensory loss and he later developed urinary retention. Repeated serum calcium determinations showed raised values associated with reduced ultrafilterability.

The serum calcium returned to normal following a course of cortisone therapy with subsequent normal values of total and ultrafilterable calcium. Conditions in which hypercalcaemia is encountered without hyperparathyroidism are summarised in a paper by Burnett, Commons, Albright, and Howard (1949).

R.W. was admitted for obstructive jaundice, a serum calcium estimation was carried out incidentally and found to be elevated. Hypercalcaemia associated with reduced ultrafilterability was found in this patient on two subsequent occasions. At autopsy normal parathyroid glands were found.

Case J.S. presented an interesting problem, he had persistent hypercalcaemia with normal or occasionally slightly raised inorganic phosphate. Reduced ultrafilterability of calcium was encountered on all eight occasions. Further investigations did not reveal any bone disease either radiologically or by serum alkaline phosphatase activity. No opaque kidney stones could be demonstrated on X-ray. However, the persistent findings of raised serum calcium was attributed to a parathyroid adenoma, but at operation no adenoma could be found. The patient continued to show hypercalcaemia. Despite all efforts the cause could not be established. These results show that reduced ultrafilterability of both calcium and inorganic phosphate in the presence of hypercalcaemia may be encountered in conditions other than hyperparathyroidism.

In all of these cases the serum inorganic phosphate was either slightly raised or within normal limits, but the percentage ultrafilterable was always found to be reduced. This simultaneous reduction in the ultrafilterability of both calcium and inorganic phosphate has been

attributed to the formation of a complex between the two and perhaps involving protein as well (Greenberg (1933); Smith (1934); and Hopkins, et. al. (1952)). The mechanism of the formation of this complex is not known. It is evident from previous studies that the suppression of parathyroid is attained at a serum calcium concentration of about 11.0 mg. per 100 ml. or more. It is also evident from the present data and that of Hopkins, et. al. (1952) that the formation of this complex always takes place when the serum calcium concentration is above 11.6 mg. per 100 ml. Therefore, it seems probable that parathyroid hormone has some inhibitory effect on the formation of this complex. The elevation of serum calcium beyond 11.5 mg. per 100 ml. is essential for the formation of such a complex. It has been reported that increasing the concentration of phosphate in vitro by Hopkins, et. al. (1952) and by intravenous infusion of phosphate in dogs by Smith (1934), in presence of normal serum calcium, did not result in the formation of such a complex.

In the present series, one atypical finding (ultrafiltration results similar to those found in hyperparathyroidism) was observed in a patient (M.M.) with hypercalcaemia associated with multiple myeloma. Four of eight cases of multiple myeloma had hypercalcaemia, but it was not very marked in one case (C.F., serum calcium 11.3 mg. per 100 ml.). Reduced ultrafilterability of both calcium and inorganic phosphate was observed in two of the cases. In this connection the hyperproteinaemia frequently found in multiple myeloma does not appear to cause reduced ultrafilterability of the serum calcium, and indeed the serum of two patients of this group J.J. and W.M. (Table XIII, P. 104) showed increased

in the ultrafilterable calcium. The reason for this is probably that the increased protein concentration is due to an increase in the globulin fraction which usually has negligible calcium binding capacity. In these particular two cases the serum albumin concentration was markedly reduced. However, in the third case M.M. ultrafiltration results comparable to those obtained in hyperparathyroidism were encountered. This atypical finding may be observed sometimes in malignant diseases. Hypercalcaemia and symptoms similar to those found in hyperparathyroidism without bone metastases has been described in neoplastic diseases. One such case is described by O'Grady, Morse, and Lee (1965) where the carcinoma of right kidney was responsible for the persistent hypercalcaemia without any bone metastases. The pre-operative hypercalcaemia in this case was corrected by the removal of the tumour. The immunological analysis of the surgical specimen demonstrated the presence of a parathyroid-like hormone. A similar substance (parathyroid-like hormone) in non-parathyroid neoplasms associated with hypercalcaemia has also been identified immunochemically by Tashjian, Levine, and Munson (1964). These findings are suggestive of excess parathyroid hormone or parathyroid-like hormone secreted by the tumour resulting in hypercalcaemia without bone metastases similar to be found in hyperparathyroidism. Hence, in presence of a functioning parathyroid adenoma associated with normal concentration of serum calcium and in majority of cases of hypercalcaemia without disorders of parathyroids, the utrafiltration results may be of some use in the differential diagnosis.

