THE RELATIONSHIP BETWEEN BONE MINERAL AND ITS IONS IN EXTRACELLULAR FLUID

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by

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ABSTRACT

It is generally believed that the mineral portion of the skeleton cannot have a thermodynamic solubility product because of the complex and variable nature of the bone salt. This opinion is examined in the light of our present understanding of bone salt composition and of previous investigations into bone salt solubility.

A simple experimental technique is described for studying the relationship between powdered whole bone and the concentrations of the ions of bone salt in solution.

It is shown that in bicarbonate-free buffer, bone powder establishes an ionic equilibrium in terms of the product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ within 24 hours. For human bone, the pK of the mean of this product is 26.39.

Studies with synthetic ultrafiltrate of plasma suggest that there is a direct relationship between the carbonate and phosphate concentrations in solutions in equilibrium with bone salt. In experiments using tris buffer with added bicarbonate, the bone salt yields constant calcium phosphate products within 72 hours, the pK of the mean being 26.30. It has not been possible to demonstrate any calcium carbonate ion product even when very high solid:solution ratios of about 500 gm bone per litre buffer are employed, but a direct relationship between the carbonate and phosphate concentrations in solution is apparent.

Equilibration studies with bone powder in normal serum suggest that the equilibrium could be operative *in vivo* provided the recently proposed views of Walser are accepted. Walser maintains that only about 53% of the total serum inorganic phosphate is in the non-associated 'free' form. With this assumption our experiments indicate that the ion product $\left[Ca^{++}\right]^3\left[PO_4^{\equiv}\right]^2$ in serum is not significantly different from that in synthetic media, the pK of the mean being 26.17.

Accepting Walser's views, which are indirectly confirmed by our experiments, it is then shown that bone mineral by a purely physico-chemical process, can maintain the concentrations of calcium and phosphate found in normal human extracellular fluids *in vivo*, provided the pH at the phase boundry is about 7.1. This value is roughly mid-way between intracellular and extracellular pH.

Brief studies with bone powder of children and several animal species suggest that there is a relationship between plasma phosphate levels *in vivo*, equilibrium phosphate concentrations with bone powder *in vitro* and the carbonate content of the bone. It is suggested that the high plasma phosphate concentrations in children may be due in part to the low carbonate content of young immature bone mineral.

A series of equilibration studies with bone from different sites in the same skeletons has yielded evidence which is compatible with the view that bone mineral behaves essentially as a calcium phosphate salt with divalent carbonate ion substitution for trivalent phosphate ion at the surfaces of the apatitic crystals. The evidence obtained is in conflict with the two other principal theories, namely that bone salt is a stoichiometric-carbonate-apatite, and alternatively that it is a two-phase system of calcium phosphate and calcium carbonate.

There are two principal biological implications of these studies. Firstly, calcium homeostasis can be explained in terms of a physico-chemical equilibrium state between the mineral of the skeleton and its ions in extracellular fluids. Secondly, slight variations in the pH of the bone environment lead primarily to changes in plasma calcium concentration, total inorganic phosphate concentration being a function of the bicarbonate concentration.

It is suggested that parathyroid hormone controls plasma calcium concentration by an effect on cellular metabolism which results in variations in the pH of the tissue fluid at the surface of the bone crystals.

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INTRODUCTION

"Qu'est-ce que ce milieu interieur? C'est la sang, non pas à la verité le sang tout entier, mais la partie fluide du sang, le plasma sanguin, ensemble de tous liquides interstitiels, source et confluent de tous les échanges elementaires".

Claude Bernard.

"The activity of cells is mediated by the ions in their environment. Many functions are directly or indirectly controlled by the electrolytes of the mammalian tissue fluids. Calcium ion in particular plays an important part in controlling the activities of membranes and produces profound effects on heart and skeletal muscle and on nervous tissues. Calcium homeostasis is essential for the integrity of the living organism".

Harold Hodge.

The relationship between bone mineral and its ions in solution in tissue fluids is of considerable importance in view of the role of calcium in mammalian physiology. Among the many functions of ionic calcium are the control of cell membrane permeability, neuro-muscular excitability and certain enzyme activities.

Calcium homeostasis is biologically essential, and disturbances leading to alterations in the concentration of ionic calcium in tissue fluids may have profound effects on the functional integrity of the individual. A fall in calcium ion concentration may result in tetany, and in severe instances, death; markedly elevated concentrations cause cardiac and intestinal disturbances and possibly serious renal damage.

The parathyroid gland plays a crucial part in the control of plasma calcium concentration. Clinical hyperparathyroidism, due to cell hyperplasia or a parathyroid tumour on the one hand, and administration of gland extract on the other are both associated with raised plasma calcium concentrations. Clinical hypoparathyroidism, which may occur as a result of accidental parathyroid extirpation at thyroidectomy, is diagnosed primarily by a fall in the plasma calcium concentration, and if the fall is substantial, there may be a consequent increase in neuromuscular excitability and tetany. The means by which the parathyroid hormone (124) brings about these changes is currently the subject of considerable speculation (92) but however it operates, the bone mineral is clearly involved.

It was thought at one time that the skeleton played a passive role in calcium homeostasis by permitting bone mineral, essentially an insoluble calcium salt of phosphoric acid (33), to dissolve up to a 'ceiling' determined by a physico-chemical solubility product. Thus a rise in plasma calcium concentration could be produced by a fall in plasma phosphate concentration (1), and vice versa. The well-established phosphaturic action of parathyroid hormone (2), which lowers the plasma phosphate concentration, was thought to be the primary action.

This relatively simple concept was discarded on three principal grounds. Firstly, Barnicot (6) and Chang (22) showed convincingly that the hormone exerted a direct effect on bone. Secondly, a solution of bone salt yielded ion products at equilibrium which varied with the experimental conditions, and no true thermodynamic solubility product could be demonstrated (77). Finally, equilibration of whole bone in serum or ascitic fluid at pH 7.4 showed that it took up calcium and phosphate until the calculated ion product in solution was below that normally found in tissue fluid, i.e. it appeared that extracellular fluid was normally 'supersaturated' with respect to the calcium phosphate of bone (80).

These observations will be discussed later, but for the moment we must note that most authorities concluded that a vital process had to be invoked to explain the anomalous concentrations of calcium and phosphate found in normal tissue fluids. The possible existence of a physico-chemical equilibrium state was repudiated.

The intention of the present work was to re-examine the question of bone solubility using better techniques than had been available in the past. It was hoped that an empirical approach to the problem might clarify the possible role of the skeleton in homeostasis, and this hope has to some extent been bourne out by events.

Evidence for a bone salt:tissue fluid equilibrium state.

The surface area of bone salt exposed to extracellular fluid is very great, and since no living membrane has ever been demonstrated between the two, it is probable that the phase boundry where salt and solution meet must be subject to some of the laws of physical chemistry.

It has been shown that calcium ion transit across this boundary is very rapid in both directions. In 1933, Hastings and Huggins (49) performed a classic experiment. They bled a dog, decalcified the blood and then returned it to the animal. They found that the blood calcium remained unchanged. In one case more than four times the extracellular fluid content of calcium was removed from the dog's blood. Even when 50% of the blood volume was replaced every ten minutes, it was extremely difficult to lower the plasma calcium to the point where tetany would occur, and the calcium concentration was restored promptly when the procedure was stopped.

In similar experiments, Patt and Luckhardt (115) have infused oxalate, Saffran and Denstedt (127) have infused citrate, and Copp

et al (23) have infused ethylene-diamine-tetraacetic acid in animals in order to reduce the plasma concentration of ionic calcium. They have all found that the skeleton gives up calcium in order to preserve homeostasis and that the normal calcium concentration is rapidly restored at the conclusion of the infusion.

There is also evidence of rapid and continuous exchange between the bone salt and its ions in the extracellular fluid (105, 106). The incorporation of bone-seeking isotope into the skeleton demonstrates that this is a continuous isoionic and heteroionic thermodynamic exchange between bone mineral and tissue fluid. These processes are quite independent of bone metabolism and can be demonstrated in dead bone <u>in vitro</u> (14).

Attempts to measure the rate of new bone formation with isotopes have been complicated by the rapid uptake of labelled calcium or strontium due to purely physical exchange processes (14, 111). The exchangeable calcium pool in man 24 hours after an injection of tracer is calculated to be 5 gms of calcium. Only about 30% of this calcium is in the extracellular fluid, and therefore approximately 0.2% of total skeletal calcium is in immediate equilibrium with the calcium of tissue fluids.

Any hopothetical calcium homeostatic mechanism must take these facts into account. Some form of physico-chemical solubility product

might well exist between bone mineral and its ions in tissue fluid, even if an independent vital process has to be postulated for the control of plasma calcium concentration.

The nature of bone salt.

McLean and Urist declare in their important monograph (96) that ...

> "the concept of a solubility product is meaningless without reference to a known and homogeneous solid phase of constant and comparatively simple ionic composition. The bone mineral does not meet these requirements."

This statement may well be true in the strictest physico-chemical sense, and it is therefore appropriate to consider at this point the nature of bone salt. This has been studied in the past by X-ray diffraction and other classical crystallographic techniques as well as the more conventional methods of analytical chemistry.

X-ray diffraction technique is a precise means of measuring the lattice parameters of crystals. In the ideal case it is possible not only to build up a general picture of the crystal structure, but also to identify the location of each major component. To obtain such resolution it is necessary to have a highly purified material, composed of large crystals, and with few faults, dislocations or other lattice defects. In the case of bone mineral we have very small, imperfect crystals of highly variable composition, contaminated

with ions and molecules adsorbed on the crystal surface or incorporated by heteroionic exchange, and which are laid down in an organic matrix. Inevitably, the resolution of the method is impaired, and although it is possible to identify the lattice type and exclude the possibility of carbonate ion exchange for hydroxyl ion within the lattice, it is not possible to distinguish between isomorphic exchange of carbonate ion for phosphate ion at the surface or in the interior of the crystal. Because of this, scope has been provided for several conflicting interpretations of bone structure.

At this point, we should note that in order to avoid some of the difficulties outlined above, crystallographers have attempted to isolate the mineral fraction of bone by extracting the organic matrix chemically, a process fraught with risk to the more labile components of the bone salt. Alternatively, they have turned to dental enamel, a material presumptively very similar in structure, but containing a much smaller proportion of organic matrix. In many instances purely synthetic analogues have been studied and the physical nature of bone inferred by analogy.

The application of X-ray diffraction technique to the study of bone mineral by De Jong in 1926 was one of the more significant advances in the study of the structure of bone salt (31). De Jong

was able to show that the mineral was crystalline in nature, and that its lattice was similar to that of the apatite minerals.

The word apatite was given to a series of naturally occurring calcium phosphate minerals by Verner in 1790, and it is a particularly apt choice in this context in that it is derived from the Greek 'to deceive'. Certainly the structure of these compounds has aroused bitter controversy, persisting until the present day.

There is general agreement that the bone salt crystals are very small, rod-like and with a diameter of about 25A to 75A, averaging about 50A. However, the lengths of individual crystals have been variously reported from 50A up to about 4,000A. This last figure is much greater than the band periodicity of the collagen fibres (640A) which form the organic matrix of bone.

These discrepancies may be explained by the work of Molnar (100) who has suggested that bone crystals are formed of chains of micro-crystals. The individual units are said to be of almost colloidal dimensions with a length of about 50A.

The chemical nature of the crystals of bone salt has been studied since the early 19th Century. The earlier findings have been recently reviewed (77) but in brief, they were found to be quite inconsistent due to continued failure to appreciate the highly variable composition of bone mineral. The lability of portions of bone salt during removal or destruction of the organic matrix was ignored, as was the intimate association of bone with substantial amounts of tissue fluid and phosphate-rich marrow (38).

It was readily shown that bone mineral was a calcium phosphate containing appreciable amounts of carbonate, magnesium and sodium as well as traces of many other inorganic substances. It was subsequently shown that citric acid represented as much as 1% of bone by weight (32).

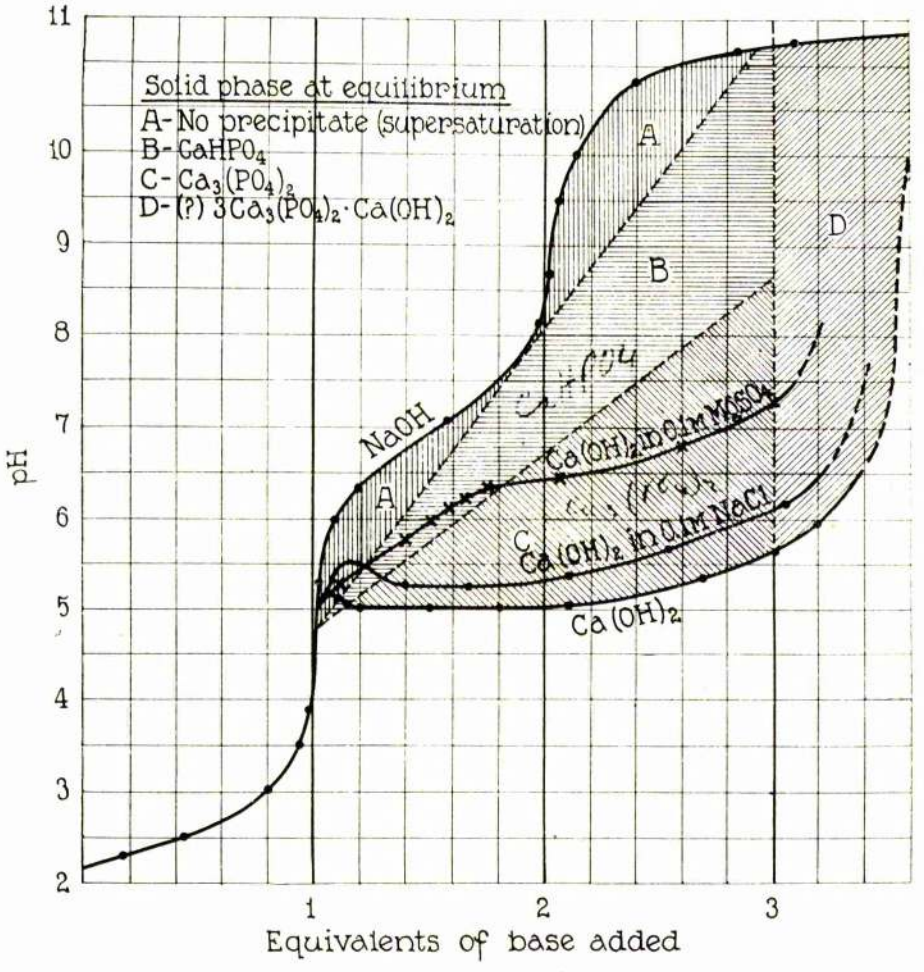
Synthetic calcium phosphates obtained by precipitation are typically characterised by determination of the Ca/P Molar ratio. As $Ca(OH)_2$ is added to phosphoric acid, the precipitates obtained become more and more basic and have higher Ca/P ratios as the pH rises. In the past it was thought that a limited number of specific stoichometric compounds could be obtained by this method, and Fig. 1 illustrates the phase diagram commonly believed to represent the properties of this system. Fig. 1 is reproduced from Holt et al (60) and shows the potentiometric titration curves for the addition of $Ca(OH)_2$ and

NaOH to phosphoric acid. It can be seen that these authors have specified the precipitated solid phases $CaHPO_4$, $Ca_3(PO_4)_2$ and "(?) 3 $Ca_3(PO_4)_2.Ca(OH)_2$". These 'salts' have theoretical Ca/P Molar ratios of 1.00, 1.50 and 1.67 respectively.

More recently this view has required modification, and it is now suggested (87, 119) that a whole series of synthetic apatites can be obtained with continuously increasing Ca/P Molar ratio of from 'octocalcium phosphate', Ca/P = 1.33 to 'calcium hydroxyapatite, Ca/P = 1.67.

Chemical analysis of whole bone for the calculation of the Ca/P ratio yielded results dependent primarily on the assumptions made. For instance, before the discovery of citric acid in bone, it was normal practice to assume that all the CO_2 liberated from bone by strong acid was from carbonates, and that the carbonate fraction of bone salt was mainly calcium carbonate. Thus the amount of calcium equivalent to released CO_2 was subtracted from total calcium to yield net calcium content. Similarly it was usual to assume that all the magnesium and sodium in bone salt existed as phosphate, e.g. $Mg_3(PO_4)_2$. Thus equivalent amounts of phosphate were subtracted to yield net phosphate contents.

It is now clear that several ions can substitute within the apatite lattice by heteroionic exchange (106,107,125,136,154,108) and



Titration curves of H_3PO_4 at 38°C.

Figure 1 **Titration curves of H_3PO_4 at 38°C. (Reproduced from HOLT et al.[2] J. biol. Chem. 64, 513, 1925).**

that whole molecules (e.g. citrate) may be adsorbed on to the surface of the crystals (106,4,143). Since the crystals are minute, the surface area relative to volume is very great and consequently surface exchange phenomena might well affect the apparent composition of the mineral.

In the main, the most satisfactory experimental evidence on bone composition comes from the X-ray diffraction studies despite the inherent difficulties in interpretation we have mentioned. In 1930, Naray-Szabo (102) characterised the naturally occurring mineral fluorapatite $Ca_{10}(PO_4)_6F_2$. In later years it was shown by Hodge (56), Neuman (106) and others that this substance was isomorphous with the 'hydroxyapatite' detected in bone salt. In 1938, Thewlis (142) in an extensive study, produced evidence to support the view that teeth were also composed principally of hydroxyapatite. This opinion on bone salt structure is dominant to-day and is widely accepted (96). However, whole bone exhibits such an apparent lack of consistent stoichiometry that several other hypotheses have been advanced with vigour. Several deserve consideration as they are supported by considerable circumstantial experimental evidence, although none of it can be said to be decisive.

Theoretically, hydroxyapatite has a Ca/P Molar ratio of 1.67: reported Ca/P ratios for whole bone have been as low as 1.5.

Dallemagne (26) has therefore postulated the existence of a hydrated tricalcium phosphate (T.C.P.H.) and has proposed the formula $3\ Ca_3(PO_4)_2.H_2(OH)_2$. This material is structurally unstable in the absence of matrix and according to Dallemagne when isolated in aqueous solution it tends to change into hydroxyapatite. Many authorities, on the other hand, deny the separate identity of T.C.P.H. (21,53,85, 96,86).

Posner has modified Dallemagne's concept to include a whole range of what he has called 'pseudo-apatites', 'calcium-deficient apatites' or 'defect apatites' with the general formula:- ... $Ca_{10-x}H_{2x}(PO_4)_6(OH)_2$ (122,117,118,119). In this notation, Dallemagne's T.C.P.H. is one special case where $x = 1$.

The view that calcium ion can exchange for hydronium ion in the apatite lattice (56) is unacceptable to Posner, and in order to explain the structure of his calcium-deficient apatites he has produced infra-red spectrophotometric data in support of hydrogen bonding as the means of maintaining electro-neutrality within the crystal (138). Winand and Dallemagne dispute Posner's interpretation of the spectrophotometric data (162).

The most controversial problem of bone salt structure concerns the location of the carbonate radicle.

Bone carbonate is labile in nature. Bergstrom (10) has shown a decrease in bone carbonate after a 4-hour intraperitoneal dialysis against ammonium chloride. Sobel et al (133) have demonstrated that the $PO_4^{\equiv}/CO_3^{=}$ ratio in bone is a reflection of the same ratio in plasma. As early as 1932, Irving and Chute (66) found that substantial amounts of carbonate were lost from the bones of rats undergoing acute acid loads. It has also been shown in vitro that weak acids remove the carbonate at a more rapid rate than the phosphate (24,27,29,71,82,105). All these findings suggest that carbonate is 'more available' than phosphate in bone.

On the other hand, there is no good physical evidence for the separate existence of a second phase of calcium carbonate in bone salt. Intimate mixtures of synthetic apatites and calcium carbonate have been studied by X-ray diffraction and the carbonate component can be readily detected at concentrations well below that of carbonate in bone (144,145). Natural occurring minerals containing admixed carbonate are rare, but when present the carbonate phase is readily detected by the polarising microscope. No such evidence has been obtained in the case of bone mineral (89).

McConnell takes the view that bone is a carbonate apatite in which the $CO_3^{=}$ radicle is an integral part of the lattice. The mineral dahlite, which exists naturally, is such a substance. In

this instance there has been intra-crystalline isomorphic exchange of $CO_3^{=}$ for $PO_4^{\equiv}$. Francolite, another naturally occurring mineral, is an example of a carbonate-fluoroapatite. McConnell's suggestion is circumstantially reinforced by the demonstration that under experimental conditions the 'precipitation' of 'hydroxyapatite' from calcium phosphate solutions can be more readily induced in the presence of free carbonate (90). The carbonate is produced by the action of the enzyme carbonic anhydrase on gaseous CO_2, and formation of the precipitate at critical concentrations of calcium and phosphorus can be suppressed by inhibiting the enzymatic hydrolysis with sulphanilamides. McConnell rests his case principally on the fact that since there are natural carbonate-fluorapatites, carbonate hydroxyapatites, and sodium and potassium containing carbonate-apatites (dehrnite, lewistonite), all of which liberate CO_2 on acidification and all of which have carbonate radicles substituted within the apatite lattice, there is at least a case for supposing that the same basic structure might occur in biological minerals. In further support, McConnell cites the evidence of Francois that bone does <u>not</u> have a pronounced ability to exchange its C^{12} for C^{14} (39).

On the other hand, Thewlis predicted on mathematical grounds that the carbonate ion was too large to exchange for phosphate <u>within</u> the lattice of hydroxyapatite without distortion (142), and his calculations have recently been verified experimentally by Trautz (146). Trautz measured the crystal parameters of synthetic carbonate-

hydroxyapatite, and claims that they are quite distinguishable from those of hydroxyapatite itself.

Neuman and Neuman (106) have reviewed most of the pertinent data and have attempted to reconcile the various hypotheses. They stated:- ...

> "We shall assume .... that all (these) investigators are essentially correct. Possibly the differences in emphasis and interpretation may be attributed to differences in the method of preparation or origin of this 'deceptive' substance. Geologic speciments .... can be expected to have few internal defects. Fusion of crystals would minimise the importance of surface reactions. On the other hand, freshly formed precipitates have a great many voids and imperfections .... Here defects and surface substitutions dominate the aberrant stoichiometry".

At the time of writing this statement would appear to be a reasonable compromise, and failing further advances in physical investigative techniques, is as much as can be said about the structurel nature of bone salt as presently understood.

<u>The solubility of bone mineral</u>.

There are many references to the subject of bone solubility. The conclusions reached have been generally unsatisfactory. The existence of a thermodynamic solubility product for bone salt has been hotly denied, yet it is desirable to have some broad concept

with which to approach the problem of normal and pathological calcification and their relationship to the plasma calcium and inorganic phosphate concentrations.

With few exceptions, it has been normal practice to study inorganic 'calcium phosphate' salts, and infer the findings to apatites in general and bone mineral in particular. Levinskas (77), states:- ...

> "Except for the presence of carbonate, the solid phase '(i.e. bone salt)' ... may be regarded as a slightly impure, basic calcium phosphate. For this reason, the chemical properties of the basic calcium phosphate system and the bone mineral are considered to be interchangeable..."

As we shall see later, bone mineral behaves quite differently from synthetic calcium hydroxyapatite and bone carbonate has a crucial effect on the ionic equilibria established in an aqueous system.

The available data have to be interpreted in the light of the modern theories of solution chemistry, which have been admirably summarised by Neuman and Neuman (106). These authors point out that the postulation of a true thermodynamic solubility product for bone mineral in plasma would require a definitive formula for the solid phase, and a knowledge of the activity coefficients of the relevant ions in plasma. As we have seen, the true nature of bone is obscure, and it is also true that the relevant activity coefficients are uncertain.

The law of mass action on which the theory of solution of sparingly soluble salts is based requires that when an ion is present in the structural formula of the solid phase in numbers greater than one, then the Molar concentration of that ion is raised to the power of that number in determining the ion product. The ion product is the product of all the ion concentrations raised to the powers corresponding to the numbers of the ions in the formula, and if a true equilibrium state exists, the product is a constant. Thus the ion product for the solid phase calcium acid phosphate, $CaHPO_4$ is $[Ca^{++}][HPO_4^{=}]$ and for tricalcium phosphate, $Ca_3(PO_4)_2$(?), the ion product is $[Ca^{++}]^3.[PO_4^{\equiv}]^2$. * To obtain the thermodynamic solubility product the 'observed concentrations' are corrected to 'effective concentrations' by incorporating activity coefficients before calculating the product. When the ion activities are unknown, the solubility equilibrium can be expressed by the product of the observed ionic concentrations, each raised to the relevant power, provided that in every case the <u>ionic strength</u> of the solution is specified and <u>constant</u>. In this way non-specific ion interaction effects on solubility are kept <u>constant</u> but not allowed for. Thus an ion product measured at $\mu = 0.15$ (the approximate ionic strength of body fluids) is of relevance only in solutions at that ionic strength.

* In all cases where square brackets are employed - they indicate concentrations in moles per litre by convention.

Many attempts have been made to calculate the solubility product of tri-calcium phosphate (11,18,35,45,75,70,81,80,99,129,136) but Holt, La Mer and Chown (57,58,59,60) were among the first to study the problem with a view to considering the physiological implications. Their results indicated the paradoxical situation that normal human plasma is supersaturated with respect to tertiary calcium phosphate "to the extent of more than 200%". Much of the apparent supersaturation (but not all) was in fact due to the protein-binding of calcium in plasma (93,95,126).

At one time, it was suggested (7) that the first phosphate salt formed by precipitation from tissue fluid was $CaHPO_4$ and that this salt subsequently underwent a re-organisation into the tri-calcium phosphate form. There were several supporters of this suggestion (59,60,129,136,130,82), which is now considered untenable at physiological pH.

Logan and Taylor (81) reported that the solubility product for bone expressed as $[Ca^{++}]^3[PO_4^{\equiv}]^2$ increased as the solid:solution ratio diminished. They suggested that the 'true value' at infinite dilution expressed as a pK_c^* was about 23.1. The biological significance of

* In this thesis, the pK_c value is the common logarithm of the reciprocal of the ion product. The pK_{sp} value includes corrections for ionic activity.

'infinite solution' was unstated. It was later shown quite convincingly that these results were produced by a failure to equilibrate due to the small amount of solid phase employed (43,72).

At solid:solution ratios greater than 200 mg per litre, Logan and Taylor (81) found that the pK_c ion product for bone was of the order of 27.71. Holt et al (59) obtained a pK_c of 27.75 with 900 mg bone per litre and Sendroy and Hastings (129) 26.36 with 10 gm per litre.

In 1950, Neuman (103) published data from which it has been possible to calculate the $[Ca^{++}]^3[PO_4^{=}]^2$ ion product in a solution bathing bone powder. The value recalculated from his one experiment (from undersaturation) was 26.11 (pK_c).

The outstanding feature of all the reported studies (see 77) is the great variety of techniques employed, and the wide range of pK_c and pK_{sp} values obtained.

In 1953, Levinskas published his studies on the solubility of a "highly characterised synthetic hydroxyapatite". His values for its 'thermodynamic solubility product (pK_{sp})" ranged from 28.11 to 37.34 in a series of 60 experiments designed to determine the effect of common ions, the effect of pH, and the effect of solid: solution ratio. His experiments covered a pH range from 5.67 to 8.59 in unbuffered NaCl and KCl solutions. He concluded that

> "Even under rigorously controlled conditions, the presumably constant, well characterised solid phase...does not follow the laws of solubility for sparingly soluble compounds".

Levinskas, however, does provide the data for 6 experiments in buffered solutions at pH 7.27 to 7.33 (77). The range of product (pK_{sp}) was only 30.52 to 30.65 although in three instances equilibrium had been approached from supersaturation. The corresponding mean value for the uncorrected ion product (pK_c) was 23.84 as recalculated from his original data. It would appear that, in a buffered system at any rate, some kind of equilibrium had been achieved and that the ion product at equilibrium was much greater than that found when whole bone was employed.

An anomalous situation now existed. It was generally accepted that plasma was supersaturated, in terms of calcium and phosphate, with respect to bone powder. At the same time it appeared that bone was less soluble than synthetic calcium phosphate. As an example, it has been noted that cloudy saturated calcium phosphate solutions clarifies when left standing over bone powder (110).

The whole question was re-opened in 1957 when Nordin (110) prompted by Neuman's experiment with bone powder from undersaturation (103), equilibrated powdered calf bone in buffers over the pH range

6.6 - 7.8. He observed fairly consistent ion products. In terms of $CaHPO_4$ the ion product expressed as a pK_c varied from 6.2 to 6.7 and in terms of $Ca_3(PO_4)_2$ from 24.7 to 26.7.

In this paper, Nordin introduced an original idea to explain the discrepancy between the ion product maintained by bone in vitro, and that found in normal tissue fluids. It had previously been assumed, when calculating the plasma ion product, that the pH to be used in determining the proportion of total inorganic phosphate present as the trivalent ion species ($PO_4^{\equiv}$), was that of circulating tissue fluid. Nordin rightly pointed out that the relevant pH was that of the bone surface. He estimated that if bone were at a pH of 6.6 to 6.8, then its ion product would be identical to that found in tissue fluids.

The present work has arisen out of this suggestion. A series of experiments were planned to confirm Nordin's findings using human bone. From there the work progressed to cover other aspects of the problem, including the effect of bone carbonate and tissue fluid carbonate on the equilibrium. The central theme has been the idea that a physico-chemical equilibrium between bone mineral and its ions in solution is a credible phenomenon. The experiments described were performed as a logical series, each intended to clarify and amplify the results of its predecessor. It has therefore been necessary to discuss each portion of the work in context. In the conclusion an

overall review of the experimental findings is presented with an attempt to place them into perspective.

E X P E R I M E N T A L

METHODOLOGY

1.1. Symbols and conventions employed.

A number of abbreviations and symbols are employed conventionally in the text as follows:-

$[\ ]$	Square brackets are used to represent concentration. Ion concentrations are usually expressed in millemoles per litre, i.e. M x 10^{-3}.
$[Ca]$	This symbol is used to indicate total calcium concentration, i.e. if referring to calcium content of plasma it includes protein-bound, associated (e.g. citrated) and ionic calcium fractions.
$[Ca^{++}]$	This symbol, strictly speaking, represents the free or ionic calcium concentration. Unless specifically indicated however it will represent, in this thesis, diffusible or dialysible calcium capable of penetrating a cellophane membrane with an average pore diameter of 24 Angstrom units.
$[P]$	This symbol is used to indicate the total inorganic phosphate concentration and includes all ion species of phosphoric acid, bound and free.
$[HPO_4^{=}]$	This symbol represents the concentration of the divalent ion species of phosphoric acid and is calculated from $[P]$, the pH and the second dissociation constant of phosphoric acid.

$[PO_4^{\equiv}]$ — This symbol represents the concentration of the trivalent ion species of phosphoric acid and is calculated from P , the pH and the third dissociation constant of phosphoric acid.

$[CO_2]$ — This symbol is used to represent the total CO_2 evolved from a solution when it is acidified to pH 1·5 with dilute sulphuric acid.

$[HCO_3^{=}]$ — This symbol represents the concentration of bicarbonate ion and is calculated from CO_2 , the pH and the first dissociation constant of carbonic acid.

$[CO_3^{=}]$ — This symbol represents the concentration of divalent carbonate ion and is calculated from CO_2 , the pH and the second dissociation constant of carbonic acid.

pK — Ion products are usually expressed as a pK for convenience. That is, the common logarithm of the reciprocal of the product is employed

e.g. $[Ca^{++}]^3[PO_4^{\equiv}]^2 = 4.1 \times 10^{-27}$

can be written as a negative logarithm

$$-\log [Ca^{++}]^3[PO_4^{\equiv}]^2 = 26.39 = pK$$

pK_c — This convention is used when a product of ionic concentrations in moles per litre, uncorrected for ionic strength by incorporation of activity co-efficients, is expressed as a negative logarithm.

pK_{sp} — This convention is used to represent an estimate of the thermodynamic solubility product and has been calculated from the pK_c by a method recommended by Nancollis, G., (personal communication) as follows:-

$$K_{sp} = K_c \ (f_z^{\,n} \cdot f_{z'}^{\,n'}) \qquad (1)$$

Where

$$-\log f_z = \frac{AZ^2 \sqrt{\mu}}{1 + BD \sqrt{\mu}} \qquad (2)$$

In which A and B are constants, z is the valency, D the molecular diameter and μ the ionic strength.

When $z = 1$ $\quad -\log f_z = + \frac{1}{2} \sqrt{\mu}$ (3)

$z = 2$ $\quad = + 2 \sqrt{\mu}$ (4)

$z = 3$ $\quad = +4.5\sqrt{\mu}$ (5)

For $Ca_3(PO_4)_2$

$$K_{sp} = \left[Ca^{++}\right]^3 \cdot \left[PO_4^{\equiv}\right]^2 \ X \ (f_2^{\,3} \cdot f_3^{\,2}) \qquad (6)$$

i.e. $$K_{sp} = K_c \ (f_2^{\,3} \cdot f_3^{\,2}) \qquad (7)$$

Take logarithms of reciprocals

$$pK_{sp} = pK_c - 3 \log f_2 - 2 \log f_3$$

Substitute for f_2 and f_3 in (4) and (5)

$$pK_{sp} = pK_c + 6 \sqrt{\mu} + \sqrt{9} \ \mu = pK_c + 15\sqrt{\mu} \qquad (8)$$

Similarly for $CaCO_3$

$$pK_{sp} = pK_c + 4 \sqrt{\mu} \qquad (9)$$

A standard correction which is a little more sophisticated replaces μ in equations (8) and (9) with the expression:-

$$\left[\frac{\sqrt{\mu}}{1+\sqrt{\mu}} - 0.2\,\mu\right]$$

N.B. No formal proof will be presented of the above expressions, but their validity in our systems will be tested empirically.

Throughout, the terms 'equilibration from undersaturation' and 'equilibration from supersaturation' will mean with respect to calcium and inorganic phosphate solely, unless otherwise specified. Undersaturation usually, but not invariably, implies that no calcium or phosphate was present in the buffer prior to addition of bone mineral.

1.2. Application of dissociation constants.

The concentrations of the free ion species $HPO_4^{=}$, $PO_4^{\equiv}$, HCO_3^{-} and $CO_3^{=}$ have been obtained by calculation from the total inorganic phosphate or total carbonate concentrations, and the pH of the samples. Dissociation constants of the relevant acids are taken from Part II, (Inorganic Ligands), of Special Report No. 7 of the Chemical Society, London, entitled 'Stability Constants of Metal-ion Complexes' compiled by J. Bjerrum, G. Schwarzenbach and L.G. Sillen.

The following constants have been employed:-

Phosphoric acid	K_1	10^{-2}	=	pK - 2.0
	K_2	10^{-7}	=	pK - 7.0
	K_3	4×10^{-13}	=	pK - 12.4
Carbonic acid	K_1	4.57×10^{-7}	=	pK - 6.34
	K_2	5.62×10^{-11}	=	pK - 10.25

The proportions of the total concentration appearing as a particular species were derived as follows:-

For phosphoric acid:-

(1) $K_1 \dfrac{\left[H^+\right] \cdot \left[H_2\ PO_4^-\right]}{\left[H_3\ PO_4\right]}$ (2) $K_2 = \dfrac{\left[H^+\right] \cdot \left[HPO_4^=\right]}{\left[H_2\ PO_4^-\right]}$ (3) $K_3 = \dfrac{\left[H^+\right] \cdot \left[PO_4^{\equiv}\right]}{\left[H\ PO_4\right]}$

and $\left[P\right] = \left[H_3PO_4\right] + \left[H_2PO_4^-\right] + \left[HPO_4^=\right] + \left[PO_4^{\equiv}\right]$ (4)

From (3) $\left[HPO_4^=\right] = \dfrac{\left[H^+\right] \cdot \left[PO_4^{\equiv}\right]}{K_3}$ (5)

From (2) $\left[H_2PO_4^-\right] = \dfrac{\left[H^+\right]}{K_2} \cdot \left[HPO_4^=\right]$ (6)

Substitute (5) in (6) $\left[H_2PO_4^-\right] = \dfrac{\left[H^+\right]}{K_2} \cdot \dfrac{\left[H^+\right]\left[PO_4^{\equiv}\right]}{K_3}$

$= \dfrac{\left[H^+\right]^2 \cdot \left[PO_4^{\equiv}\right]}{K_2K_3}$ (7)

From (1) $\left[H_3PO_4\right] = \dfrac{\left[H^+\right]}{K_1} \cdot \left[H_2PO_4^-\right]$ (8)

Substitute (7) in (8) $\left[H_3PO_4\right] = \dfrac{\left[H^+\right]}{K_1} \cdot \dfrac{\left[H^+\right]^2\left[PO_4^{\equiv}\right]}{K_2K_3}$

$= \dfrac{\left[H^+\right]^3\ \left[PO_4^{\equiv}\right]}{K_1K_2K_3}$ (9)

Substitute (5) (7) (9) in (4)

$$P = \frac{\left[H^+\right]^3 \cdot \left[PO_4^{\equiv}\right]}{K_1K_2K_3} + \frac{\left[H^+\right]^2\left[PO_4^{\equiv}\right]}{K_2K_3} + \frac{\left[H^+\right]\left[PO_4^{\equiv}\right]}{K_3} + \left[PO_4^{\equiv}\right] \quad (10)$$

and $$\left[PO_4^{\equiv}\right] = \left[P\right] \cdot \frac{K_1\ K_2\ K_3}{\left[H^+\right]^3 + K_1\left[H^+\right]^2 + K_1K_2\left[H^+\right] + K_1K_2K_3} \quad (11)$$

Substitute (11) in (5)

$$\left[HPO_4^=\right] = \left[P\right] \cdot \frac{\left[H^+\right]\ K_1\ K_2}{\left[H^+\right]^3 + K_1\left[H^+\right]^2 + K_1K\left[H^+\right] + K_1K_2K_3} \quad (12)$$

For carbonic acid:-

$$(1) \quad K_1 = \frac{[H^+][H\,CO_3^-]}{H_2\,CO_3} \qquad (2) \quad K_2 = \frac{[H^+][CO_3^=]}{[HCO_3^-]}$$

$$\text{and} \quad [CO_2] = [H_2CO_3] + [HCO_3^-] + [CO_3^=] \tag{3}$$

Substitute (1) (2) in (3)

$$[CO_2] = \frac{[H^+][HCO_3^-]}{K_1} + \frac{[H^+][CO_3^=]}{K_2} + [CO_3^=] \tag{4}$$

Substitute (2) in (4)

$$[CO_2] = [CO_3^=] \cdot \left[\frac{[H^+]^2 + [H^+]\,K_1 + K_1\,K_2}{K_1 K_2} \right] \tag{5}$$

$$\text{i.e.} \quad [CO_3^=] = [CO_2] \cdot \frac{K_1\,K_2}{[H^+]^3 + [H^+]\,K_1 + K_1\,K_2} \tag{6}$$

Similarly
Substituting (2) in (5)

$$HCO_3^- = CO_2 \cdot \frac{[H^+]\,K_1}{[H^+]^2 + [H^+]\,K_1 + K_1 K_2} \tag{7}$$

1.3. Buffers employed:specific ion effects.

The equilibration of bone powder in a buffer system similar to that as described later, results in a transfer of calcium and phosphate ions through the dialysis membrane. At equilibrium the concentrations of these ions are measured and the ion product calculated. This product is only valid, however, if, among other things, no complex has been formed between any of the ions of the solid phase in solution, and the components of the buffer. Calcium is known to form complexes readily.

The buffers employed were 0.15 M tris-(hydroxy methyl)-amino-methane adjusted to the required pH with 0.15 M HCl for the pH range 7.0 - 7.8 or alternatively 0.15 M cacodylic acid adjusted by the addition of 0.15 M potassium cacodylate for the pH range 6.2 - 7.0. The potassium salt was used rather than the sodium salt as it is known that potassium is not heteroionically exchangeable with bone salt calcium, its ionic diameter being greater than can be accommodated within the lattice, whereas sodium undergoes exchange isionically and possibly heteroionically. In both cases, the resulting ion strength is 0.15 μ, although the comparatively small concentrations of calcium and phosphate were disregarded in this calculation.

The absence of specific ion association was proven by electrometric titration of the buffers with NaOH and $Ca(OH)_2$ in succession. Samples of tris or cacodylate (5 ml 0.15 M) were titrated with 0.0253N NaOH and 0.025N $Ca(OH)_2$ respectively. The results are shown

in Fig. 2 and Table 1. It can be seen that no appreciable specific ion effect can be demonstrated with these buffers.

In later experiments a synthetic ultrafiltrate of plasma was employed. No attempt was made to determine possible ion associations in such a complex solution.

1.4. Analytical methods.

Calcium

A complexiметric titration with ethylene-diamine-tetraacetic acid (EDTA) was employed, using ammonium purpurate as indicator. There are many variants of this method, but the one employed was specially developed in the laboratory for bone equilibration studies. It is simple, very reliable and will be described in detail.

In the presence of free calcium ions, ammonium purpurate forms a weakly associated complex with a distinctive optical absorption. Titration with EDTA, a potent chelator of calcium, results in the binding of calcium, and dissociation of the complex. Thus a change in optical density (O.D.) of the dye solution can be obtained as an end-point. Protein-bound calcium, citrated calcium and dye-bound calcium are complexed in turn, and the O.D. change on completion is highly characteristic.

The details of the method are as follows:-

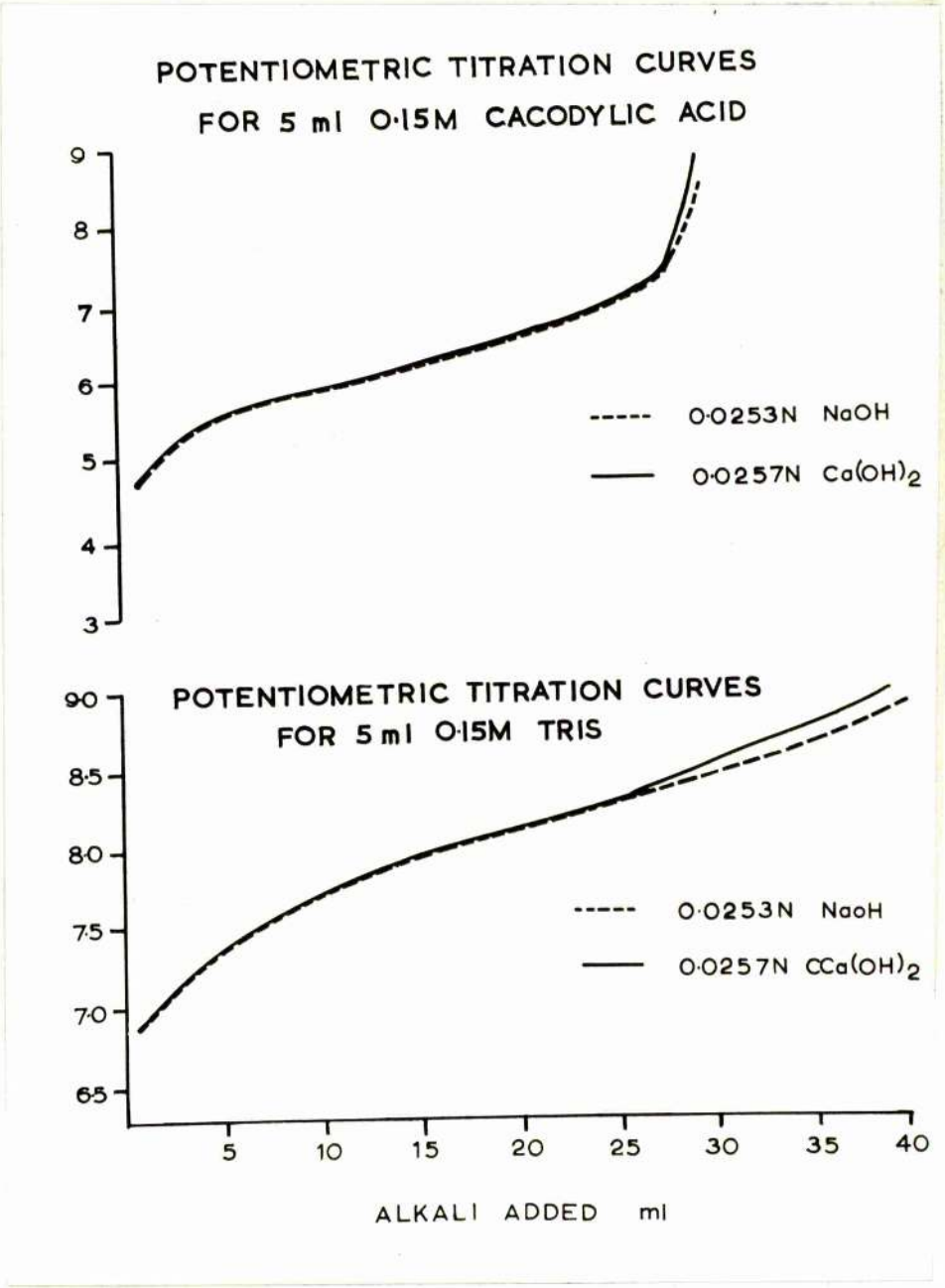


Figure 2. **Potentiometric titration curves for cacodylate and tris buffers at 18°C.**

Stock solutions.

2 N. NaOH
0.6% Ammonium purpurate (keep in refrigerator)
0.36% Ethylene diamine tetraacetic acid.

Working solutions.

0.1 N NaOH (5 ml stock diluted to 100 ml)
0.0027% Ammonium purpurate (0.75 ml stock diluted to 100 ml)
0.036% EDTA (10 ml stock diluted to 100 ml).

Procedure.

A 1 ml portion of the sample or standard solution is placed in a 20 mm. cuvette of 16 ml capacity, 6 ml of 0.1N NaOH and 3 ml 0.0027% ammonium purpurate are added from automatic burettes. The cuvette is placed in an EEL photoelectric absorptiometer with a 606 optical filter (peak absorption 5800A) and 0.036% EDTA titrated into it from a 2 ml capacity, auto-refill burette graduated to 0.02 ml. The end-point is reached when addition of a drop of EDTA no longer causes an increase in optical density, but instead a slight fall is observed. This fall indicates that maximum chelation has occurred and further addition of the colourless EDTA solution is now causing a reduction in the concentration of the dye. A calibration curve is drawn from titrations of known standard solutions and the unknowns are read off the graph.

As this is an 'end-point' colorimetric method, no great precision is required in making up the working solutions employed. Similarly, no precautions are required to control bleaching of the working solution

of the dye.

The standard error of duplicate estimations in our hands, over the range 0 to 12 mg calcium per 100 ml and not more than 4 mg inorganic phosphorus per 100 ml, was 0.016 mg/100 ml..

Inorganic phosphorus.

A modification of the method of Fogg and Wilkinson (37), which was also developed in the laboratory for bone equilibration experiments, was employed in the earlier stages of the work. This was subsequently replaced by an auto-analytical technique.

The former method is a conventional phosphomolybdic colorometric procedure which used solid ascorbic acid as reducer. Its virtue compared to the classic Fiske and Subbarow (36) method is the stability at room temperature of the dye product, the accuracy obtained with small volumes of reagents and sample, and also the fact that when unexpectedly high phosphate concentrations are encountered, the dye solution can be diluted quantitatively until an optimum O.D. is obtained. The details of the method are as follows:-

Stock solution.

10 gm crystalline ammonium molybdate are dissolved in 100 ml distilled water.
150 ml sulphuric acid (S.G. 1.84) are added to 150 ml distilled water. After cooling the molybdate solution is slowly added to the sulphuric acid.

Procedure.

A protein-free sample has first to be obtained by precipitation with 20% trichlor-acetic acid if necessary.

The sample (containing as little as 5 µg P) is made up to about 10 ml with water and 0.02 ml aqueous phenol red solution (10 mg/100 ml) are added. The pH is adjusted to neutrality with HCl or KOH. 1 ml of molybdic-sulphuric stock solution is added and also approximately 25 mg of ascorbic acid powder. The mixture is boiled for 5 minutes on a sand-bath and made up to 12.5 ml in a graduated tube after cooling. A portion is then placed in a cuvette for determination of O.D. in a photoelectric absorptiometer with a 608 filter with a peak absorption of 6800 A.

In our hands, the standard error of duplicate estimations over the range 0 - 5 mg/100 ml inorganic phosphate with up to 20 mg/100 ml calcium is $\pm$ 0.025 mg/100 ml inorganic phosphate.

At a later stage an auto-analyser was used for inorganic phosphate estimation. A modification of the Fiske and Subbarow procedure (36), using a 1 amino-2-naphthol-4-sulphuric acid reducer is employed. The standard method is published by Technicon Instruments Corporation, Chauncey, New York. This technique when carefully controlled was found to be quite satisfactory.

Carbon dioxide.

With the exception of some early analyses performed by the classical Van Slyke method (152, 153), all total carbon dioxide estimations were performed by the auto-analyser. Standard technique as published by the Technicon Instruments Corporation was employed, and found to be very reliable at concentrations greater than 5 milli-equivalents/litre. At lower concentrations, the Van Slyke method was used.

1.5. Preparation of bone powder.

The material used in the bone equilibration experiments was post-mortem bone from cases with no known skeletal abnormality nor any known metabolic disorder. In the main, the material was from adult subjects under the age of 50 dying from acute illness or violent trauma.

A length of femoral shaft was removed and freed from marrow after which it was left steeping overnight in acetone. After drying at room temperature, the material was ground manually to a fine powder and passed through a British standard graded sieve, 22 mesh. The powder had a particle diameter of not more than 700 microns. The powder was again suspended in acetone for one to two hours, washed in alcohol, rinsed in ether and dried at room temperature. In later experiments, the bone powder was finely ground in a mechanical micropulveriser (Prolabo) and the resulting powder passed through a British standard graded sieve mesh yielding a particle size of not more than 5 microns. This fine

powder was then centrifuged in carbon tetrachloride in order to separate by flotation any organic material present in the powder.

EQUILIBRATION STUDIES WITH ADULT HUMAN BONE IN TRIS AND CACODYLATE BUFFERS...

2:1 Typical behaviour of bone powder in buffer at pH 7.0.

This first experiment was preliminary and designed to demonstrate the typical behaviour of bone powder placed in a buffer system.

Experimental.

Human bone powder (1 gm) was placed with 10 ml 0.15 M tris buffer (pH 7.0) inside a dialysis bag. This bag was made by knotting the ends of a length of reconstituted cellulose tubing 2 cm in diameter. The average pore size of the tubing was 24A. Screw-capped polythene bottles of 100 ml capacity were used to contain the dialysis system; 30 ml of buffer being placed with the dialysis bag in the bottle. The system, shown diagrammatically in Fig. 3, was then placed in a specially constructed rotary device, Fig. 4, which inverted the contents of the bottles 20 times per minute and which has proved a successful and convenient method of ensuring continuous agitation.

The bottles were opened periodically to remove samples of the solution for analysis at which times the buffer pH was determined electrometrically. Chloroform was added to the buffers (6 ml per litre) as a bacterial inhibitor (77). Four experiments were performed in two of which calcium (1.5mM) and inorganic phosphate (0.16mM) were added to the buffer prior to equilibration.

Results.

The results are shown in Table 2 and Figs. 5 to 8.

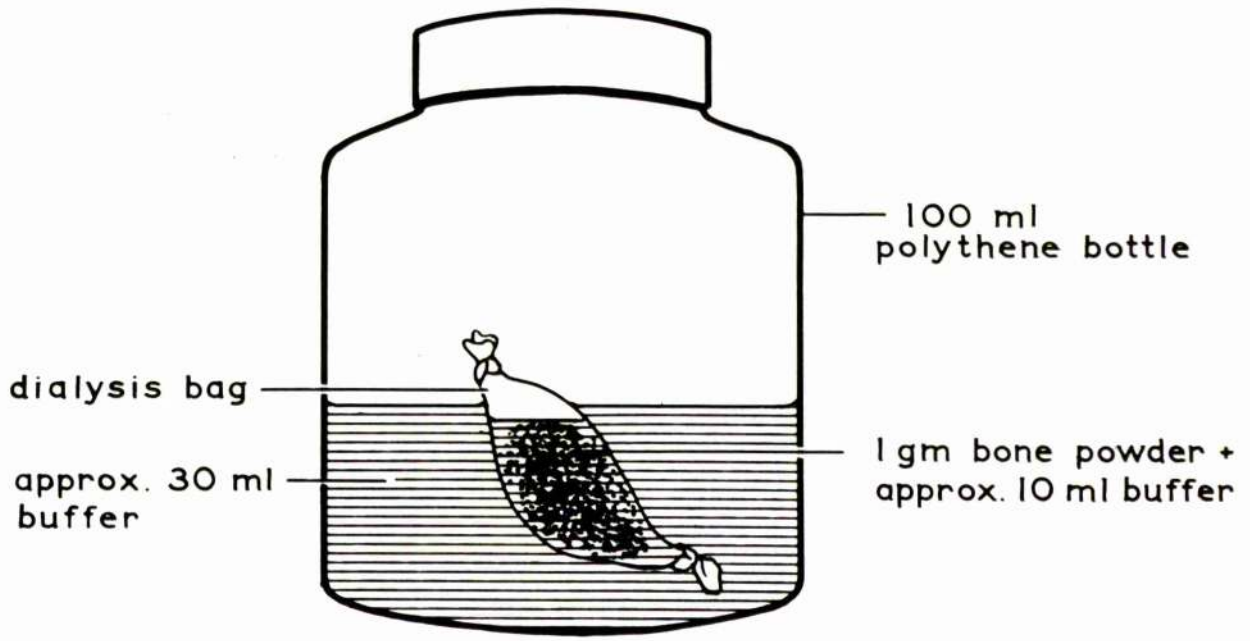


Figure 3. Diagram of the equilibration system used at 20°C. with tris and cacodylate buffers.