Serum and Ultrafilterable Calcium in Hypocalcaemia of Hypoparathyroidism.

In seven of the nine patients hypoparathyroidism developed after thyroidectomy and in only two cases (E.M., and E.B.) was it idiopathic. J.F. and W.G. were studied two days after partial thyroidectomy. The remaining five patients were on calcium and vitamin D therapy, and ultrafiltration studies were carried out several days after discontinuation of this therapy, except in the case of A.H. Patient A.H. had complications due to Addison's disease, the appropriate therapy for Addison's disease, along with calcium and vitamin D, was still being given during the ultrafiltration study, the serum total calcium concentration was therefore, only slightly decreased to 8.6 mg. per 100 ml. and the ultrafilterable calcium was within normal limits (Table XVI, P. 112). Reduced values for ultrafilterable calcium were obtained in rest of the cases except E.G. In this case a marked reduction in serum protein was also found. It was presumed that the major loss in the bound fraction was reflected in the concentration of total calcium, as the ultrafilterable calcium was essentially normal. The serum protein concentration was within the normal limits in the rest of the cases studied. Tetany was observed in three of these cases (E.M., J.F., and E.G.). Poor correlation of the extent of tetany with either the total serum or ultrafilterable calcium has been reported by Hopkins, et. al. (1953) and by Fanconi and Rose (1958). This was found to be true in the present study. Patient J.F. had severe carpopedal spasms associated with a serum calcium of 6.4 mg. per 100 ml. and ultrafilterable calcium of 4.0 mg. per 100 ml; patient E.M.

however manifested only latent tetany when the serum and ultrafilterable calcium concentrations were lower than this, and case E.G. also showed latent tetany with a serum calcium of only 6.8 mg. per 100 ml., while the ultrafilterable calcium was well within the normal limits (5.7 mg. per 100 ml.). However in this case both serum and ultrafilterable magnesium were appreciably reduced, and hypomagnesaemic tetany has been reported by Greenwald, Dubin and Cardon (1963). It is possible therefore, that the tetany observed in this case may be due to the latter cause.

Conflicting results have been reported on the effect of vitamin D on the partition of calcium during the course of treatment of these cases. Anning, Dawson, Dolby, and Ingram (1948) stated that rise in diffusible calcium during vitamin D treatment occurred first. On the other hand Terepka, et. al. (1958) followed a case of idiopathic hypoparathyroidism before and during therapy and stated that a gradual rise in the total serum calcium with a small rise in the ultrafilterable fraction occurred, so that the percentage of ultrafilterable calcium fell significantly. After two weeks of treatment they observed an increase of 2.0 mg. per 100 ml. in the total serum calcium while the increase in ultrafiltrate calcium was only 0.5 mg. per 100 ml. Hopkins et. al. (1953) reported a proportionate rise in both ultrafilterable and protein-bound fractions and they stated that vitamin D does not appear to exert any direct effect on the ultrafilterable calcium of the serum. One case of idiopathic hypoparathyroidism (E.M.) who was followed up over five weeks, (Figure 7) was treated with oral calcium and vitamin D 100, 000 units per day. The results obtained in the present study are in agreement with those of

Terepka, et. al. (1958).

Vitamin D appears to have two actions 1) the antirachitic action, which appears to be the promotion of absorption of calcium and perhaps phosphate compounds, 2) the calciokintic action (or calcium moving action) closely resembles that of parathyroid hormone, but this effect appears only when high doses of vitamin D are continued for quite a long time. Hence the major part of additional calcium made available under the influence of vitamin D by its antirachitic action (which is probably the main action) is taken up by serum protein, as the factor (parathyroid hormone secretion is reduced) which control the ionic concentration of serum calcium is lacking in these conditions. However, later as the total calcium concentration rises, it becomes distributed more uniformly under the mass action effect.