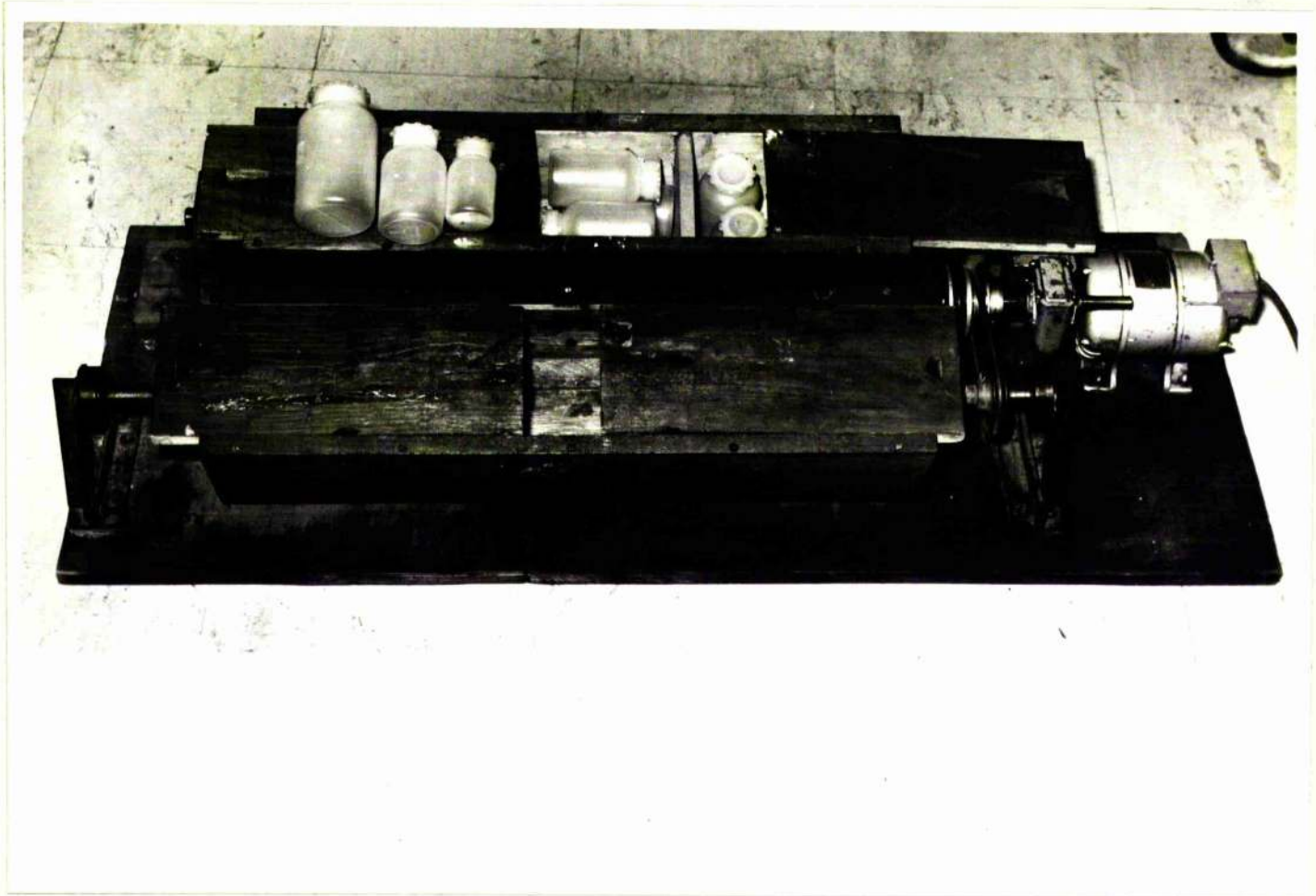


Figure 4. Rotating shaker constructed to ensure adequate agitation of equilibration bottles.

It can be seen in Fig. 5 that in the case of the experiments where no Ca or P was added initially, the pH of the solution in the bottle rose from 7.0 and stabilised at about 7.3 to 7.4 in about 24 to 48 hours.

With calcium and phosphate present initially, there was also a rise in pH, but in this case it reached a value of about 7.15 in 24 hours and then rose more slowly. The pH did not reach an equilibration value before the termination of the experiment at 72 hours.

It can be seen that in all four cases the calcium concentration reached equilibrium by 24 hours, but the experiments with no calcium or phosphate added initially had a lower equilibrium calcium concentration at a higher pH. In the case of inorganic phosphate, all experiments had the same equilibrium concentration of about 0.2mM within 24 hours of the commencement of equilibration.

In Fig. 6 we see the concentrations of the ion species $HPO_4^{=}$ and $PO_4^{\equiv}$ in the buffers, at different times during equilibration. These data were calculated from $[P]$ and pH as shown in 1:2. Due to the constant value of $[P]$ but the differing pH, the concentrations of the two species in the two buffers are quite different. The experiments with no Ca or P added initially had $HPO_4^{=}$ and $PO_4^{\equiv}$ concentrations of about $2.0Mx10^{-4}$ and $16Mx10^{-10}$ respectively, and the experiments

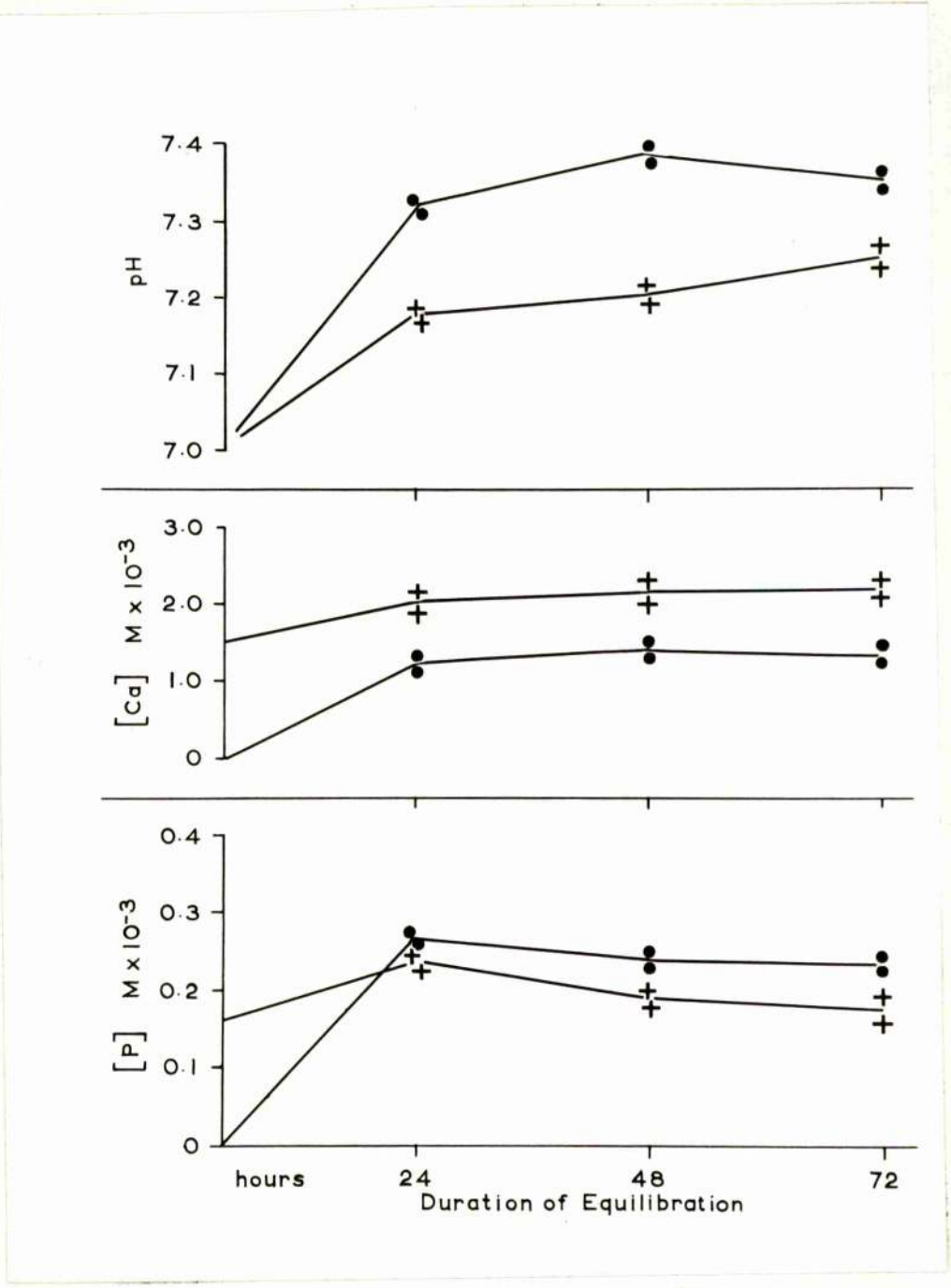


Figure 5. The relationship between duration of equilibration and pH, $[Ca^{++}]$ and $[P]$ in four experiments during which no pH adjustments were made after commencement of equilibration.

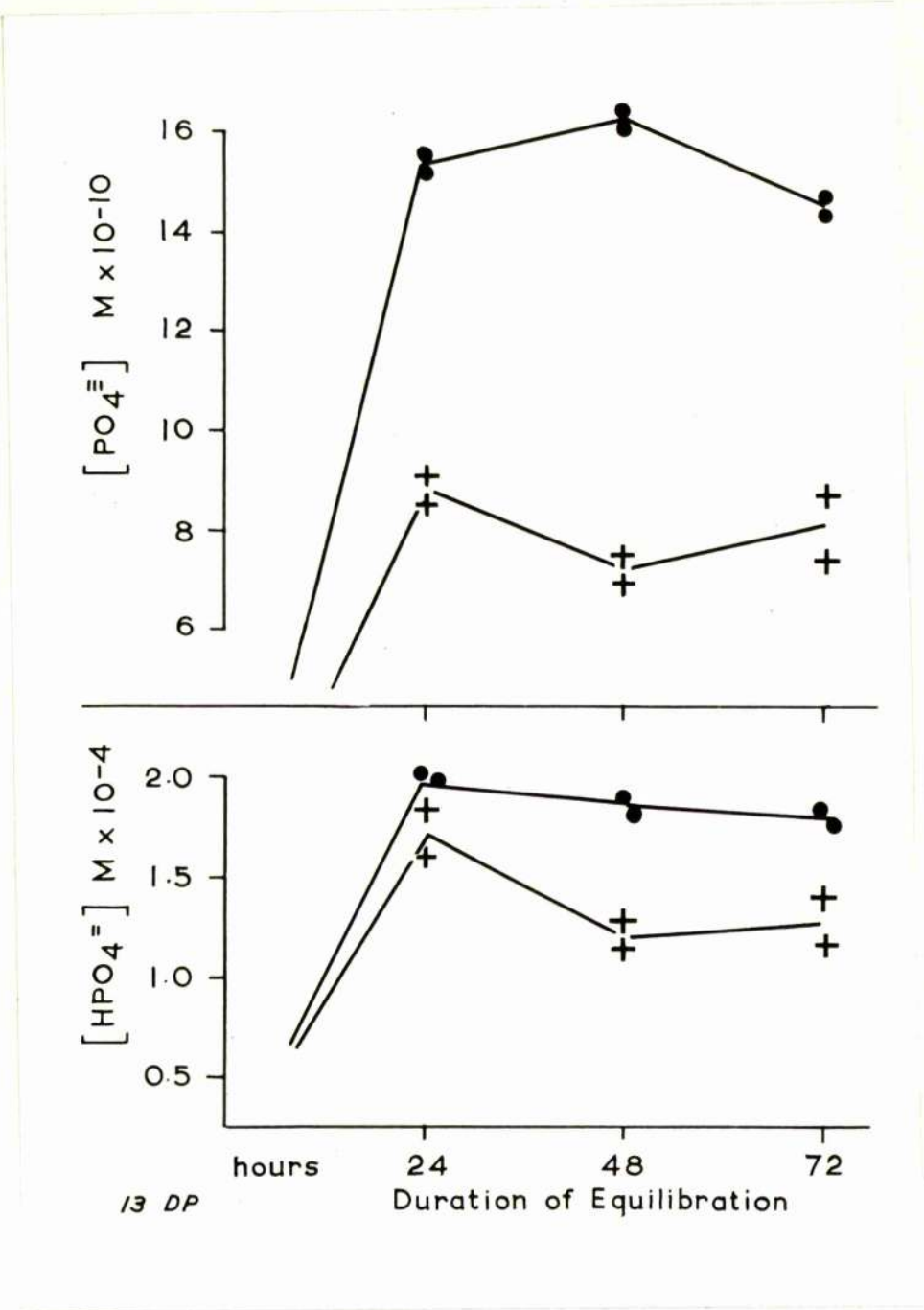


Figure 6. The relationship between duration of equilibration and the concentrations of the phosphate ion species $HPO_4^{=}$ and $PO_4^{\equiv}$.

with added Ca and P, 1.5×10^{-4} and 8×10^{-10} respectively.

The ion products $pK_c [Ca^{++}][HPO_4^{=}]$ and $pK_c [Ca^{++}]^3 [PO_4^{\equiv}]^2$ are shown plotted against duration of equilibration in Fig. 7. It can be seen that in both cases a constant value of about 6.5 and 26.2 respectively had been achieved by 24 hours.

In Fig. 8 the same ion products are plotted against pH and we can see that there is a suggestion that the $pK_c [Ca^{++}][HPO_4^{=}]$ is related to pH whereas the product $pK_c [Ca^{++}]^3 [PO_4^{\equiv}]^2$ is relatively independent of pH.

Discussion.

There are two principal points to be noted in the results of this experiment.

Firstly, when bone powder is placed in buffer at pH 7.0 the pH of the buffer tends to rise with time. It has been suggested (106), that since bone is immersed in tissue fluids normally assumed to have a pH of 7.4, then the phosphate salt might assume the role of a buffer and tend to resist change in pH by taking up or releasing ions by an exchange process. Alternatively, it has been frequently demonstrated (24,27,29,71,82) that when bone is eluted with acid it is the carbonate portion of the bone that is first mobilized. The experimental results obtained here could indicate that carbonate is being dissolved, and it

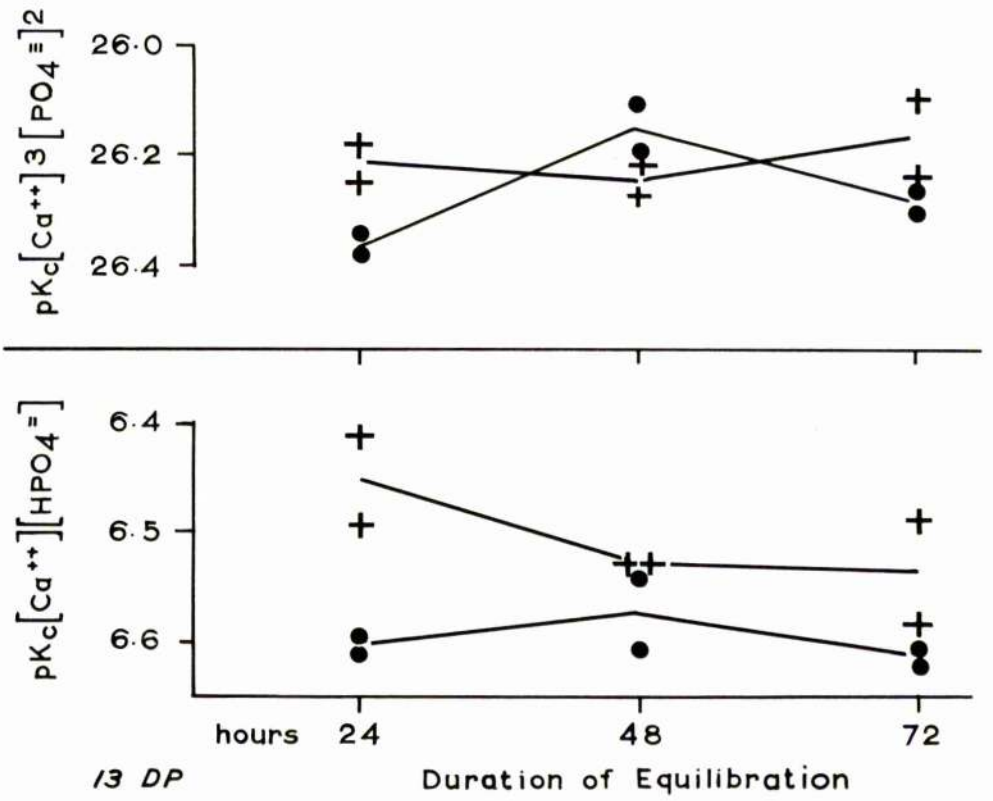


Figure 7. The ion products $pK_c[Ca^{++}][HPO_4^{=}]$ and $pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$ at different times after commencement of equilibration.

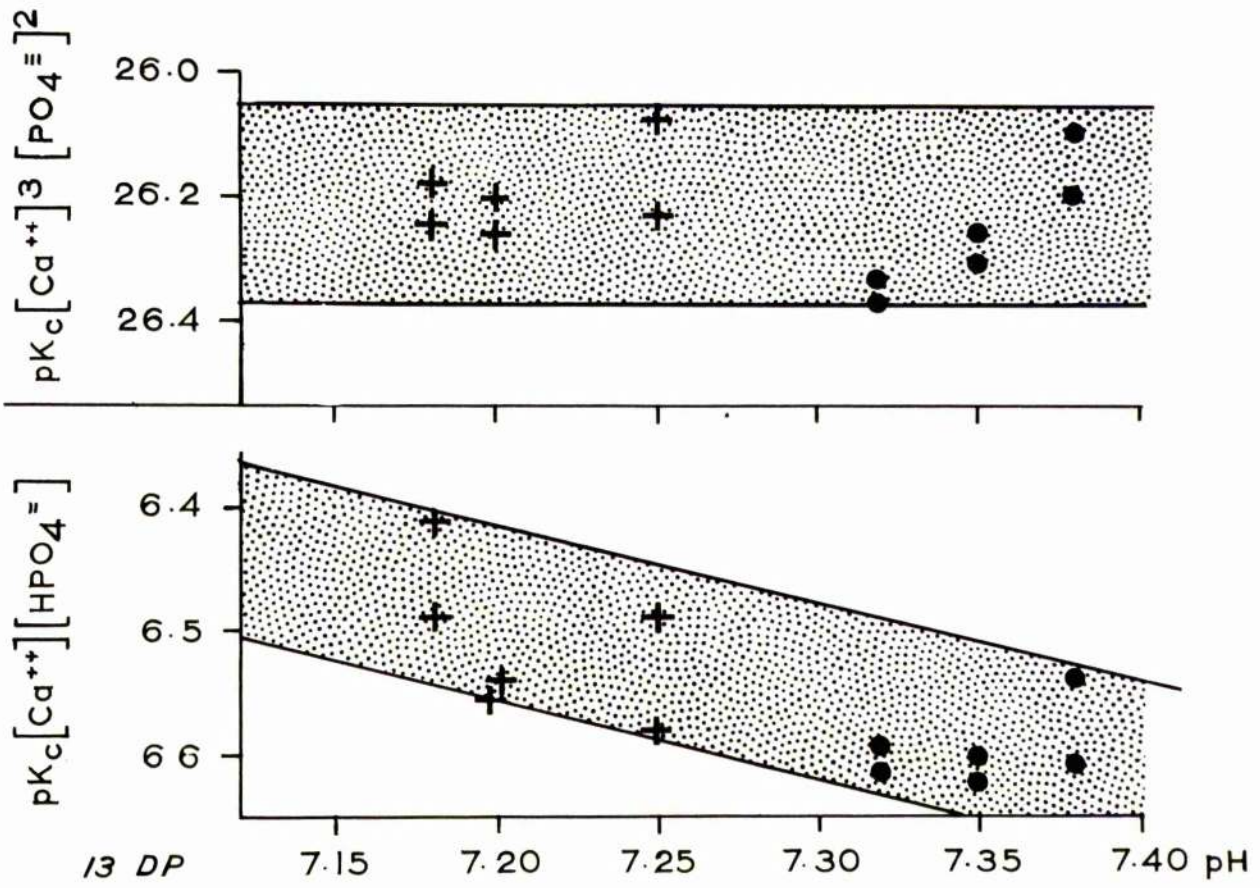


Figure 8. The relationship between the ion products $pK_c\,[Ca^{++}][HPO_4^{=}]$ and $pK_c\,[Ca^{++}]^3[PO_4^{\equiv}]^2$ and pH. The general trend is indicated by the stippled area.

may be significant that the experiments with lower initial calcium concentrations yield the highest pH values at equilibrium.

Secondly, we should note that equilibrium was established in this system by 24 hours. The criterion on which the achievement of equilibrium is assessed would be a constant pK_c, independent of pH, for the particular solid phase dissolving in the buffer. We cannot at this stage define the nature of the solid phase, but it will later be shown that $pK_c \left[Ca^{++}\right]^3 \left[PO_4^{\equiv}\right]^2$ is the ion product relevant to our problem. Even so it is obvious that neither of the calculated products varies significantly after 24 hours equilibration, and on this evidence we will accept 24-hour values as adequate 'equilibrium' values for this system.

2:2 Study of calcium phosphate ion products in bicarbonate-free buffers. Experimental.

3 gm samples of human bone powder were equilibrated at room temperature, in the dialysis system as previously described, 10 ml of buffer was placed inside the bag and a further 30 ml was sealed with the bag in a 100 ml screw-capped polythene bottle as before. Tris(hydroxymethyl)aminomethane-HCl buffer was used over the pH range 7.0 to 7.8 and cacodylic acid-potassium cacodylate over the range 6.2 to 7.0. All the buffer solutions were 0.15 M, but the effect of added calcium and phosphate salts on Molarity was disregarded as they were considered negligible.

Most equilibrations were run for 24 hours after which samples were removed for calcium and phosphate estimation. An important feature of these experiments in contrast to those reported in 2:1 was that the pH of the buffer was determined frequently during progress of equilibration, and the preselected pH was rigorously maintained by addition of the appropriate buffer component.

Results.

The results of 40, 24-hour equilibration experiments over the pH range 6.58 to 7.91, and of 10, 24-hour experiments over the pH range 6.21 to 6.50, along with 3 further 72-hour equilibrations in the latter pH range, are presented graphically in Figs. 9 to 14 and in Table 3.

Fig. 9 shows that the absolute concentration of calcium maintained by the bone in inorganic solutions is related to pH and varies from about 8×10^{-3}M at pH 6.2 to about 0.5×10^{-3}M at pH 7.8. The final concentration was independent of the initial calcium concentration in the system, equilibrations being conducted from above and below the final levels throughout the pH range.

Fig. 10 shows the concentration of total inorganic phosphate and also the absolute concentrations of the ion species $HPO_4^{=}$ and $PO_4^{\equiv}$ over the pH range studied. It can be seen that above pH 6.58 concentrations of total inorganic phosphate and divalent phosphate are relatively independent of pH and fall within the ranges .....

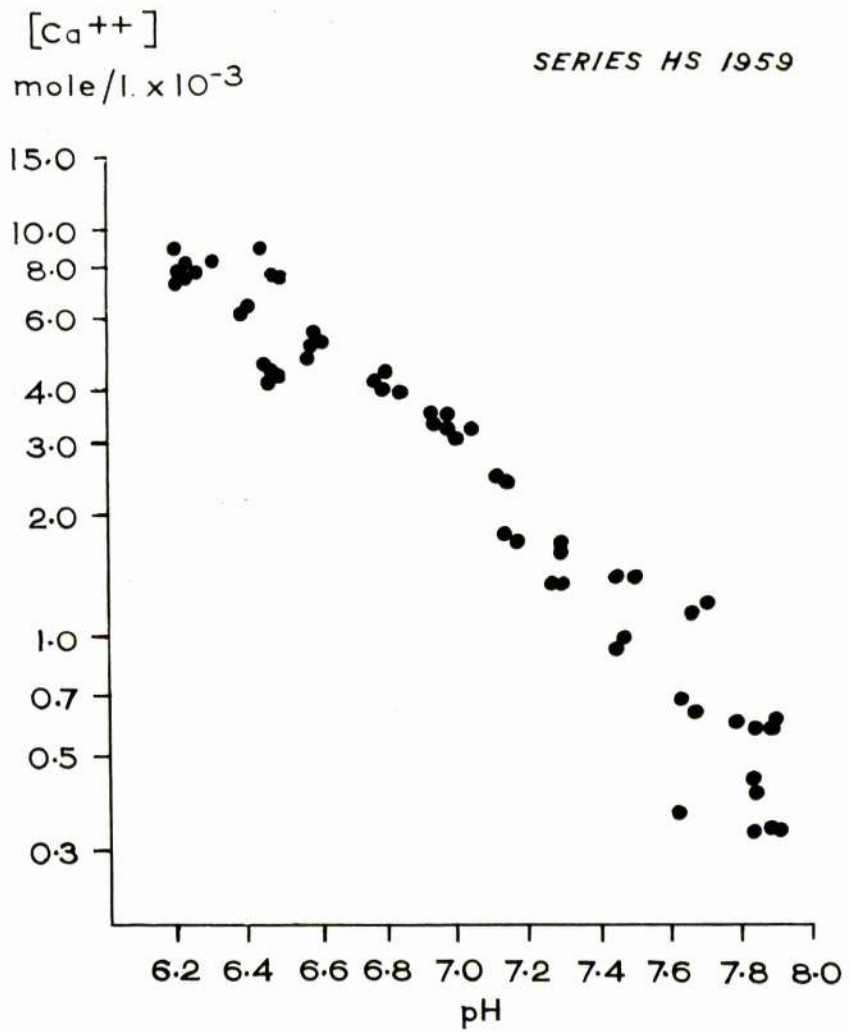


Figure 9. The relationship between pH and calcium concentration after equilibration with 0.15 M buffers at fixed pH and at 18°C.

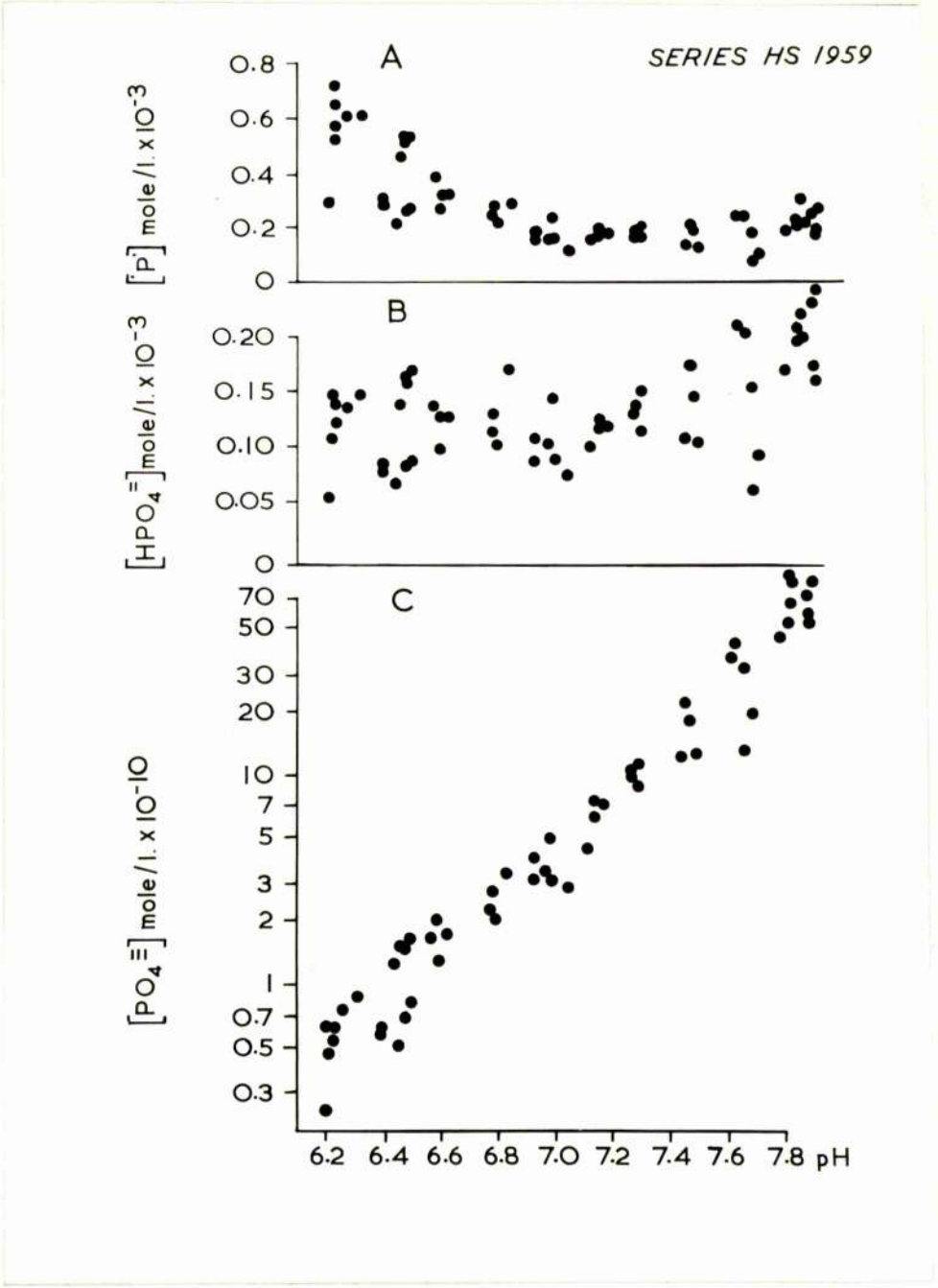


Figure 10. The relationship between pH and the concentrations of total phosphate, divalent phosphate ion and trivalent phosphate ion after equilibration with human bone powder.