Ultrafilterable and Protein-bound Calcium in Hypocalcaemia of Other Causes.

It is well known that hypocalcaemia is often associated with chronic renal disease accompanied by nitrogen retention. However, tetany is not very often seen. Although the serum protein tended to be low in half the patients studied in this group, this did not necessarily correlate with the total calcium concentrations. In all of the patients studied, the percentage of total serum calcium that was ultrafilterable was abnormally high (63 to 88%, Table XVIII, P. 116). Consequently, the concentration of ultrafilterable calcium was either normal or only slightly depressed. This probably accounts for the clinical observation that tetany occurs infrequently in renal disease. While the amount of

calcium bound to protein was decreased irrespective of the level of total calcium, the decrease in protein bound calcium may be responsible to some extent for the hypocalcaemia in cases where hypoproteinaemia was also present. Since in half of the patients the serum proteins were within normal limits, there must be other factors which may be responsible for the decreased protein bound fraction. A decrease in protein bound calcium may occur because of:-

1) a fall in concentration of serum protein, 2) a specific alteration in the binding ability of the protein and 3) changes in pH.

In the present study, the high percentage of ultrafilterable calcium whenever associated with hypoproteinaemia did not correlate well with the concentration of total protein or albumin in the serum. In contrast, hypocalcaemia in patients with hypoalbuminaemia associated with various other disorders (Table XVII, P. 115), seems to be entirely due to the loss of bound fraction, as the ultrafilterable calcium was well within the normal limits and was even slightly increased in three instances (up to 6.9 mg. per 100 ml.): with the development of hypocalcaemia the activity of parathyroid glands is increased, and the excess secretion of hormone as demonstrated in the present study and also by Lloyd and Rose (1958) should also reduce the binding capacity of plasma protein. This will also tend to raise the diffusible fraction. Parathyroid hyperplasia, and presumably increased secretory activity (secondary hyperparathyroidism) occur consistently in chronic renal disease. The accompanied acidosis in these cases also favour less binding of calcium to protein. All of these factors

are probably concerned in the maintenance of normal diffusible calcium in renal disease. The suggestion of Terepka, et. al. (1958) that the decreased protein-bound fraction was due to decreased ionised calcium, cannot be valid, as the ultrafilterable calcium is equivalent to the ionised calcium and was not lowered in these cases. Furthermore, if this was the case tetany should have been present in majority of such patients.

Serum and Ultrafilterable Magnesium in Hyperparathyroidism.

The serum and ultrafiltrate magnesium in all the pre-operative specimens from cases of hyperparathyroidism in the present study were within normal limits. Most of the patients had renal stones and radiological examination revealed skeletal decalcification in only three cases, J.R., S.L., and M.T. After removal of the parathyroid tumour patient S.L. developed hypomagnesaemia together with hypocalcaemia and tetany. The serum magnesium concentration began to fall on the third day after operation and declined progressively to reach a minimum on the tenth post-operative day (Figure 6, P. 99). A parallel decline was also noticed in the ultrafilterable fraction of magnesium. At this stage oral magnesium was started, but as this was not well tolerated by the patient, it was later administered intravenously. Both serum and ultrafilterable magnesium rose proportionally and remained within normal limits thereafter. The last estimation of serum and ultrafilterable magnesium four months after operation revealed normal values. Patient M.T. was not operated upon, and in patient J.R. only a slight fall within normal limits was observed post-operatively, this patient, however, died

a week later. None of the other nine patients developed hypomagnesaemia apart from a slight fall in the serum magnesium concentration post-operatively. However, a decreased serum magnesium concentration is frequently observed the day after an operation of any kind, which is a part of the general metabolic response to surgery, and is quickly corrected (Heaton (1963)).