0.1 to 0.4 and 0.05 to 0.25×10^{-3}M, respectively. On the other hand, the concentration of trivalent phosphate varies directly with the pH from about 10^{-10}M at pH 6.6 to about 10^{-9}M at pH 7.8.

Fig. 11A shows the relationship between pH and the product of the concentrations of ionic calcium and divalent phosphate. This product is plotted as a negative logarithm, conventially expressed as the pK. It can be seen that the pK of the ion product $[Ca^{++}][HPO_4^{=}]$ rises from about 6.3 at pH 6.6 to about 7.0 at pH 7.8. This suggests that the undissociated salt with which these ions are in equilibrium is not $CaHPO_4$.

In Fig. 11B the ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ is plotted against pH. From pH 6.58 to 6.91 the pK of this product is reasonably constant at about 26.4.

Fig. 12 shows the crude product $[Ca^{++}]^3 . [P]^2$ plotted against pH. The value of the corresponding product calculated from the concentrations of ionic calcium and total inorganic phosphorus in normal tissue fluid is also shown, and is equivalent to the product obtained with bone powder at pH 6.8.

Fig. 13 shows the data plotted to demonstrate the reciprocal relationship between $[Ca^{++}]^3$ and $[PO_4^{\equiv}]^2$. The line indicates the arithmetic mean product of all the values illustrated.

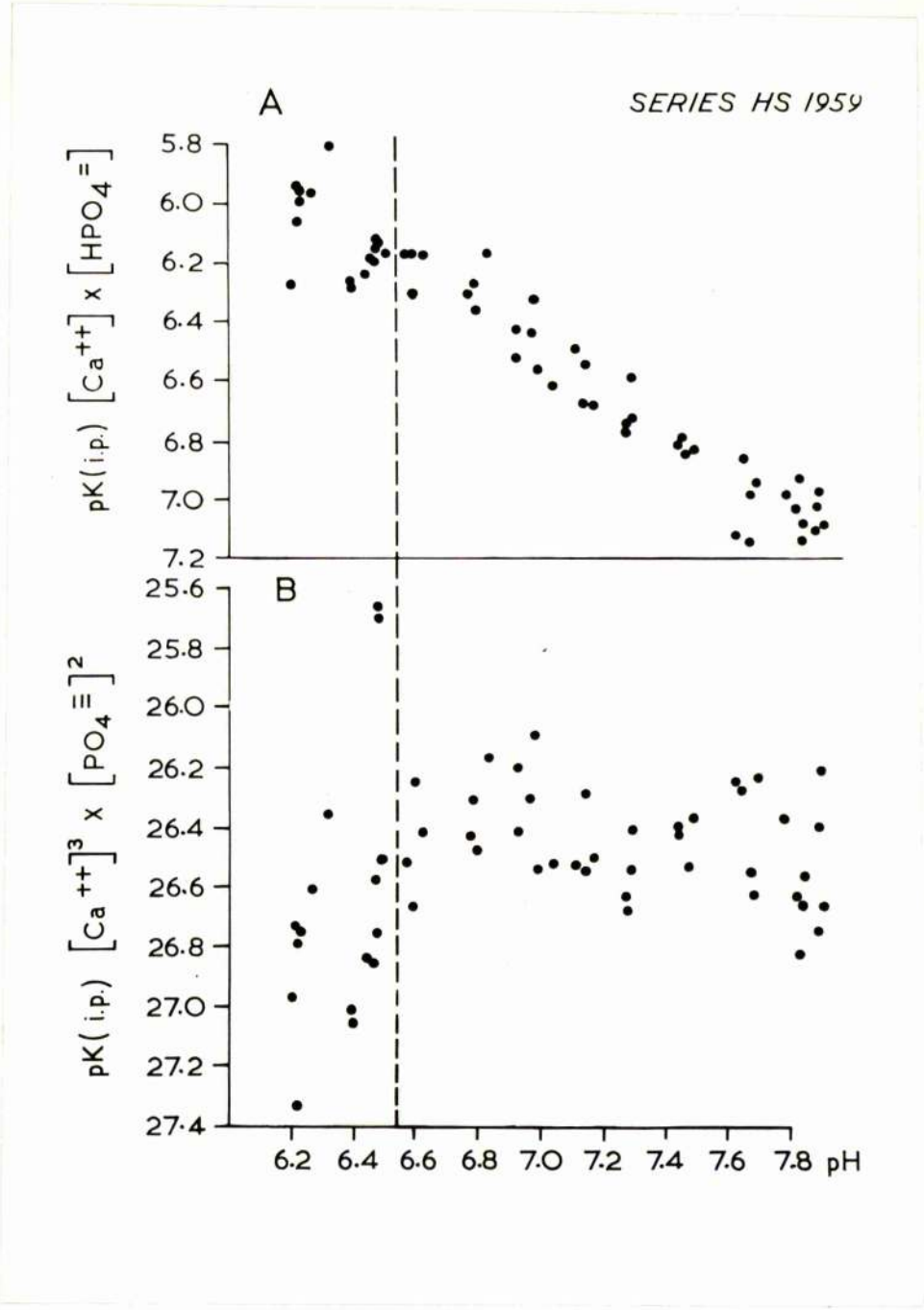


Figure 11A. The ion products $[Ca^{++}][HPO_4^{=}]$ and $[Ca^{++}]^3[PO_4^{=}]^2$ expressed as negative logarithms (pK) and plotted against pH.

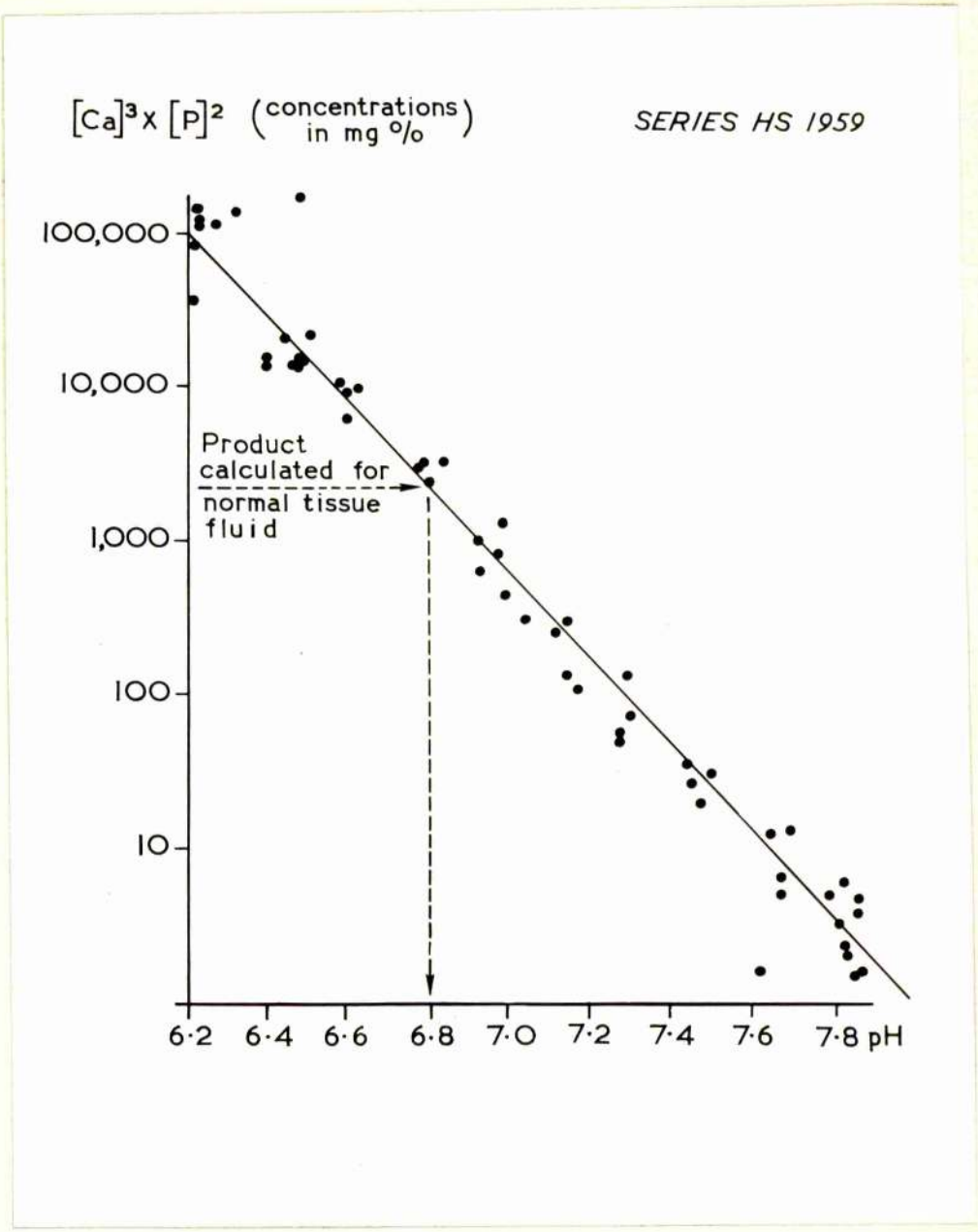


Figure 12. The relationship between the crude product in normal human tissue fluid (Ca^{++} = 6 mg/100 ml and P = 3 mg/100 ml) indicated and corresponds to an equilibrium with bone at pH 6.8.

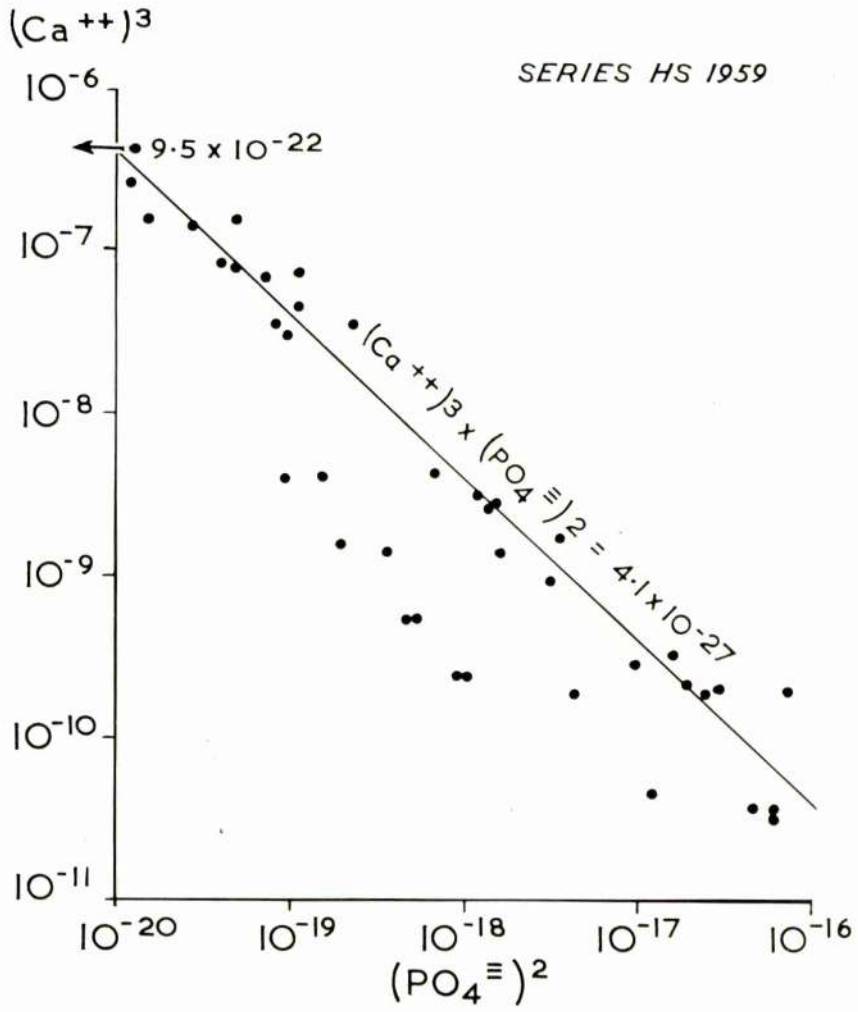


Figure 13. $[Ca^{++}]^3$ plotted against $[PO_4^{\equiv}]^2$ to show the reciprocal relationship between these values. The line represents the arithmetic mean of the product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ calculated from all experiments above pH 6.58.

The data obtained in experiments below pH 6.58 are somewhat anomalous, as are shown in Fig. 11B. This may be due to the relatively large changes in the concentration of calcium ions that are produced by small pH fluctuations. As a result of this, the system is less stable in the low pH range. Since the pH values are lower than any presumed physiological range, these results, while presented, will not be considered in interpreting the data.

Although the product $[Ca^{++}]^3 [P]^2$ obtained at pH 6.8 was equivalent to that of tissue fluids, it can be seen from Figs. 9 and 10 that the Molar ratio of calcium to phosphate at this pH was about 20 to 1, and very much higher than the 1.5 to 1 Molar ratio normally found in plasma. An experiment was therefore designed to establish whether a Ca:P ratio nearer to that in tissue fluid could be reproduced <u>in vitro</u>, by sustaining the phosphate concentration of the system. This experiment is illustrated in Fig. 14. After 24 hours equilibration at pH 7.00, inorganic phosphate was added to raise the phosphate concentration of the buffer to about 6.5 mg/100 ml, followed by hourly additions to maintain this concentration. Under these circumstances there was an appreciable fall in $[Ca^{++}]$ which continued for 24 hours.

<u>Discussion</u>.

The data appeared to confirm the existence of a calcium phosphate product in buffer solutions equilibrated with human dead bone.

This product is dependent on pH when expressed in terms of $[Ca^{++}][HPO_4^{=}]$ suggesting that the relevant phosphate ion species governing the equilibrium between bone and the bathing fluid is trivalent rather than divalent phosphate (Fig. 11). This can also be seen by comparing Figs. 9 and 10 which show that there is a reciprocal relationship between the concentrations of $[Ca^{++}]$ and $[PO_4^{\equiv}]$ over the whole pH range, whereas no such relationship exists between $[Ca^{++}]$ and $[P]$ or $[HPO_4^{=}]$. A product involving tribasic phosphate fits the data better than one involving divalent phosphate whether equilibrium is approached from high or low concentrations of either or both ions. The suggestion (7,59,60,129,136,110,130,82) that the salt formed on precipitation of calcium phosphate is calcium acid phosphate, is therefore not substantiated with this technique.

The effect of pH on the dissociation of phosphoric acid and hence on the concentration of the tribasic ion might explain the relationship between the concentrations of calcium ion and hydrogen ion in inorganic systems as previously noted by Hodge (56).

The mean ion product observed, $[Ca^{++}]^3\ [PO_4^{\equiv}]^2 = 4.1 \times 10^{-27}$ ($pK_c = 26.39$) is lower than that obtained in tissue fluid at pH 7.4 but corresponds quite closely to the theoretical product in normal tissue fluid at a pH of 6.8. The calculation is as follows:-

Normal plasma ionic calcium $[Ca^{++}]$	$= 1.5 \times 10^{-3}$ mole/litre
Normal plasma inorganic phosphate $[P]$	$= 1.12 \times 10^{-3}$ " "
Fraction of $[P]$ as $[PO_4^{\equiv}]$ at pH 6.8	$= 9.8 \times 10^{-7}$
Concentration of $[PO_4^{\equiv}]$	$= 1.10 \times 10^{-9}$
Therefore $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$	$= 4.1 \times 10^{-27}$

This calculation confirms Nordin's observations (110), and if the pH in bone is less than that in tissue fluid, it implies that the pH in bone will be about 6.8 (Fig. 12) or approximately equivalent to intracellular pH (20,155).

However, although the 'solubility' of the bone preparation would account for the calcium phosphate product in tissue fluid if the pH governing the equilibrium were 6.8, the ratio of calcium to phosphate in these experiments is always very much higher than that in tissue fluid. The ratio of calcium to phosphate in the media in equilibrations below 7.4 is far higher than the Ca:P ratio of bone itself, whatever the ratio in the solutions may have been initially. In fact, in experiments which started in solutions containing no calcium or phosphorus, the amount coming out of bone did not correspond to the ratio of these elements in bone itself, the Ca:P ratio below pH 7.4 always being much higher than 2:1 by weight. Furthermore, in the experiments starting with moderate amounts of calcium and phosphate in the solutions, the calcium tended to rise and the phosphate to fall until equilibrium was established. A possible explanation of these findings is that a basic

salt of calcium and phosphate with a high Ca:P ratio is dissolving, and that a less basic one with a lower ratio is forming.

Although a calcium phosphate product has been demonstrated in relation to bone, the experiments discussed so far do not indicate that this product is other than fortuitous. Neuman (103) has suggested the possibility that the relationship between calcium and hydrogen ion concentrations in solution equilibrated with hydroxyapatite, could be explained by exchange of calcium for hydronium ion. Clearly if this were the case, variations in the pH of the solutions would influence the concentrations of Ca^{++} and $PO_4^{\equiv}$ in a reciprocal manner and might result in a fortuitous product. None of the experiments presented in Figs. 9 to 13 necessarily imply that either the calcium or phosphate ion influence directly the concentration of the other.

The experiment shown in Fig. 14 may help to resolve the problem. By maintaining a high concentration of phosphate in the fluid artificially the calcium concentration was reduced without changing the pH, and this possibly indicates the existence of a true reciprocal relationship between these two ions. It is possible that the concentration of phosphate in tissue fluid is normally maintained by extraskeletal mechanisms, such as absorption of phosphate from gut or as a result of protein catabolism. These vital processes lower the Ca:P ratio to the physiological level of

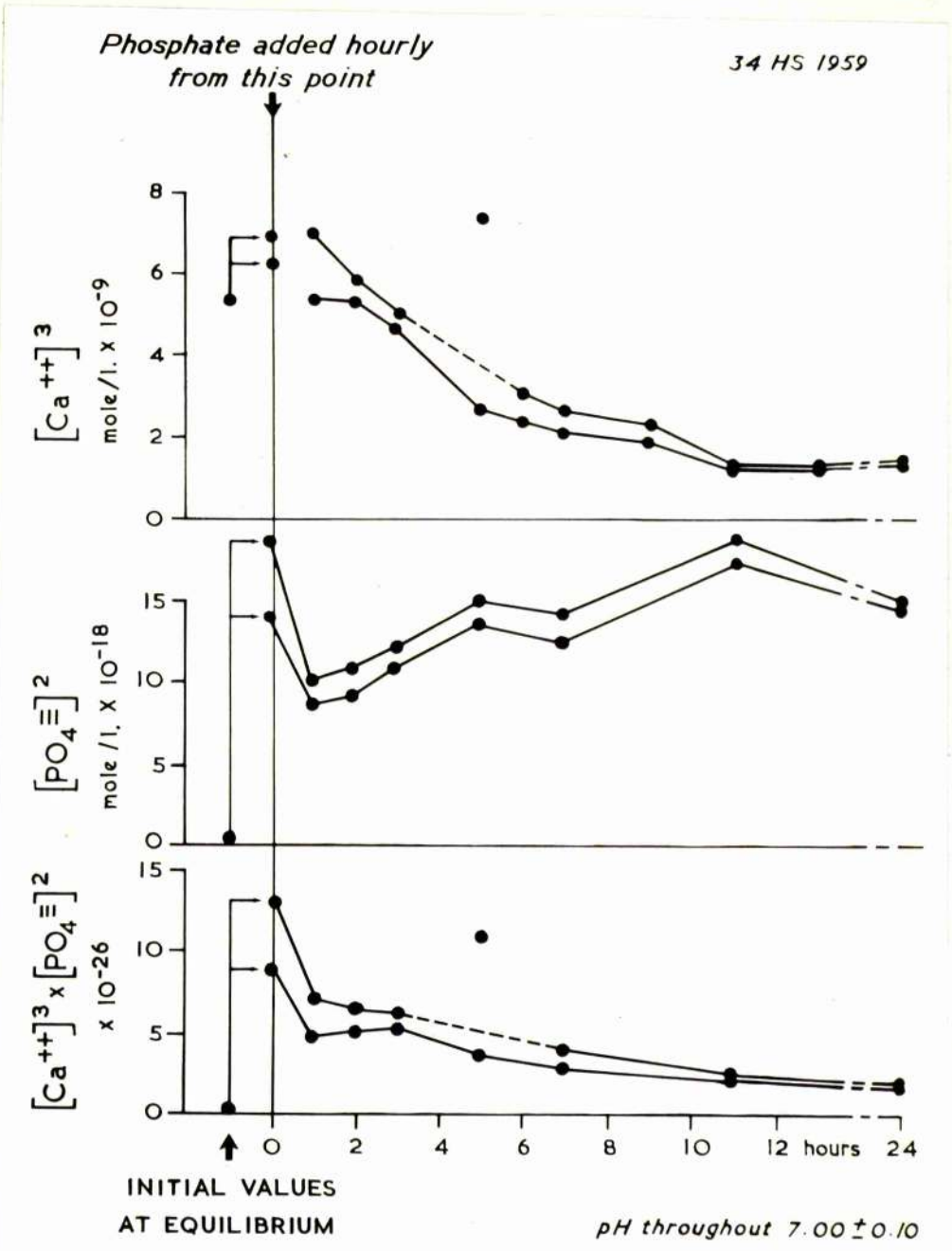


Figure 14. **The effect of adding inorganic phosphate to equilibrated systems to maintain an absolute phosphate concentration similar to that found in plasma. There is a fall in the concentration of calcium.**

2:1 by weight. Thus may be explained the Ca:P ratio in tissue fluid which is very much lower than that observed in our inorganic system at equilibrium, even when the ion product is the same.

It has been shown that the ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ best expresses the behaviour of bone mineral in buffer, and it is convenient to express the dynamic equilibrium in this form on <u>empirical</u> grounds <u>without necessarily implying that the solid phase is tri-calcium phosphate</u>.

2:3 <u>The relationship of solid:solution ratio to ion product</u>.

Logan and Taylor (81) have shown that in solution in equilibrium with 'bone salt', the ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ was a function of the solid:solution ratio, and increased as the ratio diminished. They explained their findings by postulating an ion adsorption effect which increased in magnitude as the relative surface area increased. Increased adsorption was said to result in a fall in ion product. This observation is widely quoted in current texts (96), despite contrary experimental findings elsewhere (45, 46, 72).

It was therefore decided to investigate this feature of bone equilibria although it was realised that in physiological terms only the higher solid:solution ratios could be of significance.

Experimental.

A series of 30 equilibration experiments were set up as in 2:2. In this case the buffer employed was 0.15M tris at pH 7.4 in all cases.

Five groups of experiments had various amounts of bone powder together with 10 ml of buffer placed inside the dialysis bags, and 30 ml of buffer placed outside. The amount of bone powder employed was such that the solid:solution ratios in the various groups were 25, 12.5, 6.25, 2.5 and 0.25 gm bone powder per litre of buffer. In each group four experiments were run from undersaturation (no Ca or P in buffer initially) and two from supersaturation (3mM Ca, 1.3mM P) with respect to calcium and inorganic phosphate. The equilibrations were conducted for 96 hours.

Results.

The results of the 30 experiments are shown in Table 4 and Figs. 15 to 18 where the values of $[Ca^{++}]$, $[P]$, $[PO_4^{\equiv}]$ and $pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$ are shown respectively after 1, 2, 3 and 4 days equilibration in the five groups of experiments with varying solid:solution ratios.

In Fig. 15 it can be seen that the calcium concentration either rose from zero or fell from 3mM to a value dependent on the solid:solution ratio. In experiments from undersaturation it can be seen that although in each case the range of values observed within each group was very

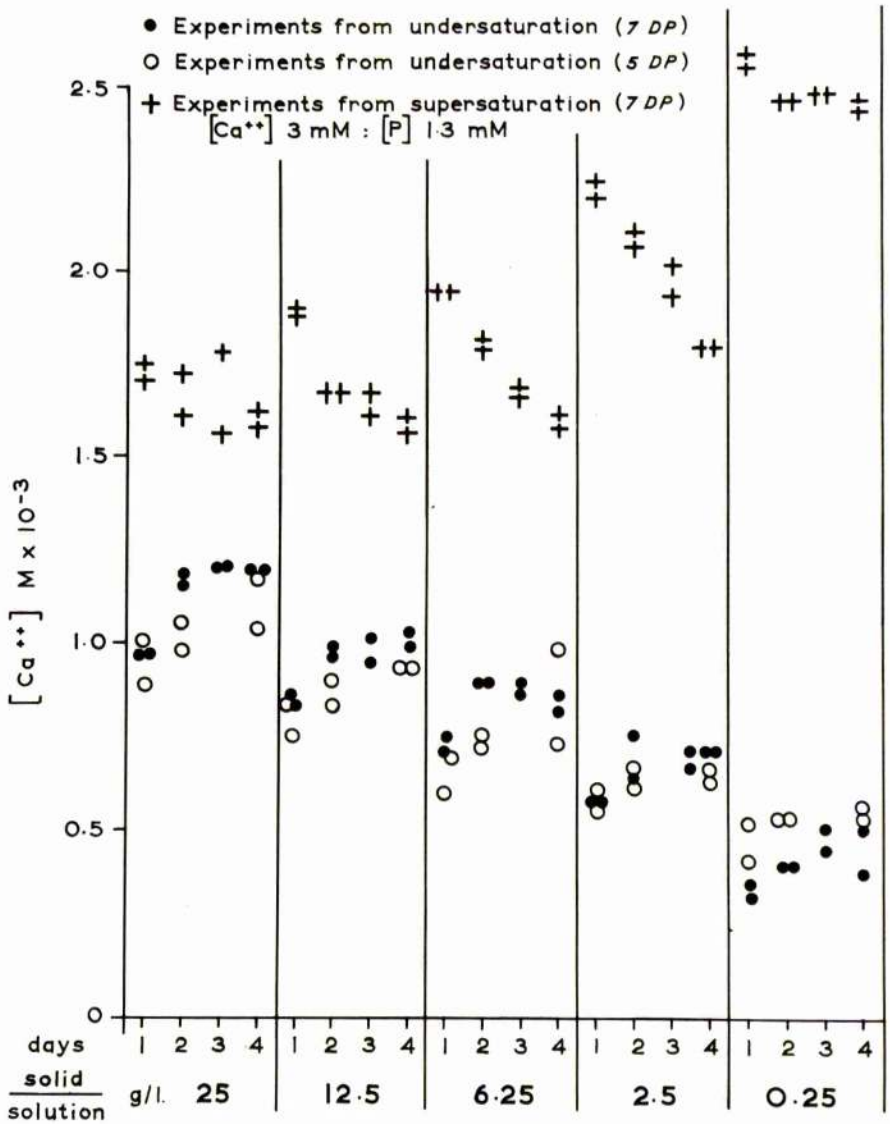


Figure 15. $[Ca^{++}]$ at 1,2,3 and 4 days in equilibration experiments with bone powder in 0.15 M tris buffer at pH 7.4 from undersaturation and supersaturation with respect to Ca and P and where the solid:solution ratio varied from 25 to 0.25 gm bone powder per litre of buffer.

similar for 48, 72 and 96 hours, the equilibrium concentration of the various groups fell consistently from just over 1mM at 25 g/litre to 0.5mM at 0.25 g/litre.