Patients studied by Potts and Roberts (1958) and Hanna, et. al. (1961) who were found to develop hypomagnesaemia after partial parathyroidectomy had evidence of bone decalcification. As the skeleton contains about 65% of the total magnesium present in the body, the hypomagnesaemia, like hypocalcaemia can be satisfactorily explained in these patients by the transfer of mineral from the extracellular fluids to bone during the phase of remineralisation which follows removal of the tumour. However, as the ratio of calcium to magnesium in bone is approximately 50:1, the requirement for magnesium is very much less than for calcium and the hypomagnesaemia therefore is more readily corrected than hypocalcaemia. Apart from case S.L. where it seems also that this post-operative hypomagnesaemia is an indirect effect, the parathyroid glands do not appear to have an important function in the maintenance of normal magnesium metabolism. Heaton and Pyrah (1963) performed magnesium balance studies before and after the removal of parathyroid adenomas. An abnormally high urinary excretion of magnesium was found in three out of their nine patients with primary hyperparathyroidism. On this observation, together with findings of a general tendency for a reduction in the urinary magnesium excretion after parathyroidectomy without a significant change

in the serum magnesium concentration, they stated that an overactive parathyroid gland may affect magnesium metabolism. However, since the results in their three cases of hypoparathyroidism were within the normal limits, they were forced to the conclusion, as in the present studies, that the parathyroids may not be important for the maintenance of magnesium metabolism. In patients with hypomagnesaemia reported by Harmon (1956) and Agna and Goldsmith (1958), the hyperparathyroidism appears to be more severe than in the present series, as judged from the high serum calcium values.

Serum and Ultrafilterable Magnesium in Hypercalcaemia of Other Causes.

Hypomagnesaemia was encountered in 5 of the 8 cases of multiple myeloma and in only four of these five cases was the ultrafilterable magnesium decreased, so that the percentage ultrafilterability remained constant. In the fifth case J.J. (Table XIII, P. 104) hypomagnesaemia was associated with normal ultrafilterable magnesium, and elevated percentage ultrafilterability. A marked reduction in the serum albumin was also found. Therefore, hypomagnesaemia in this case was probably entirely due to the loss of bound fraction. Apart from this case, hypomagnesaemia was associated only with those cases where hypercalcaemia was present. It seems that this hypomagnesaemia is probably the direct result of hypercalcaemia, as reciprocal relationship between these two elements has been described by Hanna, MacIntyre, Harrison, and Fraser (1960). It seems likely that the renal excretion of magnesium was increased, which would tend to decrease the ultrafilterable fraction initially. Later,

since the magnesium-protein relationship obeys the law of mass action, the protein-bound magnesium would decrease to establish a new equilibrium with respect to the ultrafilterable fraction. If this process continued a low total and ultrafilterable magnesium would be expected. There is evidence to suggest that excess renal excretion of magnesium may be expected in hyperparathyroidism, alcoholism, and in multiple myeloma with accompanied hypercalcaemia (Prasad, et. al. (1961)). This explanation does presume that the equilibrium between ultrafilterable and bound magnesium is being reached slowly. This is also supported by the results obtained when hypercalcaemia was acutely produced in a normal human subject by the intravenous infusion of calcium gluconate (Table XV, P. 110). With increase in serum calcium a gradual decrease in serum magnesium occurred. The lowest concentration of serum magnesium (1.7 mg. per 100 ml. compared with a control concentration of 2.0 mg. per 100 ml.) was found when the serum calcium reached the peak value of 13.1 mg. per 100 ml. However, the concentration of magnesium in the ultrafiltrate was decreased appreciably (a drop of 0.3 mg. per 100 ml. in serum magnesium while 0.5 mg. per 100 ml. in ultrafiltrate magnesium occurred), and this decrease was far more than calculated on the basis of the mass action effect. As it has been stated above, a decrease in ultrafilterable magnesium will be expected initially and since the equilibrium between the protein-bound and ultrafilterable fraction is reached slowly, in such acute conditions the decrease in ultrafilterable magnesium should be evident. Normal values of both serum and ultrafilterable magnesium were found 24 hours later.

The same conclusions can be drawn from the results of Alcock

and MacIntyre (1964) who found that magnesium supplements to magnesium depleted rats increased the urinary excretion of calcium and produced a 20% depression of plasma concentration of calcium. Similar results were also reported by Smith (1961). In humans also, the administration of oral magnesium to magnesium deficient patients was followed by an increase in urinary calcium excretion (Hanna, et. al. (1960)).

In the present series in cases of hypercalcaemia without hyperparathyroidism (Table XIV, P. 106) the serum and ultrafilterable magnesium was within normal limits except in case J.S. who showed a slight increase. In these cases, results similar to above would be expected. There is evidence that magnesium absorption is increased slightly with vitamin D therapy (Meintzer and Steenbock (1955)), and it seems likely that in cases J.R., M.K., and M.W. receiving high doses of vitamin D, increased intestinal absorption of magnesium was presumably adequate to compensate for the increased urinary excretion. In case E.E. these findings may be due to renal impairment, as urinary retention occurred at one stage. However, these findings could not be accounted for in the case of J.S. and R.W. Renal involvement may be a possibility but apparently there was no such evidence.

Serum and Ultrafilterable Magnesium in Hypocalcaemic States.

As already discussed under hyperparathyroidism, the parathyroid glands are not concerned in magnesium metabolism to the same extent as in calcium metabolism. In hypoparathyroidism, normal values for both serum and ultrafiltrate magnesium were encountered in all the cases studied,

except case E.G. (Table XVI, P. 112), where decreased values for both serum and ultrafilterable magnesium were obtained. In this case the decrease in serum magnesium was probably mainly due to a marked decrease in the bound fraction, as the serum albumin concentration was also grossly reduced. Tetany was also present and since the ultrafilterable calcium was within normal limits, this was regarded as due to the hypomagnesaemia. Tetany was also present in one case O.B. of the group of patients with hypoalbuminaemia from various causes (Table XVII, P. 115). In this case also the ultrafilterable calcium was within normal limits but the serum and ultrafilterable magnesium were grossly reduced to 0.6 and 0.7 mg. per 100 ml. respectively. The tetany was again regarded as due to the hypomagnesaemia, particularly as both cases responded well to the magnesium supplements. In the rest of cases in this group the ultrafilterable magnesium was within normal limits and as serum total magnesium was slightly decreased due to the loss of protein-bound fraction the percentage ultrafilterability was elevated in some of these cases. Determination of serum and ultrafilterable magnesium and subsequent magnesium therapy may be worthwhile in these cases. The serum magnesium concentration did not correlate well with serum albumin concentrations. Although the hypoalbuminaemi effects the percentage ultrafilterability of magnesium, the absolute concentration of magnesium in the ultrafiltrate is also influenced by other factors such as renal function, thyroid function and dietary intake (Prasad, et. al. (1961)).

Variable results were obtained in the group of patients with chronic renal disease. In six of the cases serum magnesium was decreased

but the ultrafilterable magnesium was within normal limits. This decrease in serum magnesium has been attributed partly to the reduction in the protein-bound fraction due to the decrease in serum albumin concentration (where this occur) and partly to the renal tubular damage which might interfere with magnesium reabsorption (Prasad, et. al. (1961)). The maintenance of ultrafilterable magnesium can again be explained by low pH as in case of calcium.

In the other eight cases the serum magnesium ranged from normal to slightly elevated values. Increased concentration of ultrafilterable magnesium was always associated with increased serum magnesium. Hypermagnesaemia has been described in renal disease and has been attributed to the reduction in glomerular filtration rate (Robinson, Murdaugh, and Peschel (1959)): similar results in nephrosis in children and chronic glomerular nephritis has been reported by Silverman and Gardner (1954).

Hence several factors seems to be responsible for the miantenance of magnesium concentration in the serum and the ultrafiltrates i.e. serum protein, thyroid, and renal function are important factors in the presence of normal dietary intake. However, apparently in addition to above mentioned factors, other unknown factors are operating to maintain the concentration of ultrafilterable magnesium within a narrow range.