In experiments from supersaturation, apart from the experiments at 25 g/litre and 0.25 g/litre, the calcium concentration appears to fall steadily for 4 days tending to an equilibrium value of about 1.6mM. The anomalous result is at 0.25 g/litre where there is a constant $[Ca^{++}]$ of 2.5mM from 48 to 96 hours suggesting an equilibrium state or an inability of the solid to 'take up' any further calcium.

Fig. 16 shows analogous data for the concentrations of inorganic phosphate. The experiments from undersaturation all appear to demonstrate an equilibrium phosphate concentration of about 0.2 to 0.3mM by 24 hours. The experiments from supersaturation, however, show equilibrium values only down to ratios of 12.5 g/litre within 4 days. Below this ratio an apparent 'equilibrium' concentration is reached at higher concentrations extending to about 1.0mM at 0.25 g/litre.

Fig. 17 shows the trivalent phosphate concentrations. Exactly similar conclusions can be drawn from this data as were drawn from those of total phosphate (Fig. 16). The equilibrium $PO_4^{\equiv}$ concentration from undersaturation is about $15Mx10^{-10}$ at pH 7.4.

Fig. 18 shows the calculated values of the $pK_c [Ca^{++}]^3 [PO_4^{\equiv}]^2$ obtained from the data in Figs. 15 and 17. The experiments from

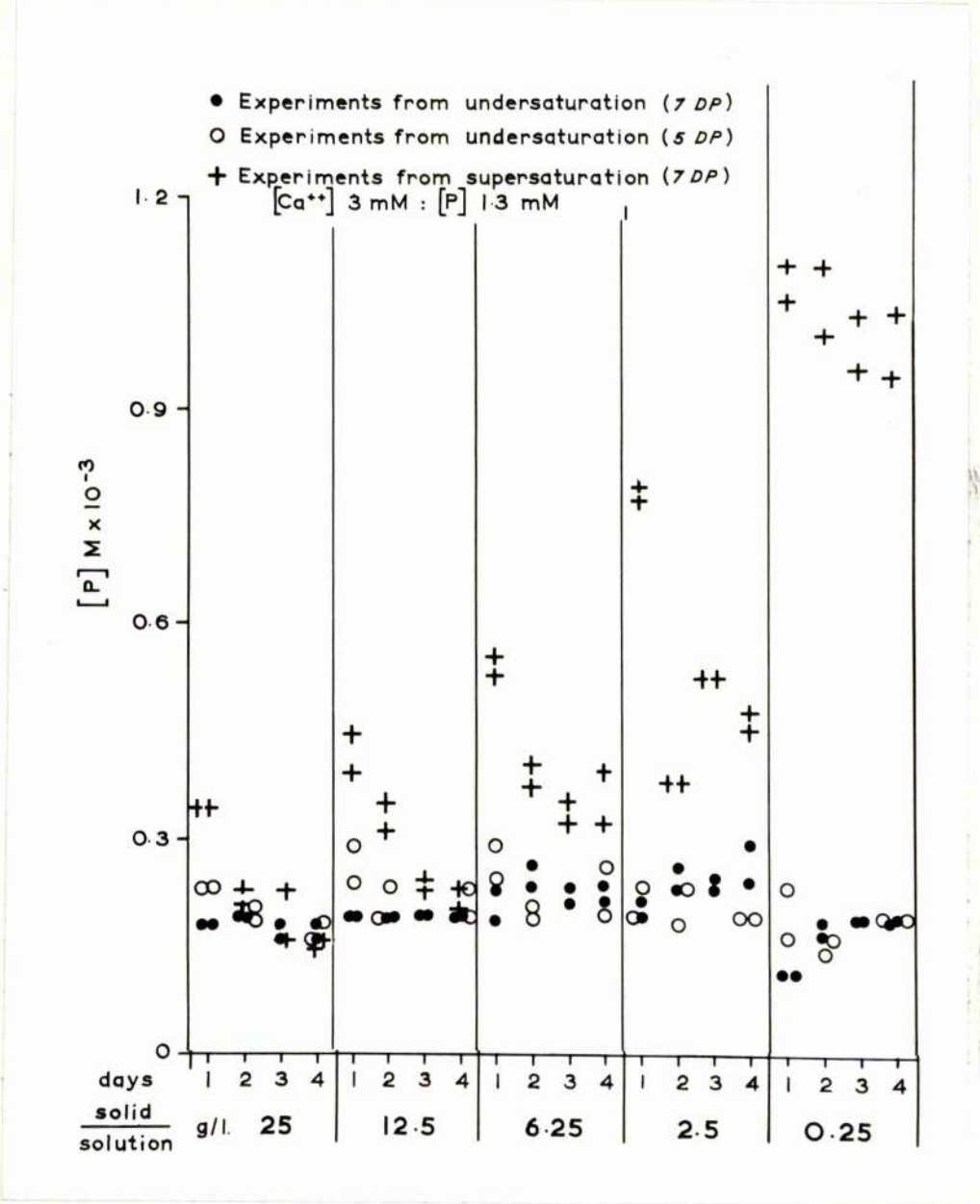


Figure 16. Total inorganic phosphate concentration at 1,2,3 and 4 days in equilibration experiments with bone powder in 0.15 M tris buffer at pH 7.4 from undersaturation and supersaturation with respect to Ca and P and where the solid: solution ratio varied from 25 to 0.25 gm bone powder per litre of buffer.

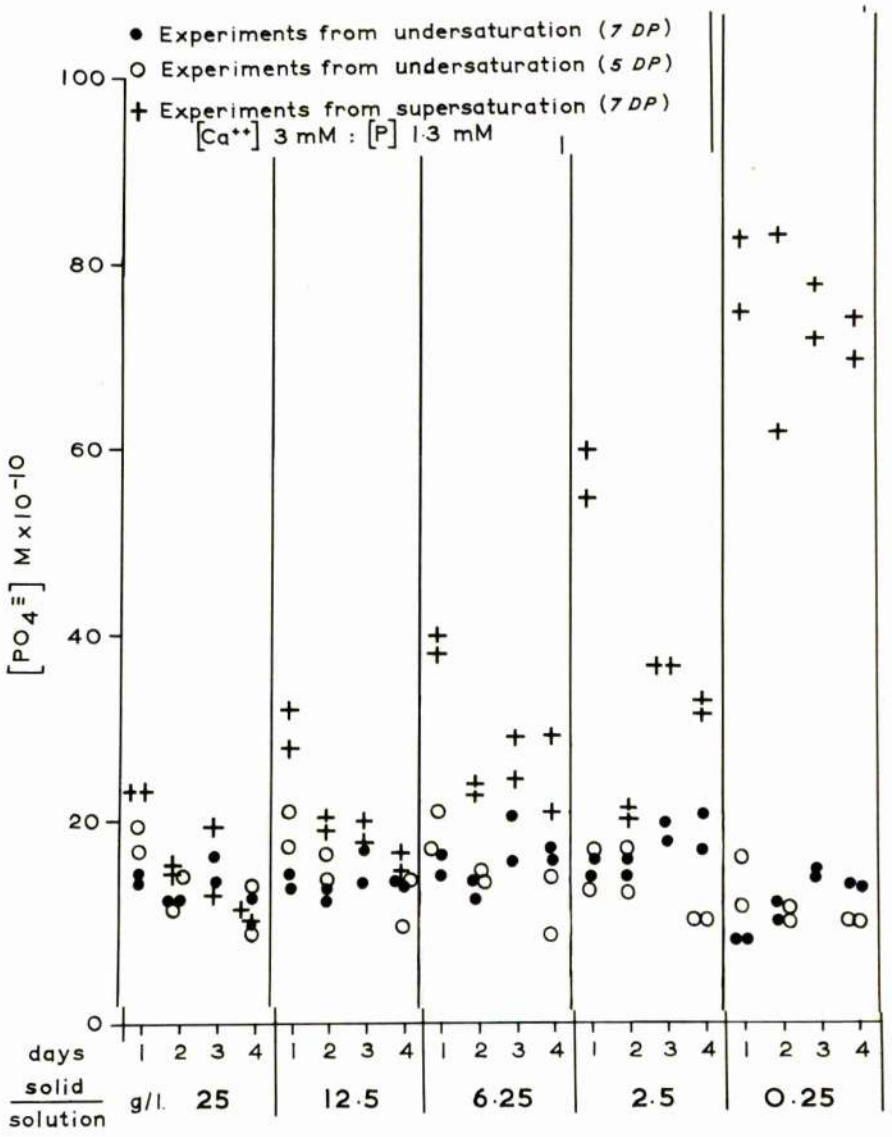


Figure 17. Trivalent phosphate concentration at 1,2,3 and 4 days in equilibration experiments with bone powder in 0.15 M tris buffer at pH 7.4 from undersaturation and supersaturation with respect to Ca and P and where the solid:solution ratio varied from 25 to 0.25 gm bone powder per litre of buffer.

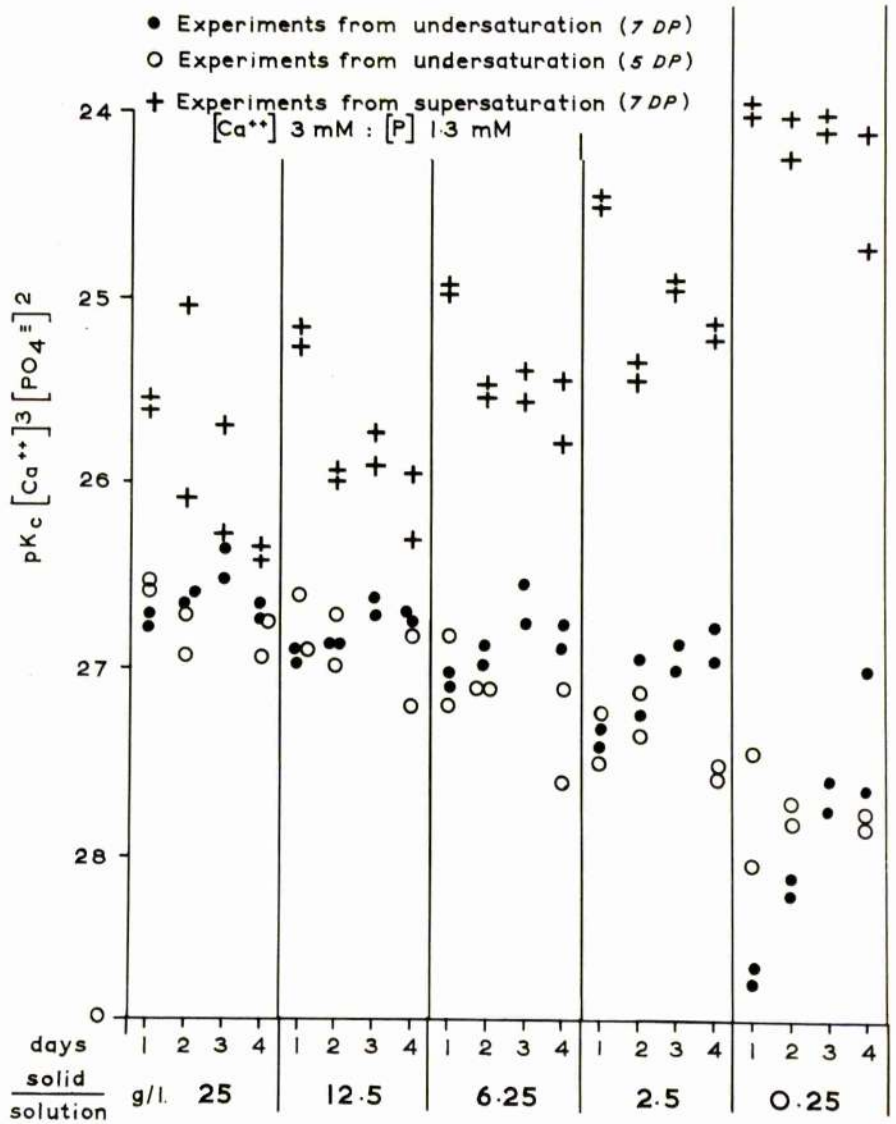


Figure 18. Values of $pK_c [Ca^{++}]^3 [PO_4^{=}]^2$ at 2,3, and 4 days in equilibration experiments with bone powder in 0.15 M tris buffer at pH 7.4 from undersaturation and supersaturation with respect to Ca and P and where the solid:solution ratio varied from 25 to 0.25 gm bone powder per litre of buffer.

undersaturation and supersaturation both demonstrate an unwillingness to come to the equilibrium value at solid solution ratios below about 12.5 g/litre.

In experiments from undersaturation it appeared that an equilibrium product could be achieved within 4 days down to a solid:solution ratio of about 6.25 g/litre, but below that ratio, although the ion product increased with time, it had neither reached the equilibrium value nor become constant by the fourth day, and the lower the solid:solution ratio, the lower the maximum product (or highest pK) observed.

Experiments from supersaturation appeared to show the development of a constant product by about 48 hours, but the product observed varied from a pK_c of 26.0 at 25 g/litre to about 24.0 at 0.25 g/litre.

The ion products (pK) observed by the fourth day with a solid:solution ratio of 25 g/litre were well within the range 26.1 to 26.9 observed in the previous series of experiments (2:2).

Discussion.

Logan and Taylor (81) stated ...

> "The ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ increases as the amount of bone or tricalcium phosphate, equilibrated with solutions of their ions, decreases below 150 mg per litre".

The experimental evidence for this unlikely statement consists of three series of equilibrations (17 experiments) with calcium phosphate precipitates, and 5 experiments with bone powder. Three different techniques were employed in order to test the postulated adsorption of calcium and phosphate ions onto the precipitate at ion products below the 'solubility product' of 'bone salt'. In all their experiments, however, the solid phase had either been recently formed by precipitation, or the solid phase had been placed in a solution supersaturated in terms of the ion product subsequently to be demonstrated. Klement (72) and Greenwald (45,46) have shown that the high ion products (pK = 23.1) observed by Logan and Taylor with small amounts of solid phase, were probably due to the inability of the very small amounts of solid present to reduce the 'supersaturated' solution to equilibrium levels. We have already seen titration data with precipitate analysis (Fig. 1) in which it can be shown that it is relatively easy to produce stable 'supersaturated' solutions of calcium and phosphate. This state is readily obtained by increasing the pH of a mixture of $Ca(OH)_2$ and sodium phosphate.

Klement and Greenwald's findings are substantiated by the results of the present experiments. Although the pK_c does fall as the solid:solution ratio falls in experiments from supersaturation, the reverse is true in experiments from undersaturation indicating the inability of small amounts of bone powder to take up or give up calcium and phosphate.

It is interesting to note in these experiments that the phosphate appears to be much more 'mobile' than the calcium. In Fig. 16 it can be seen that the equilibrium phosphate concentrations were achieved from undersaturation within 24 hours in all but the lowest solid:solution ratio experiments, and in the supersaturation experiments equilibrium concentrations were achieved within 3 days at 12.5 g solid/litre solution. This is in marked contrast to the limited mobility of the calcium concentration.

As the pH is relatively constant throughout this series the trivalent phosphate concentrations show characteristics similar to those of the total phosphate concentrations.

As in the previous series (2:2) it is apparent that the inorganic phosphate concentration in the buffers in contact with bone powder is consistent at about 0.2mM.

Summary.

It has been demonstrated that bone behaves in a very reproducible manner when placed in buffer solutions. Apparently it will dissolve either with liberation of base, or with the conversion of a basic radicle to a more acid one and consequent removal of H^+ from solution. The process is readily controlled by addition of appropriate buffer components.

Bone in solution yields a relatively constant ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ over the pH range 6.58 to 7.80 both from undersaturation and supersaturation and the mean value of this product is 4.17×10^{-27} ($pK_c = 26.39$). The possibility of the solid phase $CaHPO_4$ being involved in equilibria at these pHs is repudiated.

The ion product obtained is believed to be a genuine equilibrium of 'biological' significance, as it appears that the conditions employed (viz. more than 25 g solid/litre solution) are likely to be those of physiological bone mineral *in vivo*.

The suggestion that bone salt at high solid:solution ratios can absorb ions in such a way as to reduce the observed equilibrium ion product is not substantiated.

The results may indicate the existence of a physico-chemical property of skeletal mineral which controls the concentrations of tissue fluid calcium and phosphate, possibly by a

mechanism involving pH.

At this stage of the work it is of note that the phosphate concentrations appear to behave in rather a different manner from the calcium concentrations.

EQUILIBRATION STUDIES WITH HUMAN ADULT
BONE IN SYNTHETIC ULTRAFILTRATE OF PLASMA ...

3:1 Short-term experiments.....(4 - 6 hours)

3:2 Long-term experiments.....(24-30 hours)

It has been shown that when powdered human bone was allowed to come into equilibrium with tris and cacodylate buffer solutions containing no bicarbonate, it reproduced a relatively constant ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ over a pH range of 6.6 to 7.8. The constancy of this empirical product was an experimental observation suggestive of a solubility product for bone mineral, although the precise nature of the solid phase was not known. The arithmetic mean value of the ion product was 4.1×10^{-27} M and would correspond to the product of the ion concentrations observed in normal tissue fluids if the pH at the interface was 6.8.

Although the ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ at equilibrium at pH 6.8 was equivalent to that in tissue fluid at pH 7.4, the Ca:P Molar ratio was much higher than that of extracellular fluid (1.5 to 1) approaching a value of 16 to 1. These systems were therefore less similar to the <u>in vivo</u> situation than consideration of the 'product' alone might suggest. However, the buffers employed were not 'biological' buffers and further experiments were therefore performed with a modified Krebs Ringer bicarbonate solution of electrolyte composition similar to that of normal tissue fluid.

A further reason for studying the properties of this medium was the fact that the 'solubility product' of calcium phosphate was known to be markedly affected by the presence of other electrolytes

in the solvent.

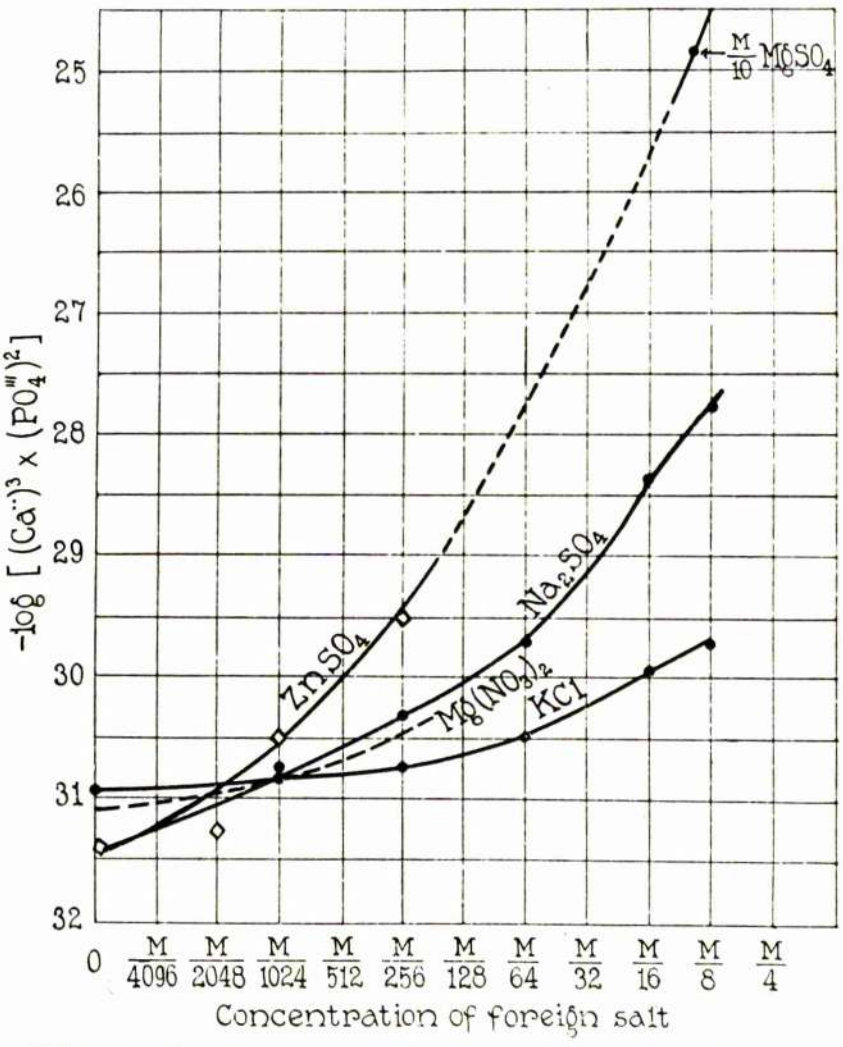
Fig. 19 is reproduced from Holt et al (59) and shows the variation in pK ion product for (?) $Ca_3(PO_4)_2$ in solutions containing a number of salts at various concentrations.

The temperature of equilibration in these experiments was 37°C in an attempt to simulate <u>in vivo</u> conditions.

<u>Experimental</u>.

Two series of experiments were performed .....

In the first series the limitations of the equipment imposed a time-limit of about six hours on the equilibration. The second type of experiment was therefore planned to run for 24 hours unattended, and the equipment was designed and built accordingly. Unfortunately, technical difficulties resulted in a rather cumbrous set-up of limited stability. The results obtained, however, were of great interest in view of their implications, and in their turn led to a simplification in experimental design.



Effect of foreign salts on the solubility of $Ca_3(PO_4)_2$ at 38°C.

Figure 19. **Effect of foreign salts on the solubility of $Ca_3(PO_4)_2$ at 38°C. Reproduced from Holt et al (59).**

3.1 Short-term experiments (4 - 6 hours).

Three-gram samples of bone powder in about 3 ml of buffer fluid (see below) were placed in sealed lengths of seamless regenerated cellulose tubing with ...

Modified Krebs Ringer Bicarbonate					
Na^+	114.4	mille-equiv.	per	litre	
K^+	5.0	"	"	"	"
Cl^-	102.7	"	"	"	"
HCO_3^-	16.7	"	"	"	"
Glucose	160.0	mg/100 ml.			

an average pore diameter of 24 Angstrom units, and attached to the vertical limbs of the gassing assembly (Figs. 20 and 21). The dialysis tubes were immersed in about 10 ml of the same buffer in glass equilibration vessels in a water bath at 37°C. The gassing assembly was attached to a 'Vibromix' unit which vibrated the samples at approximately one hundred cycles per second.

The pH was adjusted by controlled mixing of CO_2 and air. Each gas was fed through a rotameter to a humidifier at 37°C. containing buffer solution of similar chemical composition to that used in the equilibration vessels. After mixing, the gas was passed into the dialysis tubes via the gassing assembly and also through fine-bore polythene tubes to the fluid in the equilibration vessels. The pH was recorded electrometrically with a probe

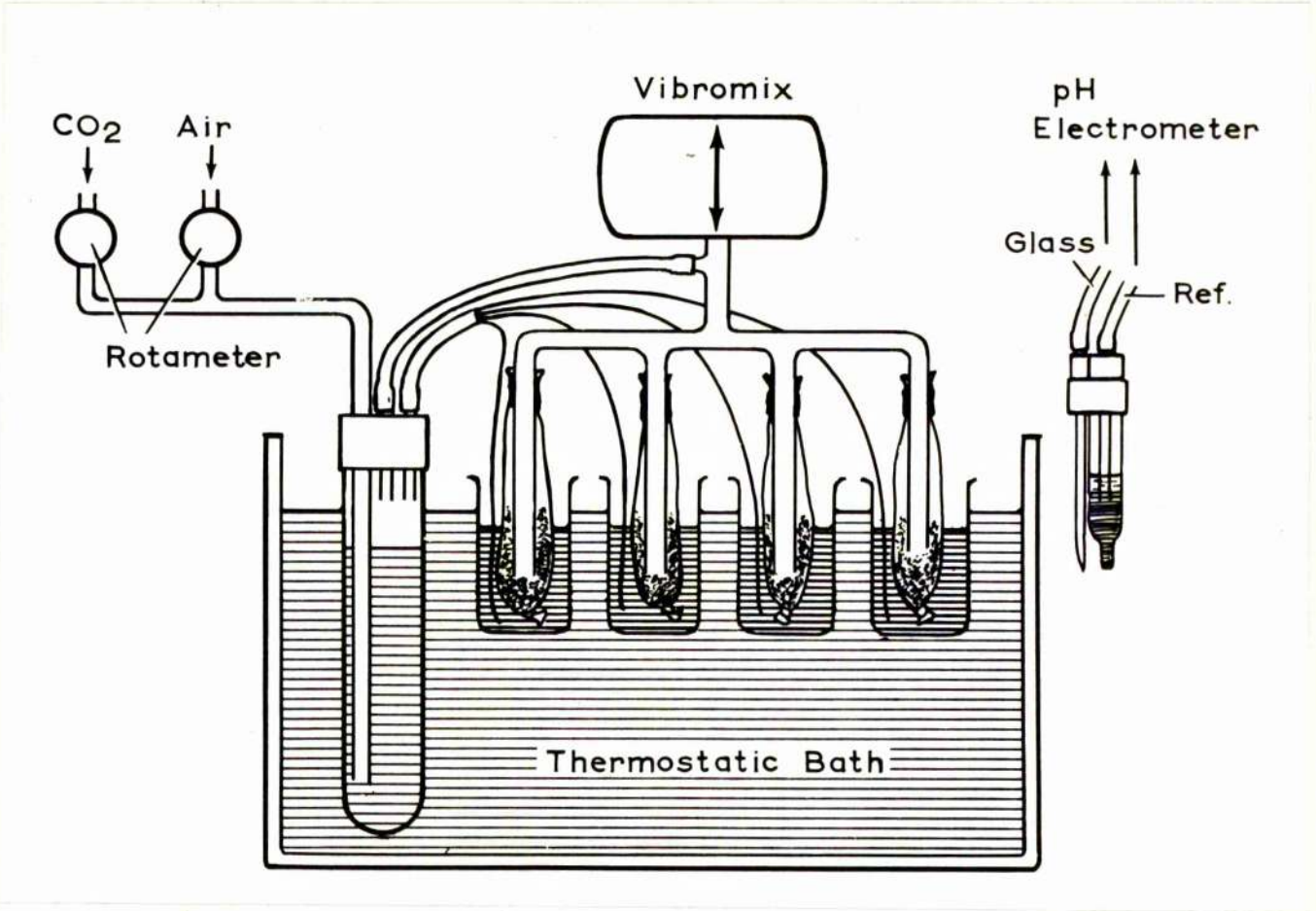


Figure 20. Diagram of equipment constructed for 4 to 6 hour equilibration studies with bone powder in synthetic ultrafiltrate of plasma.