PART I

SUMMARY

The serum osmolality has been determined in 101 cases of hypochloraemia The cause of chloride depletion was due to vomiting, less often to continuous gastric suction, and rarely to diarrhoea or fistulae, associated with various clinical disorders. Patients were divided into three groups according to whether the serum osmolality was normal, decreased or increased.

The patients in group I succeeded in maintaining the osmotic pressure within normal limits in spite of chloride depletion by the compensatory retention of bicarbonate and urea.

This compensatory mechanism failed to operate in the group II patients where the loss of chloride eventually resulted in lowering the osmotic pressure.

In group III patients, the serum osmolality was elevated in spite of low chloride, partly due to bicarbonate retention but mainly due to urea retention. There was a relationship between the increased serum osmolality and urea concentration.

Although the chloride ions contribute 35% of the total osmotic pressure, there was no relationship between the serum chloride and the osmotic pressure in any of the three groups.

The patients in group III may simply be the extension of group I cases. The transition of one group to the other probably coincides with the onset of renal dysfunction. This kidney involvement is a reversible process and, therefore, must be distinguished from renal disease. The patients in group II are quite distinct from the cases in the other two groups; and were not in the early stages of biochemical

upset, and had not passed through the stages corresponding to other groups. Failure to retain urea and to maintain the osmotic pressure of extracellular fluids may be due to a defect in some mechanism presumably related to osmoregulatory centres, abnormal secretion of antidiuretic hormone or to renal tubular damage.

PART 2

SUMMARY

A new simple ultrafiltration technique has been described and the procedure outlined for ultrafiltration of blood serum.

The importance of the method of sample collection, duration of ultrafiltration and pH was investigated. The ultrafiltration results were affected considerably by large changes in pH, while duration of ultrafiltration had no effect.

For 20 healthy human subjects, the range for ultrafilterable calcium was found to be 55-66% of the total serum calcium.

Ultrafiltration data on sera from patients with hyperparathyroidism before and after removal of parathyroid adenomas, hypoparathyroidism, vitamin D therapy, multiple myeloma, hypercalcaemia due to other causes, and renal disease has been presented.

The serum ultrafilterable calcium was raised before operation in every patient with a functioning parathyroid adenoma even though the serum total calcium was within the normal limits. After removal of the adenoma, the serum ultrafilterable calcium always fell below normal. This fall was proportionately greater than the fall in the protein bound fraction. It was inferred that parathyroid hormone reduces the ability of serum protein to bind calcium. This was supported by the observation of the effect of intravenous calcium on a human subject where the suppression of parathyroid glands resulted in an increase in the binding ability.

The ultrafilterable calcium was reduced in cases of hypercalcaemia due to causes other than hyperparathyroidism. This was also associated with reduced ultrafilterability of inorganic phosphate, possibly due to the formation of non-filterable calcium-phosphate-protein complex.

Hence difficult cases such as those with functioning parathyroid adenoma associated with normal calcium values and cases with hypercalcaemia without parathyroid adenoma, the ultrafiltration results may be of some aid in the differiential diagnosis.

The serum ultrafilterable calcium was found to be diminished in hypoparathyroidism, but poor correlation of the extent of tetany with either total serum calcium or the ultrafilterable calcium was found to exist.

Hypoproteinaemia was generally associated with hypocalcaemia, which was accompanied by normal or, more frequently, a high percentage ultrafilterability of calcium. The hypocalcaemia present was due to the loss in the bound fraction.

In renal disease although the total serum calcium was low the percentage of ultrafilterable calcium was almost invariably high, regardless of the concentration of serum protein. The absence of tetany in these cases was not due to acidosis alone. Other factors e.g. hypoalbuminaemia and the specific alteration in binding capacity of serum protein by the excess secretion of parathyroid hormone also contribute.in maintaining the ultrafilterable calcium.

Normal values for both serum and ultrafilterable magnesium were found in hyperparathyroidism and in hypoparathyroidism. It was concluded that parathyroid glands play no part in magnesium metabolism.

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