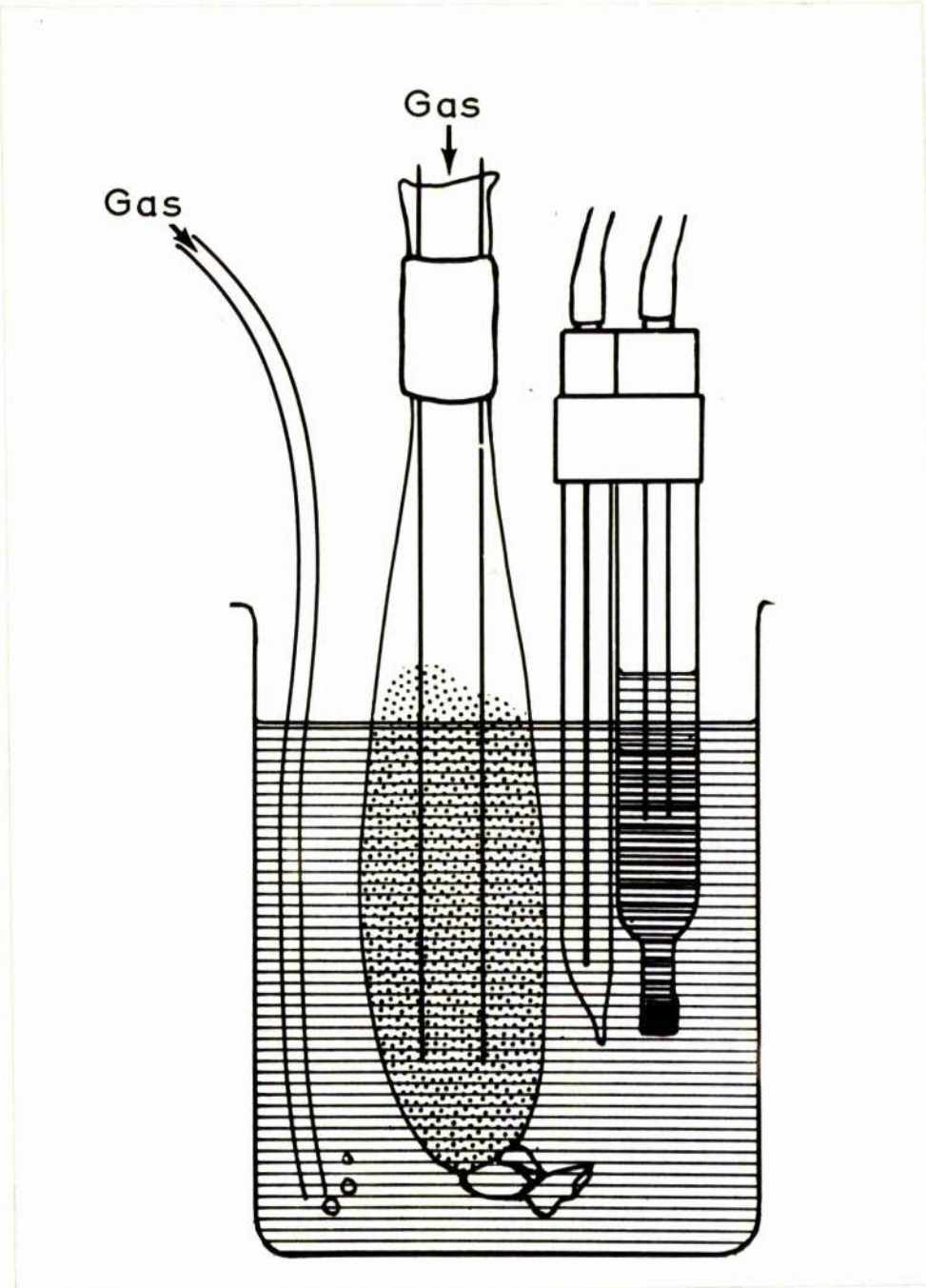


Figure 21. Diagram of equilibration vessel showing bone powder inside a dialysis bag attached to the descending limb of the gassing assembly.

consisting of one spearpoint glass electrode, and one standard calomel(KCl) electrode. The equilibration vessels were not sealed and the probe was dipped into the vessels in succession. The pH range of 6.7 to 7.4 was obtained by varying the partial pressure of CO_2 from 50% to 5.4% of ambient pressure.

The 42 short-term experiments performed were from undersaturation, and had no calcium or phosphate present initially in the buffer.

Samples of the equilibration fluid were withdrawn from the cells at four and six hours after commencement of the experiment and the concentrations of calcium and inorganic phosphate determined. In some cases the total carbonate concentration was also determined.

3:2 Long-term experiments (24-30 hours)

Samples of bone powder (0.5 gms) were placed in knotted lengths of dialysis tubing with about 1 ml of synthetic ultrafiltrate solution. The dialysis bags were placed with 20 ml of the buffer in a specially constructed cell (Fig. 22) and allowed to equilibrate for 24 to 30 hours in an incubator at 37°C.

The pH was continuously recorded electrometrically, and maintained at a constant level by adjusting the partial pressure of CO_2 in the air supply to the cell. Using physiological concentrations of bicarbonate in the buffer, it was found possible to maintain a constant pH in the range 6.7 to 7.6.

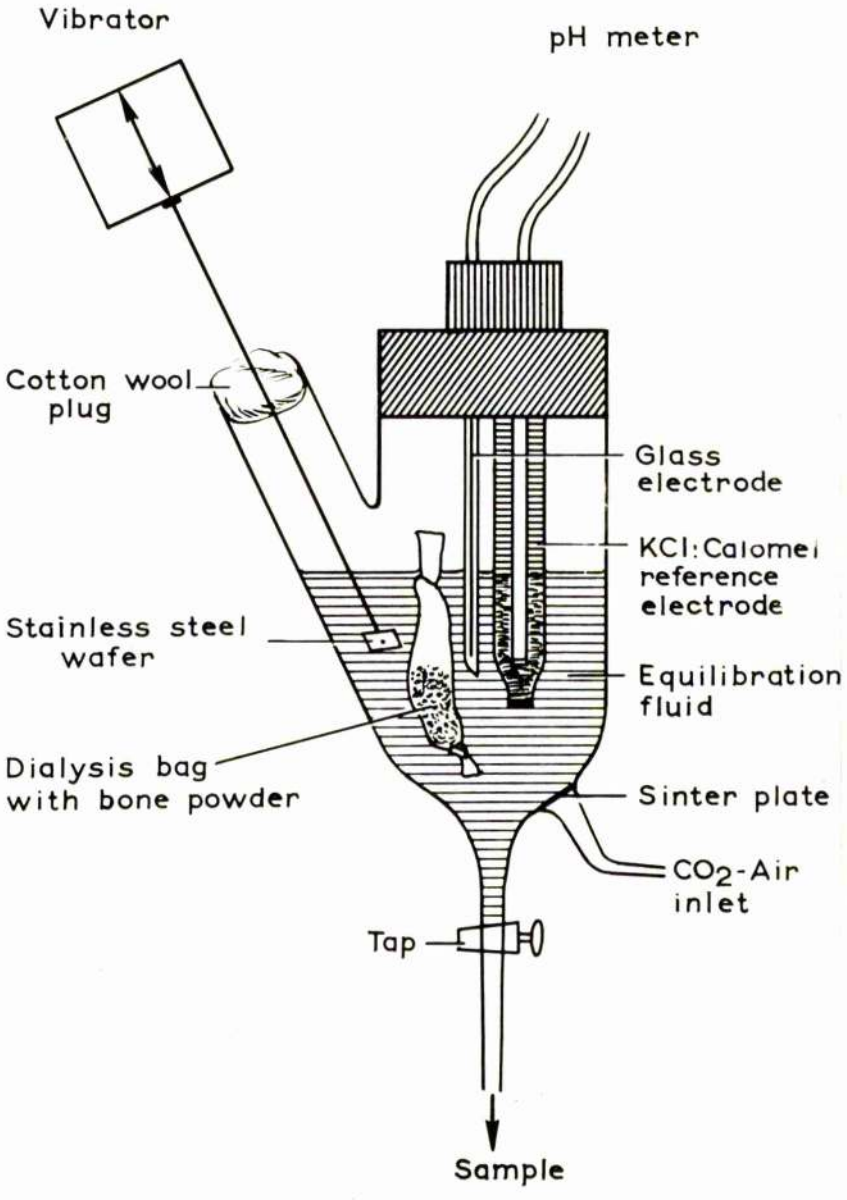


Figure 22. Diagram of equipment constructed for 24 to 30 hour equilibration studies with bone powder in synthetic ultrafiltrate of plasma.

The fluid in the cells was subjected to continuous vibration by a 'Vibromix' attached to a stainless steel wafer, and it was possible to remove samples of buffer for analysis without unsealing the cell and reducing markedly the CO_2 tension.

Results.

Short-term experiments (3:1)

The results of 42 experiments are shown in Figs. 23 to 26 and Table 5.

Fig. 23 shows that the concentration of calcium at 4 and 6 hours is related to pH. It averages from about 1.4mM at pH 6.8 to about 0.6mM at pH 7.3. These values are rather less than those found in bicarbonate-free buffers (about 4.0mM and 1.4mM respectively).

Fig. 24 shows that the concentration of inorganic phosphate at 4 and 6 hours ranges from about 0.3 to 0.7 $Mx10^{-3}$ and is influenced little, if at all, by pH. It is clear that over the pH range 6.6 to 7.4, the presence of bicarbonate raises the phosphate concentrations appreciably over the range observed in bicarbonate-free solutions which is indicated by the broken lines on the figure.

Fig. 25 shows that the pK of the ion product $[Ca^{++}]^3[PO_4^{=}]^2$ is fairly constant over the pH range 6.7 to 7.4, but after 4 and 6 hours equilibration the concentration observed is generally lower than that

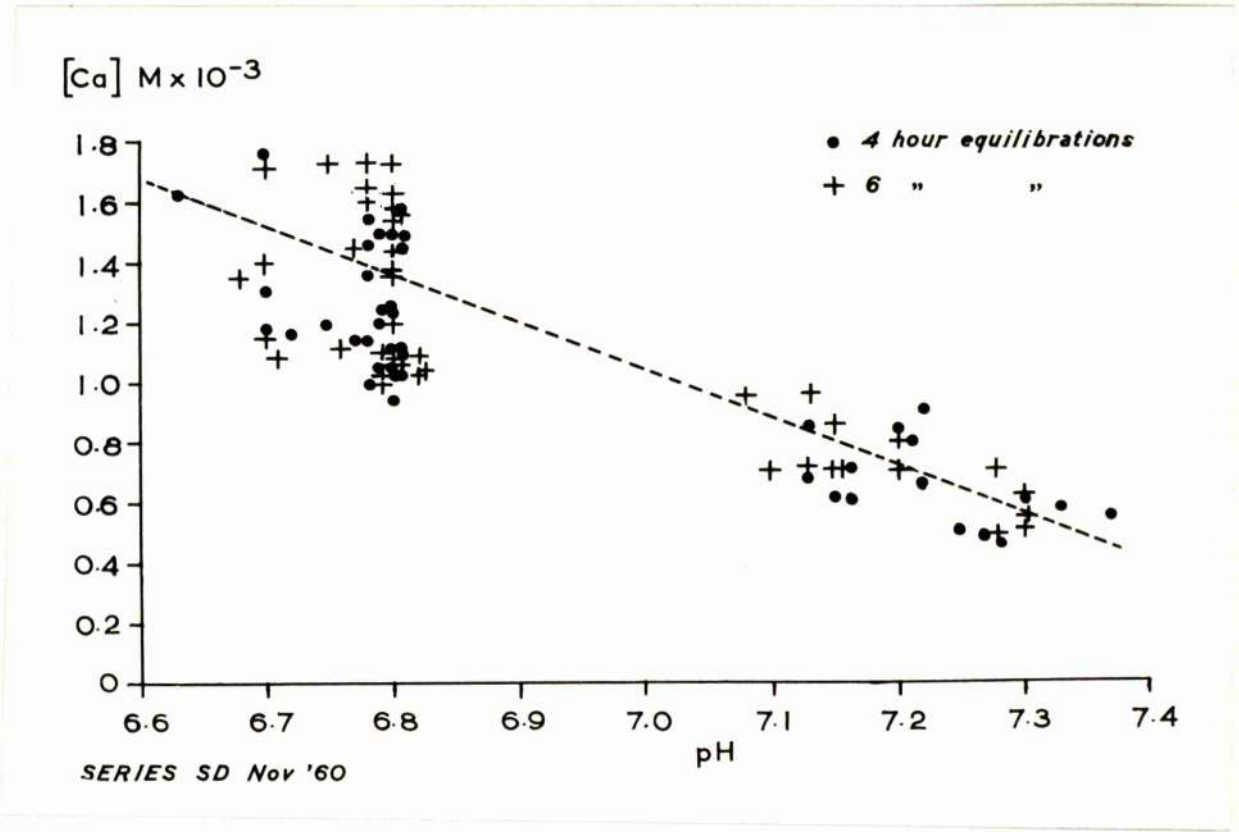


Figure 23. $[Ca^{++}]$ plotted against pH in the 4 to 6 hour studies.
The general trend of the relationship is indicated.

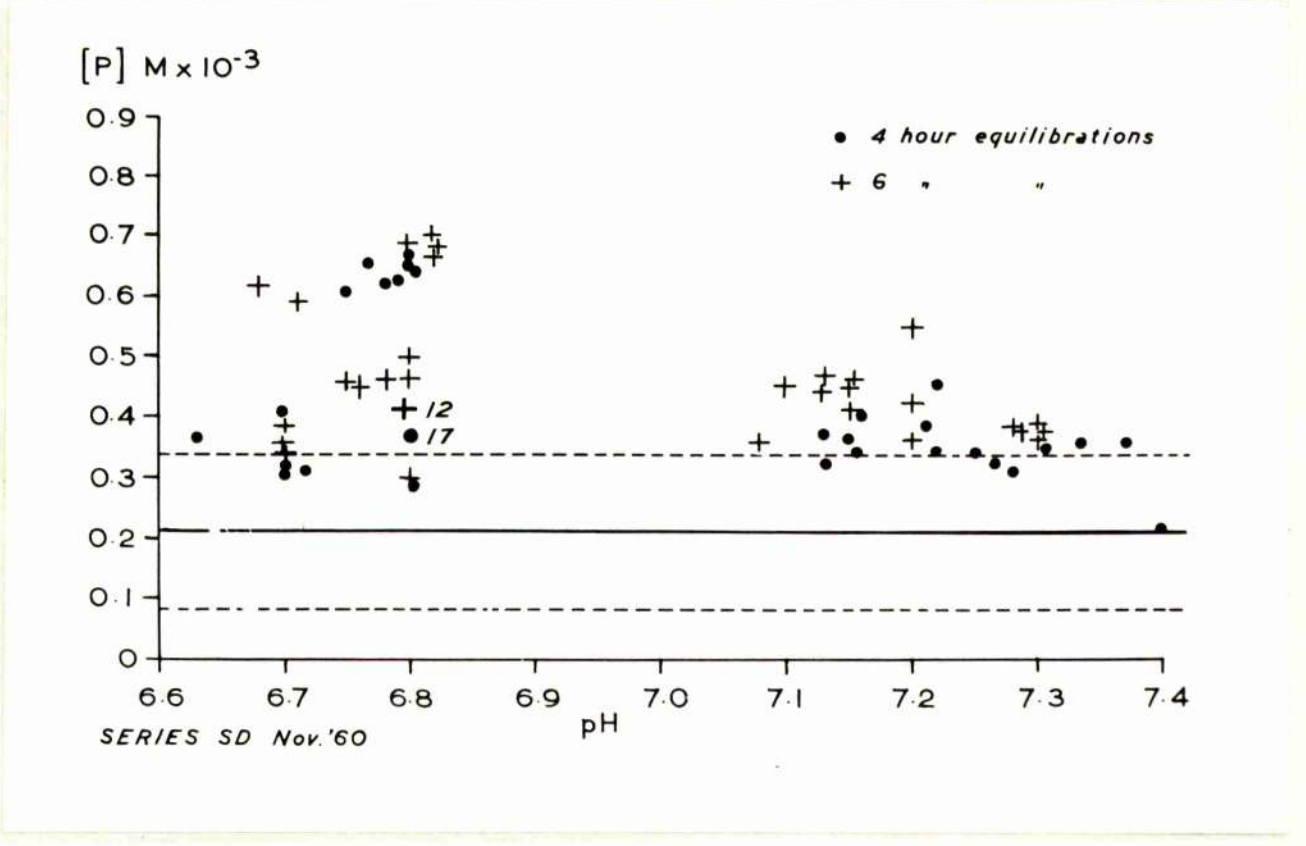


Figure 24. **[P] plotted against pH in the 4 to 6 hour studies. At that point shown by the numbers on the Figure there are 17 4-hour and 12 6-hour observations too close together to be plotted individually. The lines indicate the mean and range of [P] previously observed in bicarbonate-free media.**

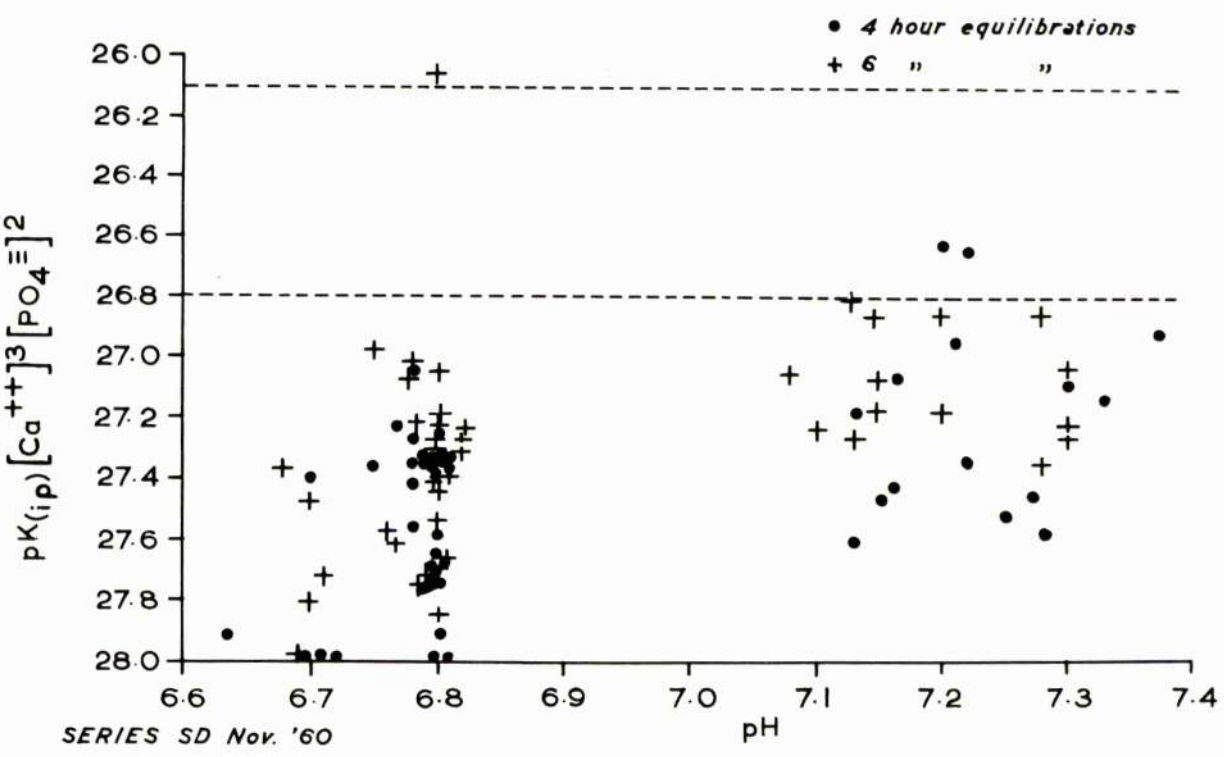


Figure 25. Values of $pK_c [Ca^{++}]^3 [PO_4^{\equiv}]^2$ plotted against pH in the 4 to 6 hour studies in synthetic ultrafiltrate of plasma.
The lines indicate the range of values observed in bicarbonate-free media.

obtained in bicarbonate-free buffers at 24 hours as shown by the broken lines on the figure. The observed product (pK) ranges from 28.0 to 26.6.

Fig. 26 shows the relationship between the phosphate and bicarbonate concentrations in those bicarbonate experiments at pH 6.75 - 6.80. It can be seen that the concentration of inorganic phosphate varies directly with that of bicarbonate. The figure also shows the range of phosphate concentration observed in 56 previous experiments with bicarbonate-free buffer over the pH range 6.58 to 7.91 (2:2).

24-30 hour experiments (3:2)

Figs. 27 to 29 show the results of sixteen 24 to 30 hour equilibration experiments in the pH range 6.7 to 7.6 (22 observations).

Fig. 27 shows that the calcium concentration at equilibrium ranged from about 1.5×10^{-3} M at pH 7.6 to about 3×10^{-3} M at pH 6.7. At the lower pHs, these values are smaller than those previously obtained. The lines on the figure indicate the mean and range of calcium concentration previously observed with tris and cacodylate buffers.

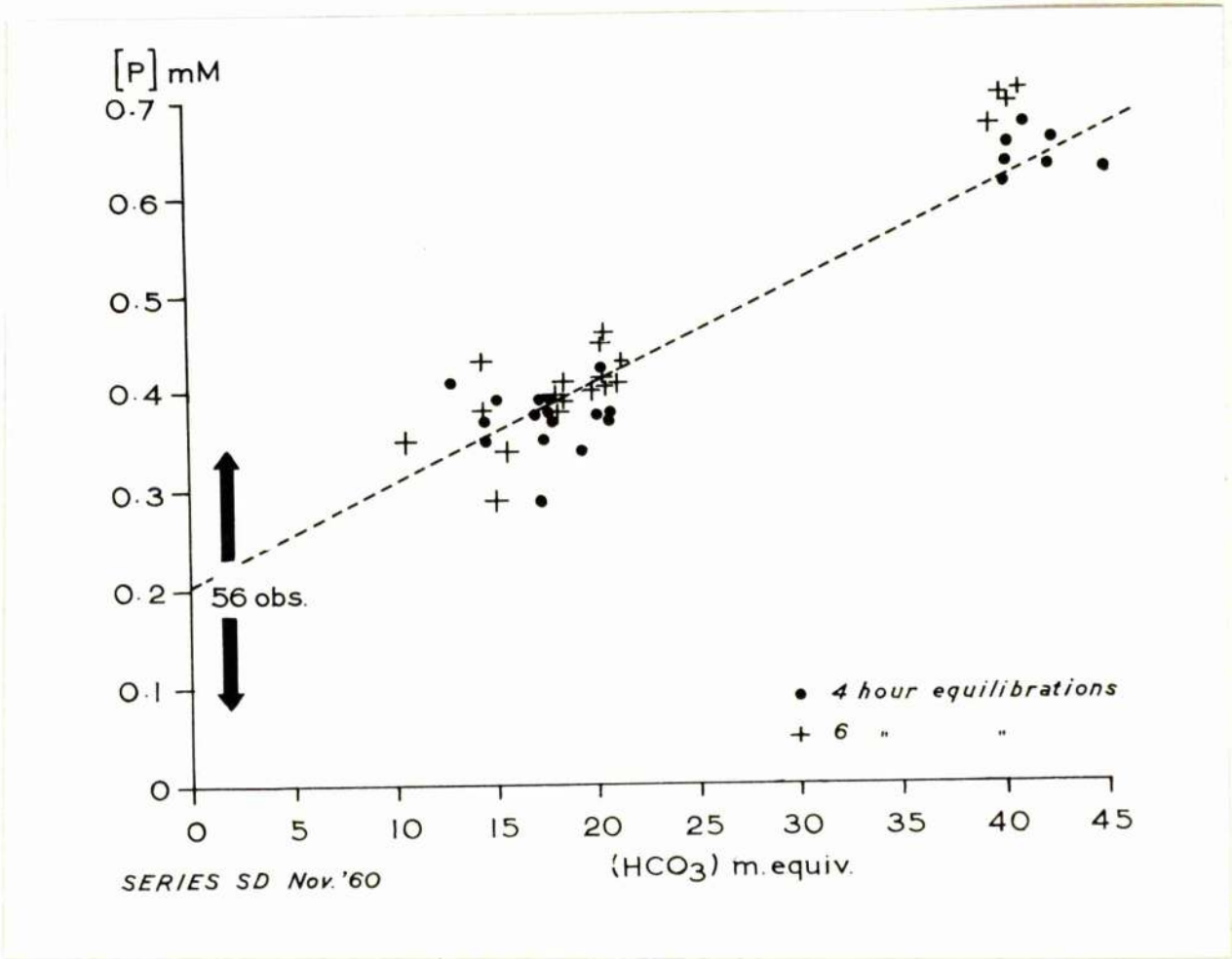


Figure 26. The concentration of total inorganic phosphate in 4 to 6 hour studies is shown plotted against bicarbonate concentration. Only the results in experiments in pH range 6.75 to 6.80 are shown. The arrows indicate the limits in [P] found previously in equilibrations in bicarbonate-free media over the pH range 6.58 to 7.91.

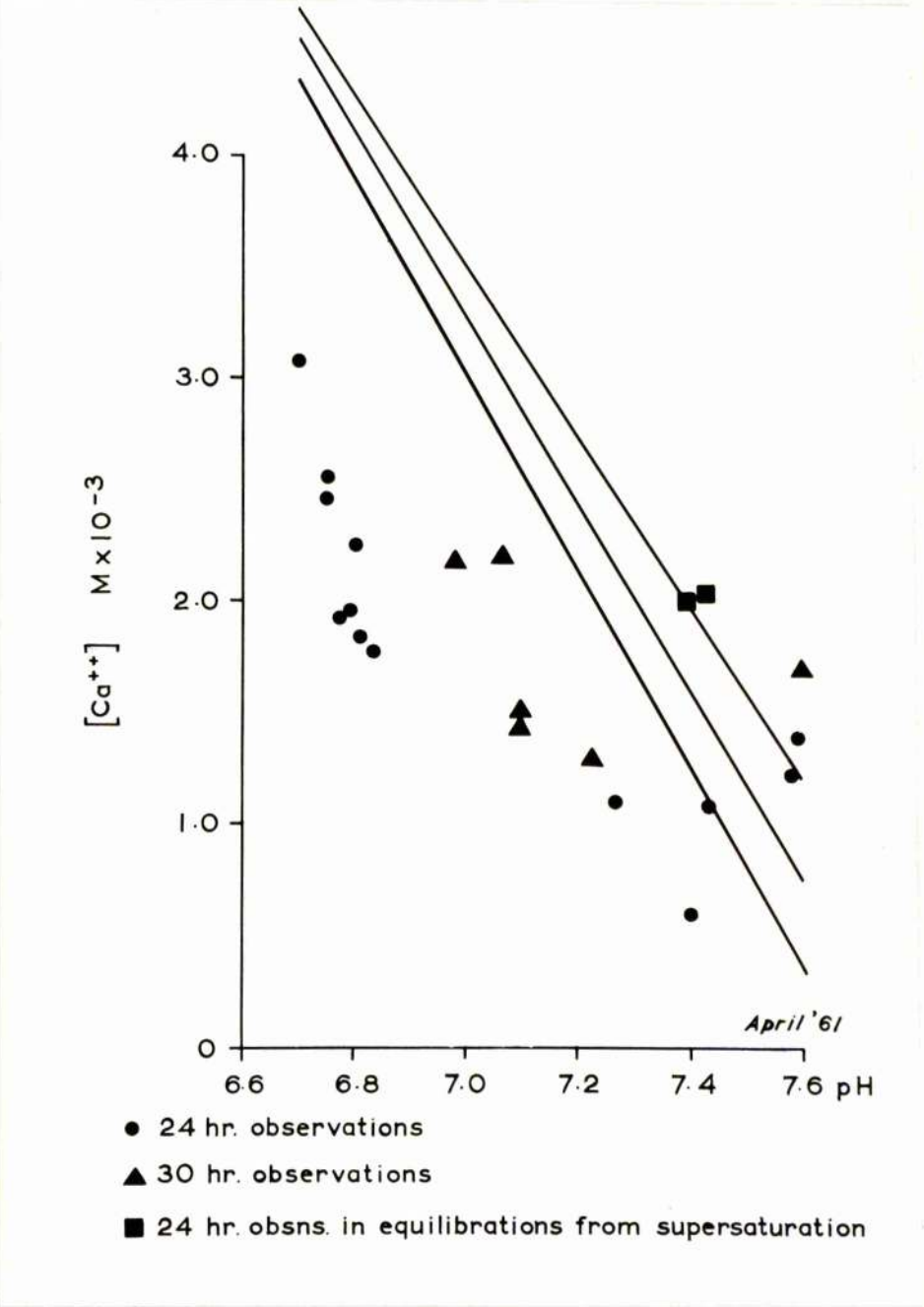


Figure 27. $[Ca^{++}]$ plotted against pH in the 24 to 30 hour studies.
The lines indicate the range of calcium concentration previously observed in bicarbonate-free media.

Fig. 28 shows that the equilibrium concentrations of total inorganic phosphate were consistently higher than those previously obtained with bicarbonate-free solutions.

Fig. 29 shows the values of pK for the ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ in the various systems studied so far. In the bicarbonate experiments, the 6-hour product was rather lower than that obtained with bicarbonate-free solutions: this was probably due to incomplete equilibration since the 24-30 hour ion products did not differ significantly from those previously obtained, although the range was rather greater.

Discussion.

This series of experiments demonstrates fairly conclusively that when human bone powder is allowed to come into equilibrium with buffered bicarbonate solutions, the concentration of inorganic phosphate in the buffer is a function of the amount of bicarbonate present. At pH 6.8, the total inorganic phosphate concentration in a solution containing about 25mM bicarbonate was about twice as high as that found in bicarbonate-free solution. The phosphate concentrations found at 4, 6 and 24 hours at pH 6.6 to 7.6 were all higher than those obtained with bicarbonate-free buffers despite the probability that equilibrium was not achieved in the series of short-term experiments. The values obtained at these different times, however, did not differ significantly, and so the analysis of the phosphate-bicarbonate

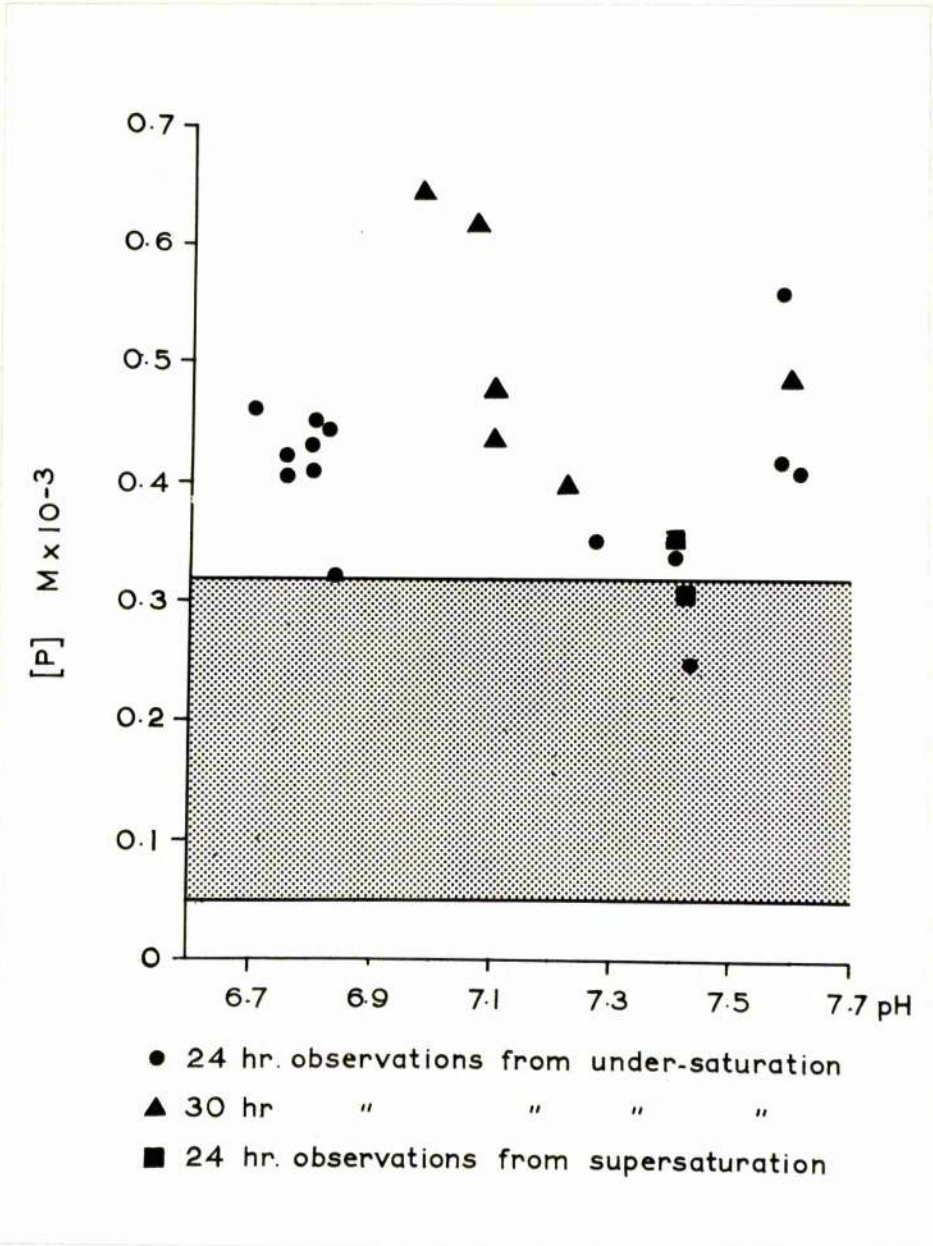


Figure 28. **[P] plotted against pH in the 24 to 30 hour studies.**
The range of values previously observed in bicarbonate-free media is indicated.

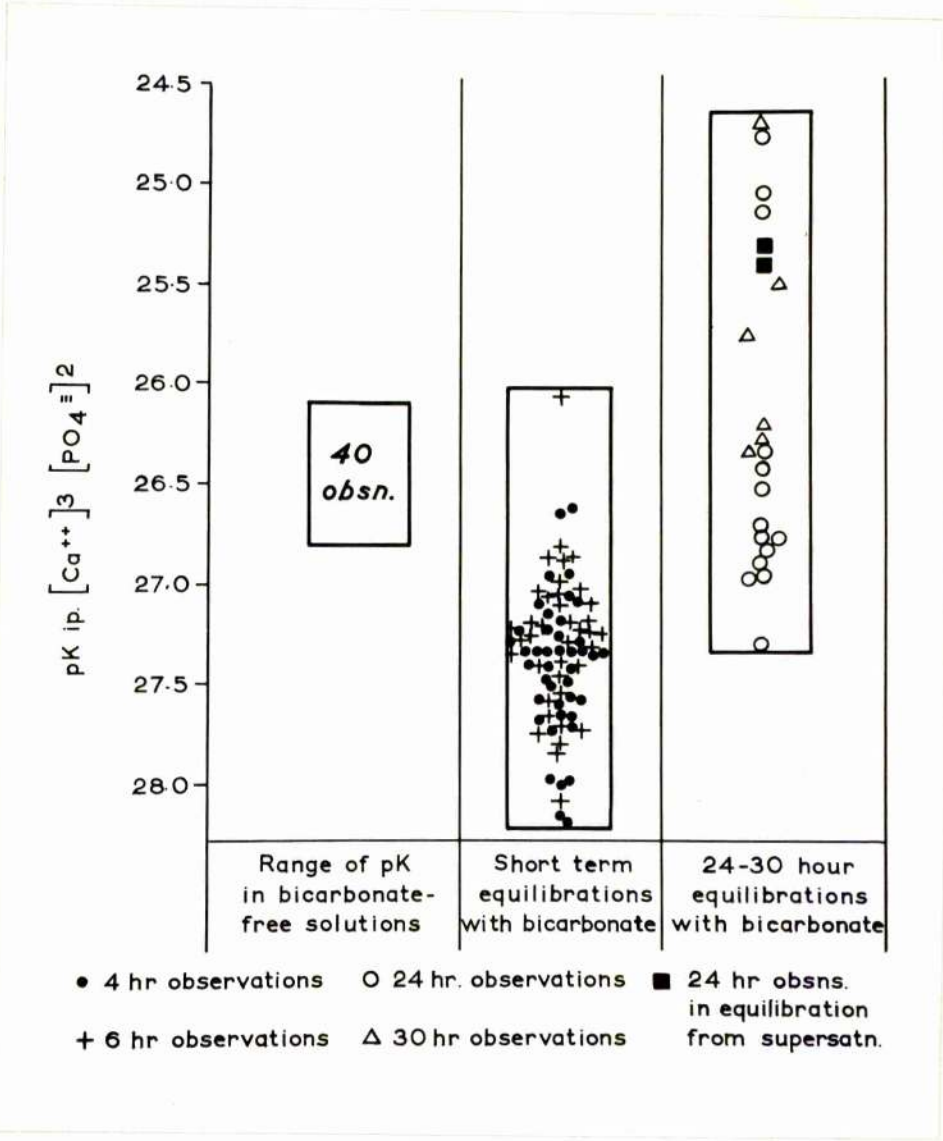


Figure 29. Summary of pK_c $[Ca^{++}]^3[PO_4^{\equiv}]^2$ values observed in the short-term and 24 to 30 hour studies in synthetic ultrafiltrate of plasma, and in 40 experiments in bicarbonate-free media.

relationship is based on all the data available.

The calcium concentrations observed were lower than those observed in bicarbonate-free solutions at the same pH. The values observed at 24 hours were higher than those obtained at 4 and 6 hours, again suggesting that the latter therefore did not represent equilibrium conditions. At 24 hours, the mean ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ did not differ significantly from that previously reported for bicarbonate-free solutions, although the range was much greater. The greater variability was due to the difficulties inherent in a system employing CO_2 at high partial pressures for the maintenance of a constant pH. In this system there was a general tendency for the pH to rise as the equilibration progressed, possibly due to elution of carbonate.

These results can be interpreted in at least two ways. The first and simplest explanation would be a heteroionic exchange of carbonate or bicarbonate for phosphate. Such a phenomenon has been reported by Neuman to occur in hydroxyapatite (105,107), and since it is now generally accepted that the larger ionic radius of carbonate prevents it from occupying the phosphate locus within the lattice without distortion (142,146,147), this exchangeable phosphate would represent material adsorbed at the crystal surface.

An alternative explanation could be based upon the hypothesis of bone salt composition originally propounded by Dallemagne (25,26,28), who claims that bone mineral is a two-phase system of calcium carbonate and tri-calcium phosphate hydrate. If this were the case, bone mineral would behave as a mixture of two sparingly soluble salts with calcium as the common ion. In such a system, the elevation of the carbonate ion concentration in the liquid phase would reciprocally depress the calcium ion concentration. In turn this would allow the phosphate ion concentration to rise. Such a system would not only explain the present data, but might serve to explain the 'unphysiological' calcium:phosphate ratios at equilibrium which we have previously found in bicarbonate-free solutions in equilibrium with bone powder.

It was previously suggested that the high calcium:phosphate ratio obtained at pH 6.8, which might be the effective pH at the crystal surface, could be explained by the continuous solution of a basic salt of calcium phosphate with simultaneous precipitation of an acidic form with uptake of hydrogen ion and release of calcium ion.

The concept that bone mineral comprises two distinct phases - a phosphate and a carbonate - suggests the possibility that in certain circumstances the dissolution of calcium carbonate might occur simultaneously with the precipitation of calcium phosphate with resulting high Ca:P ratio. In support of this hypothesis, it has been claimed

that the preferential mobilization of calcium carbonate has been demonstrated by elution experiments with dilute hydrocholoric acid (24,27,29,71,82,105). High calcium:phosphate ratios might thus represent primarily the dissolution of the carbonate fraction of bone with release of carbon dioxide by the acid which is usually added in order to maintain a constant pH. The calcium concentration would thus continue to rise until it reached a 'ceiling' determined by the $[Ca^{++}]^3[PO_4^{\equiv}]^2$ ion product and the prevailing concentration of inorganic phosphate. The mechanism which tends to 'fix' the concentration of the latter is unexplained.

This series of experiments which was initiated with the intention of confirming the constant ion product for bone salt in synthetic biological media has brought to light a most important physico-chemical relationship between phosphate and carbonate ions. This relationship was considered to require further detailed study.

EQUILIBRATION STUDIES WITH ADULT HUMAN BONE IN BICARBONATE MEDIA

4:1. Equilibrations in tris:bicarbonate buffers at increasing ionic strengths.

4:2. The effect of ionic strength on bone equilibria in general.

4:3. 24-hour equilibrations in tris:bicarbonate buffers at constant ionic strength.

4:4. 72-hour equilibrations in tris:bicarbonate buffers at constant ionic strength ($\mu = 0.15$) both from undersaturation and supersaturation with respect to calcium and inorganic phosphate.

4:5. Equilibrations at very high solid:solution ratios.

SUMMARY.

In view of the findings reported in the previous section it was decided to confirm the interpretation by isolating the effect of carbonate ion concentration on the equilibrium phosphate concentration. The original simple experimental design was employed with the addition of potassium bicarbonate to the tris buffers.

Five series of experiments are reported .....

4:1 Equilibrations in tris:bicarbonate buffers at increasing ionic strengths.

Experimental.

The 16 equilibrations reported were performed as before (2:2) with the exception of changes to the buffer formulae. Tris buffer, 0.15 M was brought to pH 7.00 and then 10, 20, 30 and 40mM $KHCO_3$ was added to litre aliquots. All equilibrations were from undersaturation and $[Ca^{++}]$, $[P]$, $[CO_2]$ and pH were determined at 24 hours.

Results.

The results are shown in Table 7 and Figs. 30 to 32.

Fig. 30 shows the calcium, total inorganic phosphate and total carbonate concentrations at 24 hours. Over the pH range 7.35 to 7.60 $[Ca^{++}]$ falls from about 1.0 to 0.45mM, $[P]$ rises from about 0.35 to 0.7mM and $[CO_2]$ rises from about 9.0 to 40mM respectively.

Fig. 31 shows the $pK_c [Ca^{++}]^3 [PO_4^{\equiv}]^2$ ranging from about 26.4 to 26.1. The ion strength varies from 0.16 at pH 7.35 to 0.19 at 7.60. An estimate of the thermodynamic solubility product pK_{sp} $Ca_3(PO_4)_2$, employing the correction previously detailed (1.1), is shown and all the calculated values lie between 30.3 and 30.0 - a reasonably narrow range.

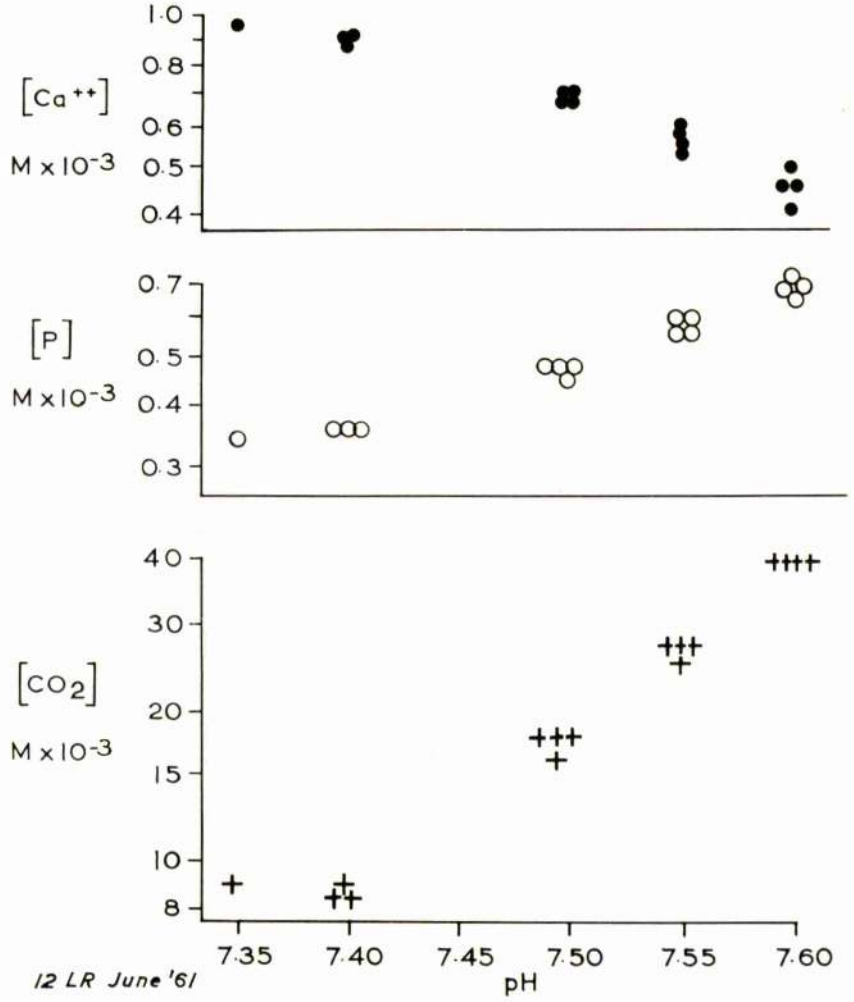


Figure 30. $[Ca^{++}]$, $[P]$ and $[CO_2]$ plotted against pH in 24-hour equilibration studies with human bone powder in tris buffer with added bicarbonate. The ionic strength of the buffer ranged from 0.16μ to 0.19μ.

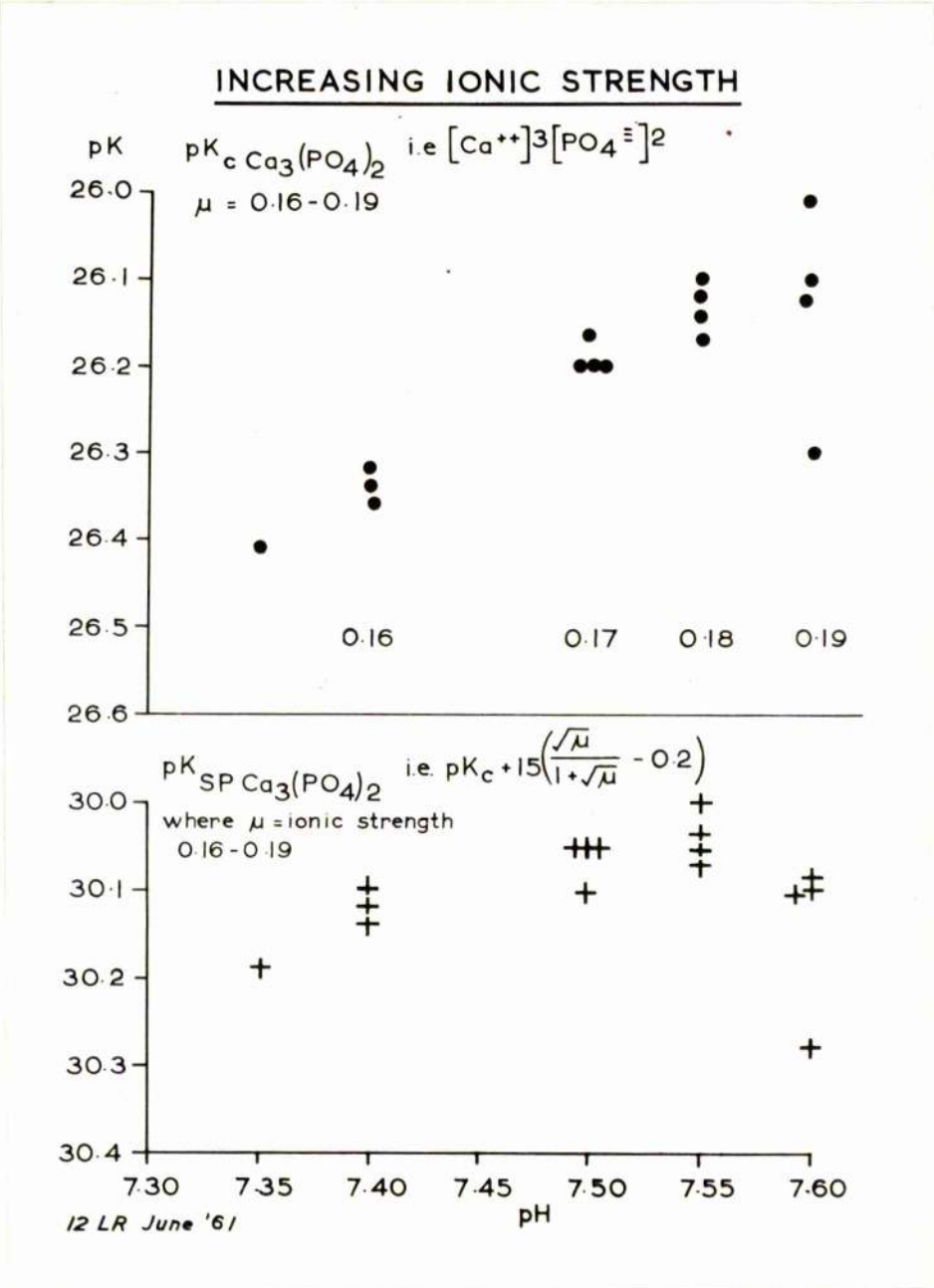


Figure 31. The pK $[Ca^{++}]^3[PO_4^{\equiv}]^2$ plotted against pH in 16 experiments in tris-bicarbonate buffer. An estimate of the thermodynamic solubility product (after Nancollis , 1:1) is also shown plotted against pH.

Fig. 32 shows the corresponding data for the pK_c and pK_{sp} of the ion product $[Ca^{++}][CO_3^{=}]$. Both appear to be correlated with pH, the former ranging from 8.0 to 7.4 and the latter from 9.0 to 8.4 over pH range 7.35 to 7.60.

Discussion.

As seen in the synthetic plasma ultrafiltrate experiments, the phosphate concentration at equilibrium increased as the carbonate concentration increased.

The $pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$ values all lie within the accepted range but there is a suggestion of a correlation with pH. Unfortunately, in this series of experiments, the ionic strength of the buffer increased with added bicarbonate. An approximate estimate of the pK_{sp} was obtained by correcting for non-specific ion effects by the expression previously described (1:1).

It can be seen that the range for the thermodynamic solubility product is slightly diminished by this correction, the best estimate probably being about 30.1.

The ion product $[Ca^{++}][CO_3^{=}]$ can be seen to be highly correlated with pH and the correction for non-specific ion effects appears to make little difference.

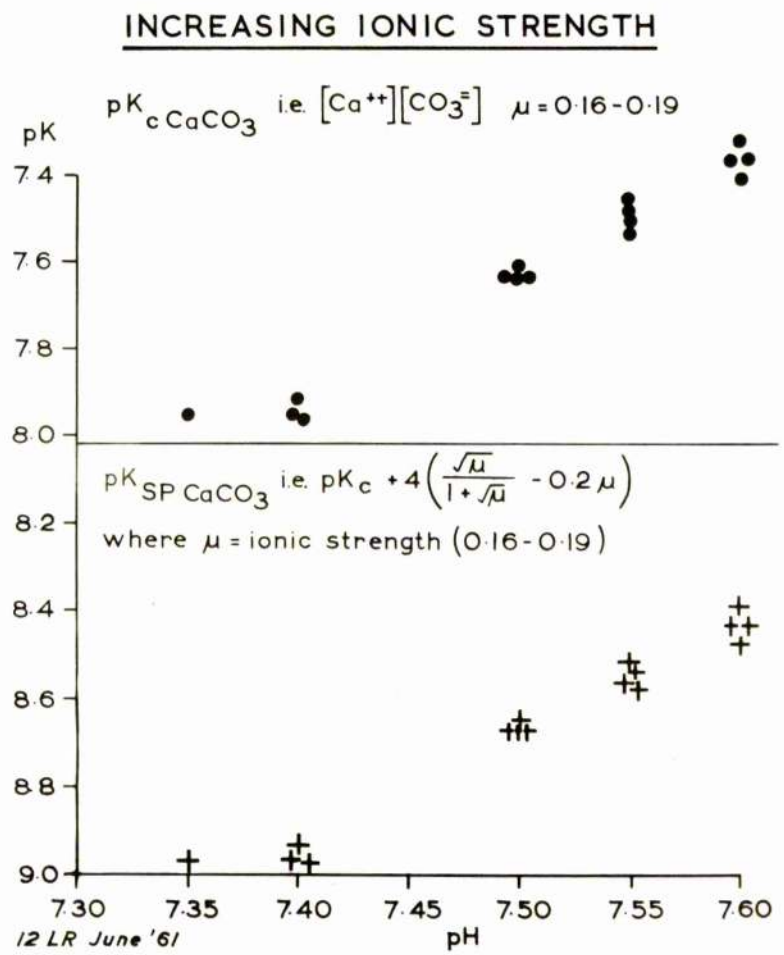


Figure 32. The pK_c $[Ca^{++}][CO_3^{=}]$ and an estimate of pK_{sp} $[Ca^{++}].[CO_3^{=}]$ plotted against pH in 16 equilibration experiments in tris:bicarbonate buffers at varying ion strengths.

In view of one apparent failure of the ionic strength correction to eliminate the pH dependence of the products, it was decided to test the ionic strength correction empirically and then to repeat the equilibrations with varying carbonate as described above at constant ionic strength.

4:2. The effect of ionic strength on bone equilibria in general.

Neuman has maintained that one cannot calculate a thermodynamic solubility product for a substance (bone mineral) the activity of whose ions in biological media is unknown (106). In 1:1 a correction for non-specific activity effects suggested by G.H. Nancollis (personal communication) has been reproduced, and this correction has been tested in the equilibration system.

Experimental.

A series of 24 experiments in six groups was prepared as in 2:2. On this occasion the buffer employed was 0.01M, 0.05M, 0.10M, 0.20M, 0.30M and 0.45M tris, initially at pH 7.2. Of the 4 experiments at each ion strength, two were from undersaturation and two had 8 mg calcium and 0.8 mg inorganic phosphate per 100 ml (2mM Ca:0.26mM P) added to the buffers. The experiments were terminated at 24 hours and $[Ca^{++}]$, [P] and pH determined.

Results.

The results are shown in Table 8 and Figs. 33 to 36. Since $[Ca^{++}]$ is known to vary with pH, only those values within the pH range 7.3 to 7.4 are plotted in Fig. 33. It can be seen that although there appears to be some difference between experiments from undersaturation and those from supersaturation, within each group $[Ca^{++}]$ increases quite markedly as ionic strength of the buffer increases, despite a constant pH.

Fig. 34 shows the corresponding data for $[P]$. Since normally there is no correlation between $[P]$ and pH, all $[P]$ values are plotted. In this case there is a decided correlation between $[P]$ and the ionic strength of the buffer.

Fig. 35 shows the relationship between $pK_c [Ca^{++}]^3 [PO_4^{\equiv}]^2$ and ionic strength. The ion product increases from a pK_c of about 27.5 at $\mu = 0.01$ to about 26.5 at $\mu = 0.15$. At high ionic strengths the increase is less marked reaching a pK_c of about 26.0 at $\mu = 0.45$.

Fig. 36 shows the ion products corrected for non-specific ion effects by the procedure outlined in section 1:1. It can be seen that the pK_{sp} value increases from about 28.7 at $\mu = 0.01$ to about 30.5 at $\mu = 0.20$. At ionic strengths greater than 0.2 the corrected ion product appears to be about 30.6.

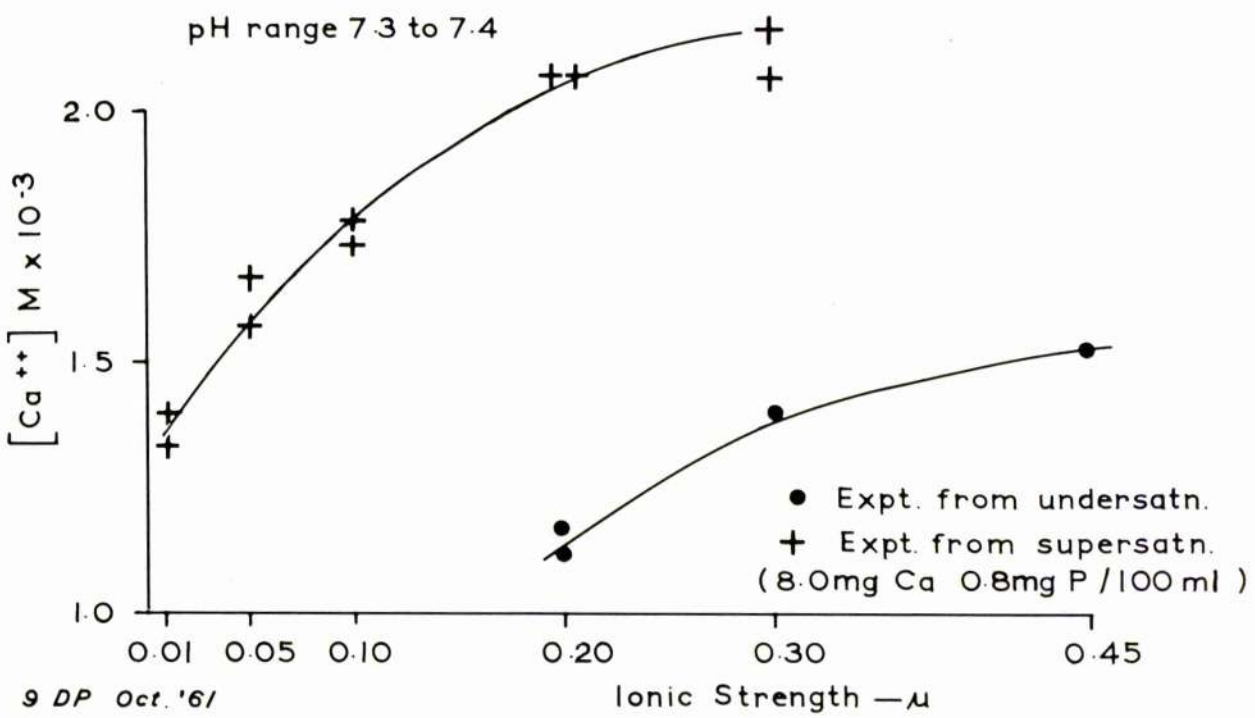


Figure 33. **[Ca^{++}] plotted against ionic strength in equilibration studies in tris buffer over pH range 7.30 to 7.40.**

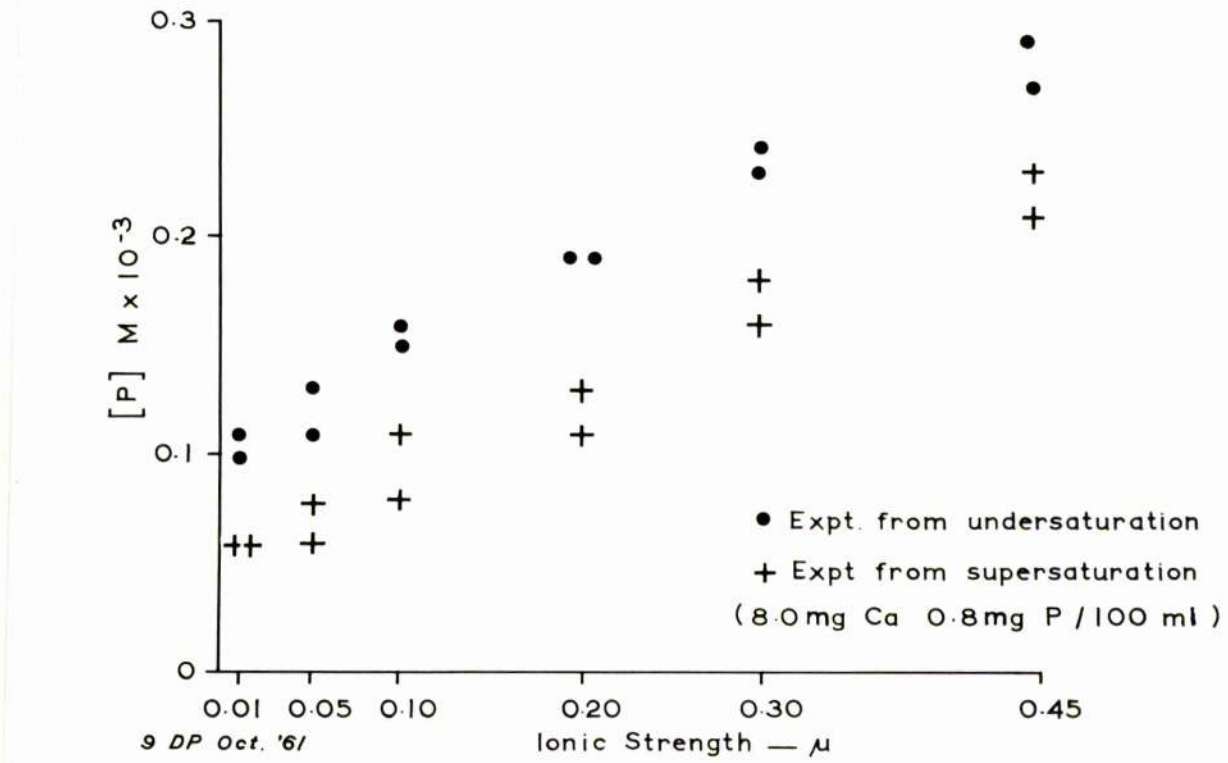


Figure 34. [P] plotted against ionic strength in equilibration studies in tris buffer over pH range 7.30 to 7.40.

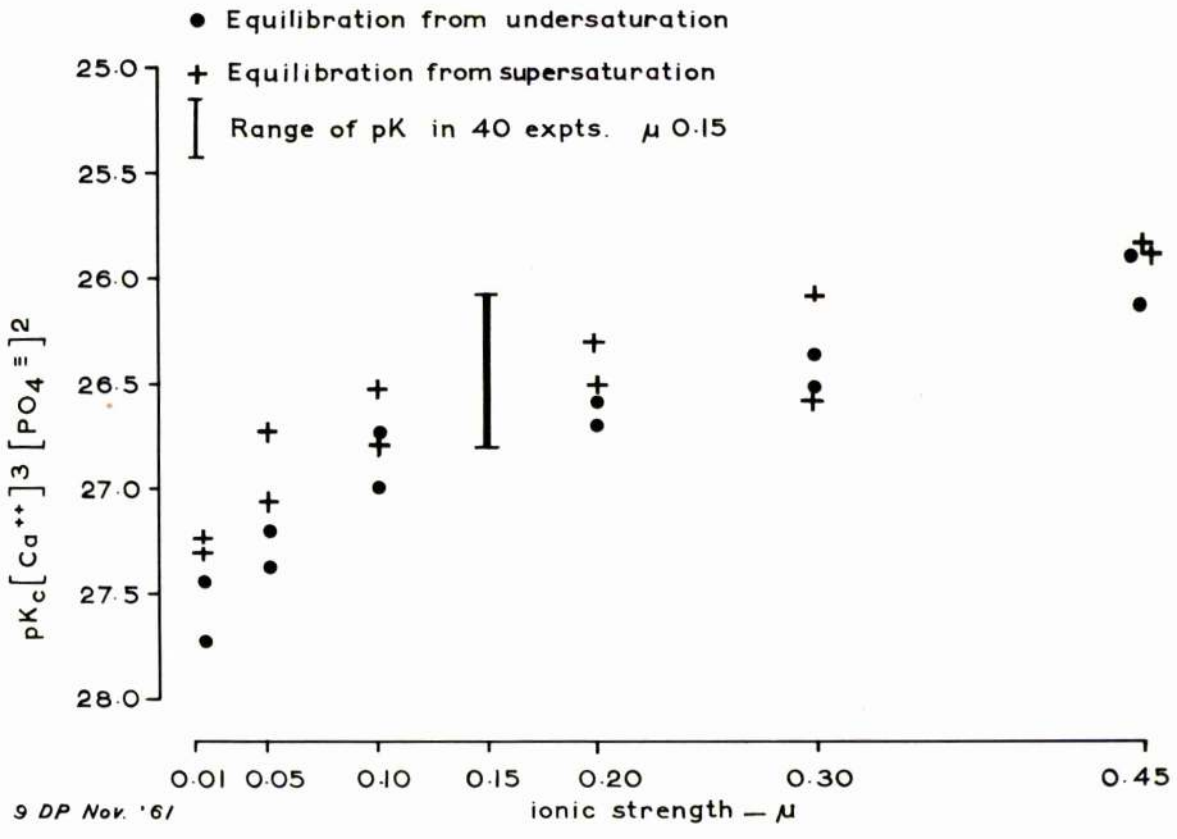


Figure 35. Values for $pK_c [Ca^{++}]^3 [PO_4^{\equiv}]^2$ plotted against ionic strength in equilibration studies in tris buffer in the range of values previously observed in tris at $\mu = 0.15$, is indicated.

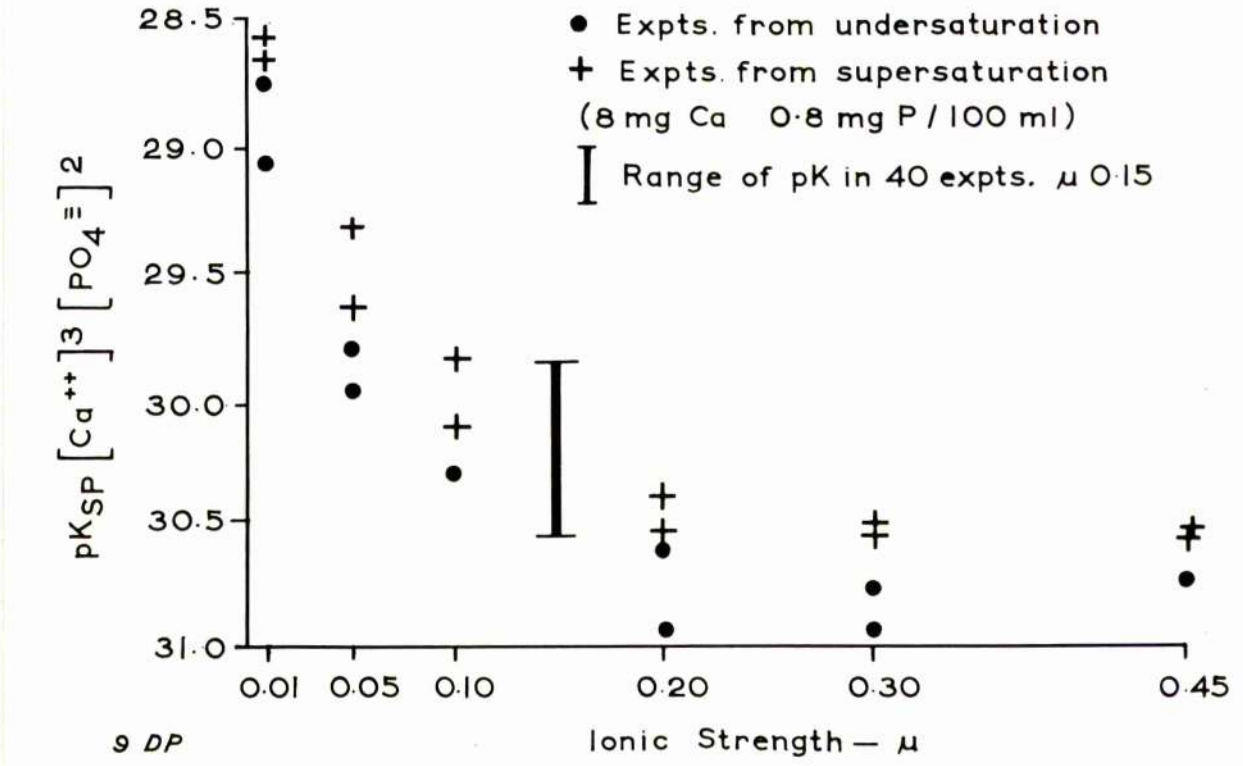


Figure 36. Values for $pK_{sp}[Ca^{++}]^3[PO_4^{\equiv}]^2$ (after Nancollis) plotted against ionic strength in equilibration experiments in tris buffer. The corresponding estimates for 40 experiments previously performed in tris buffer at $\mu = 0.15$, are indicated.

Discussion.

It is apparent from the data, that although ionic strength undoubtedly has an effect on the equilibrium concentrations of calcium and inorganic phosphate, the ion products observed between μ = 0.10 and 0.30 are scarcely significantly different. This is undoubtedly due to the fairly wide scatter of pK_c values obtained with this experimental technique.

The correction for non-specific ion effects would appear to be applicable to all solutions of ionic strength in excess of 0.20. From the data presented here it would seem that at μ = 0.15 the correction is not valid for our system, and on empirical grounds one can say that all experiments must be conducted at constant ionic strength in order to eliminate non-specific ion effects.

4:3. Equilibrations in tris:bicarbonate buffers at constant ionic strengths.

Twenty experiments were performed exactly as reported in 4:1 with the exception that the buffers employed were 0.15, 0.14, 0.13, 0.12 and 0.11M tris with 0, 10, 20, 30 and 40 mM added $KHCO_3$ respectively. The buffers therefore were all 0.15 M with increasing concentrations of bicarbonate.

Results.

The results are shown in Table 7 and Figs. 37 to 39 and can be seen to be very similar to those observed in 4:1. Over the pH range 7.32 to 7.58 $[Ca^{++}]$ falls from about 1.0 to 0.4mM, $[P]$ rises from about 0.3 to 0.8mM and $[CO_2]$ rises from about 7 to 35mM respectively.

Fig. 38 shows pK_c and pK_{sp} values for the ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ ranging from 26.5 to 26.1 and 30.3 to 29.8 respectively.

Fig. 39 shows pK_c $[Ca^{++}][CO_3^{=}]$ ranging from 8.0 at pH 7.35 to about 7.50 at pH 7.4 and the corresponding estimate of pK_{sp} $[Ca^{++}][CO_3^{=}]$ ranging from 9.0 at pH 7.35 to 8.5 at pH 7.55.

Discussion:

It is clear from these results that the calcium phosphate ion product is very reproducible in the presence of bicarbonate, and does not appear to be pH dependent.

The same cannot be said of the calcium carbonate ion product. Not only does its pK_c vary with pH but at constant ion strengths the results tend to have a sigmoid distribution when plotted against pH. One possible explanation could be that whereas 24 hours was adequate for phosphate equilibration, this might not be true of carbonate. A further series of experiments was therefore designed to test this possibility.

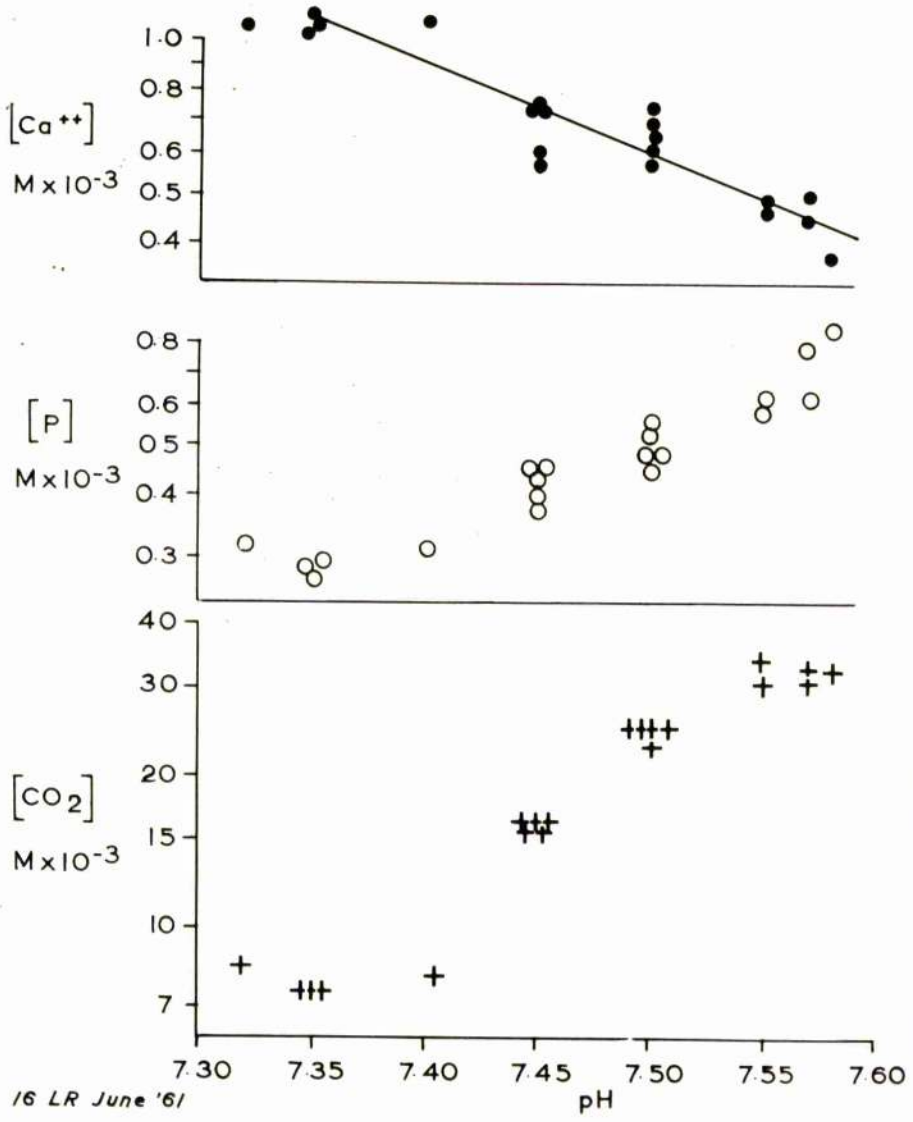


Figure 37. $[Ca^{++}]$ $[P]$ and $[CO_2]$ plotted against pH in 20 equilibration studies in tris buffer with added bicarbonate.
In all cases $\mu = 0.15$.

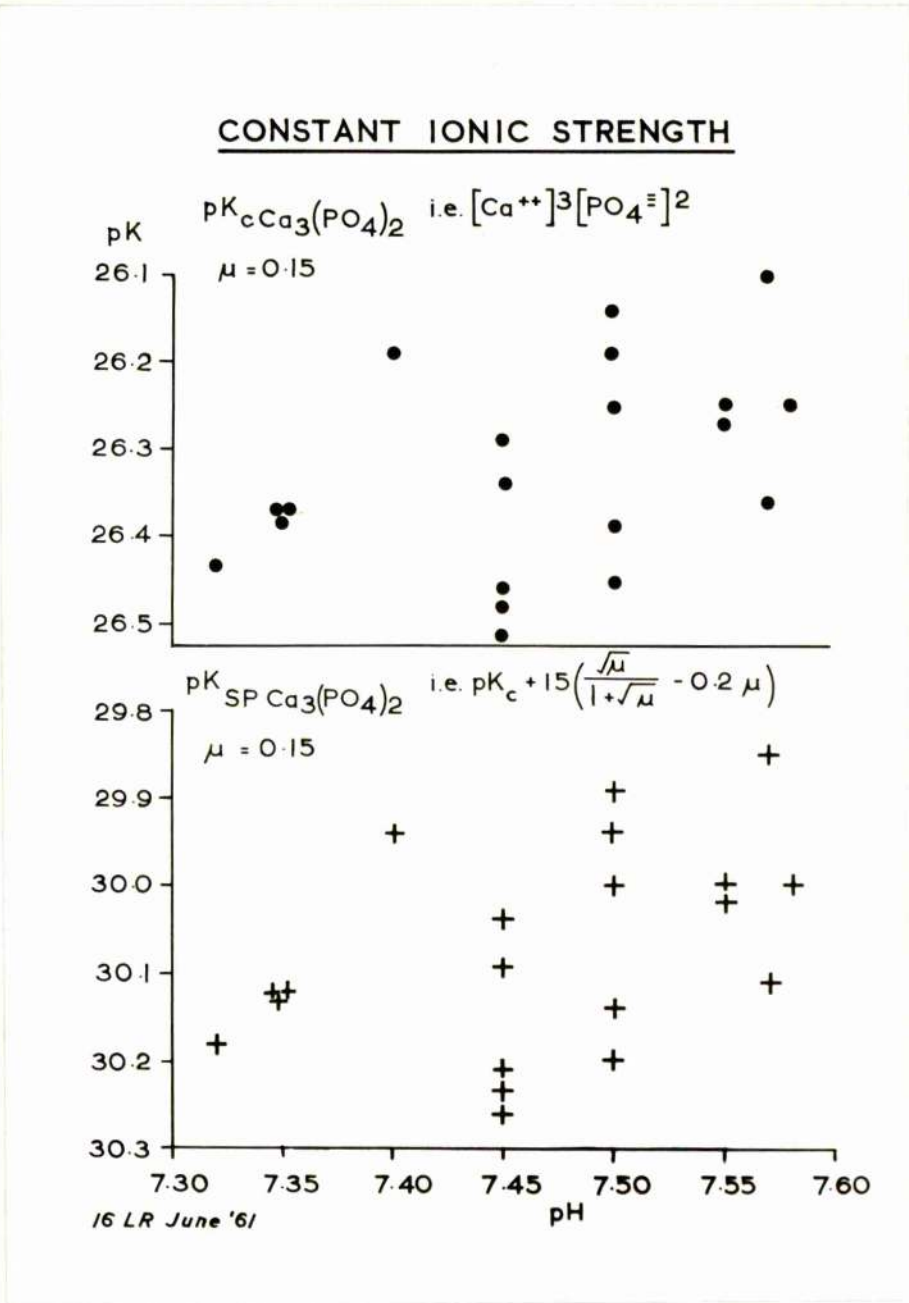


Figure 38. The pK_c and an estimate of the pK_{sp} values for the ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ plotted against pH in equilibration studies in tris buffer with added bicarbonate at $\mu = 0.15$.

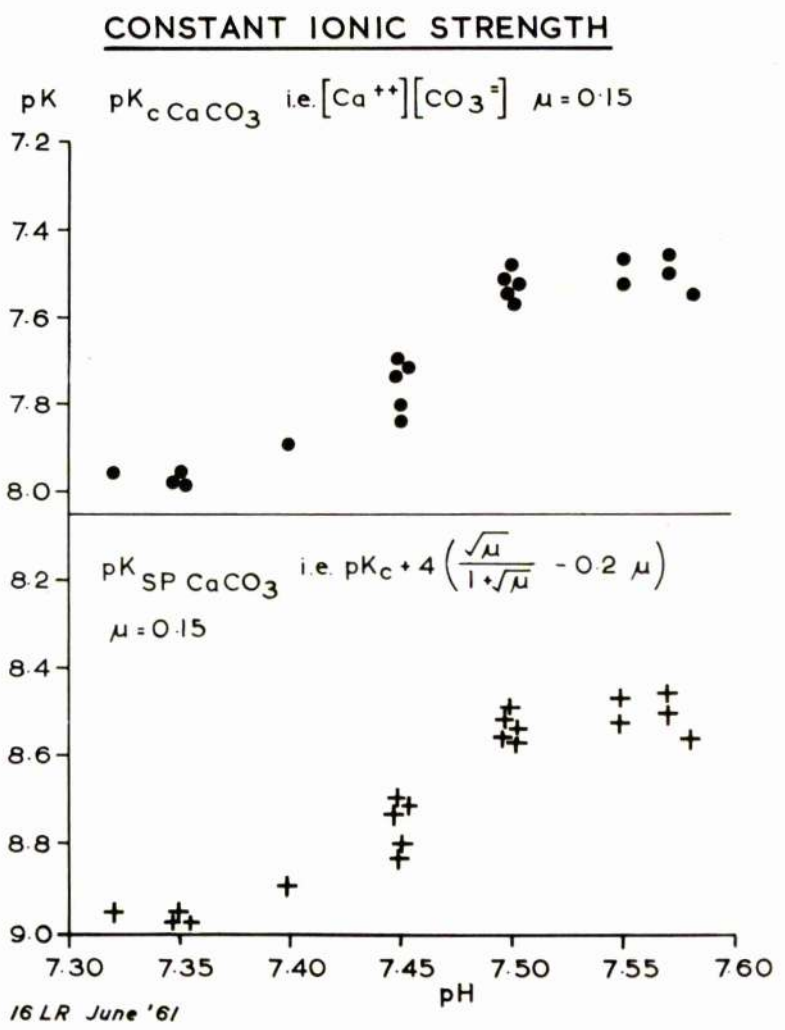


Figure 39. The pK_c and an estimate of pK_{sp} values for the ion product $[Ca^{++}][CO_3^{=}]$ plotted against pH in equilibration studies in tris buffer with added bicarbonate at $\mu = 0.15$.

4:4 72-hour equilibrations in tris-bicarbonate buffers at constant ionic strength (μ = 0.15) both from undersaturation and supersaturation with respect to calcium and inorganic phosphate.

Experimental.

Samples of bone powder (1 gm) were placed in dialysis bags with 10 ml of tris buffer containing different concentrations (10 to 40mM) of potassium bicarbonate. The Molarity of the tris was adjusted prior to the addition of the bicarbonate so that the final ionic strength of the buffer was 0.15μ. In half of the experiments, 4mM Ca and 1.9mM P was added to the buffers to allow equilibration to occur from supersaturation. The sealed bags were placed in polythene bottles with a further 30 ml of buffer solution and the system allowed to equilibrate for 72 hours. The pH of the original tris buffer was about 7.2 but each system was left to find its own equilibration pH value after addition of the bicarbonate, and the final pH values varied from 7.2 to 7.8.

Twenty experiments are reported. Five pairs of experiments were conducted from undersaturation with respect to calcium and phosphate, i.e. the initial buffer was tris + 10, 20, 30 or 40mM bicarbonate, with no calcium or phosphate present. A second series of 10 experiments used identical buffers with the addition of calcium (4mM) and inorganic phosphate (1.9mM) i.e. experiments from supersaturation with respect to calcium and inorganic phosphate.

Results.

The results are shown in Table 9 and Figs. 40 - 45.

Fig. 40 shows that the calcium concentration varied with pH. At 72 hours, it ranged from 2.08mM at pH 7.28 to about 0.23mM at pH 7.82.

Fig. 41 shows that the total inorganic phosphate concentrations also varied with pH. At 72 hours, it ranged from about 0.16mM at pH 7.28 to about 0.81mM at pH 7.82.

Fig. 42 shows the relationship between the ion product $pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$ and the pH at 24, 48 and 72 hours in 20 experiments. At 24 hours it ranged from 27.68 to 25.11, but at 72 hours the range had narrowed to 26.73 to 25.96 and was independent of pH. The arithmetic mean value for this ion product at 72 hours expressed as a pH was 26.30.

Fig. 43 shows the relationship between total $[CO_2]$ and pH. The initial range of $[CO_2]$ was 0 to 40mM and the initial pH 7.2. It can be seen that the $[CO_2]$ at 72 hours ranged from 1.5mM to 30mM and that apart from the four experiments at the lowest pH, there was rather less CO_2 in the buffers than had been added to the systems originally.

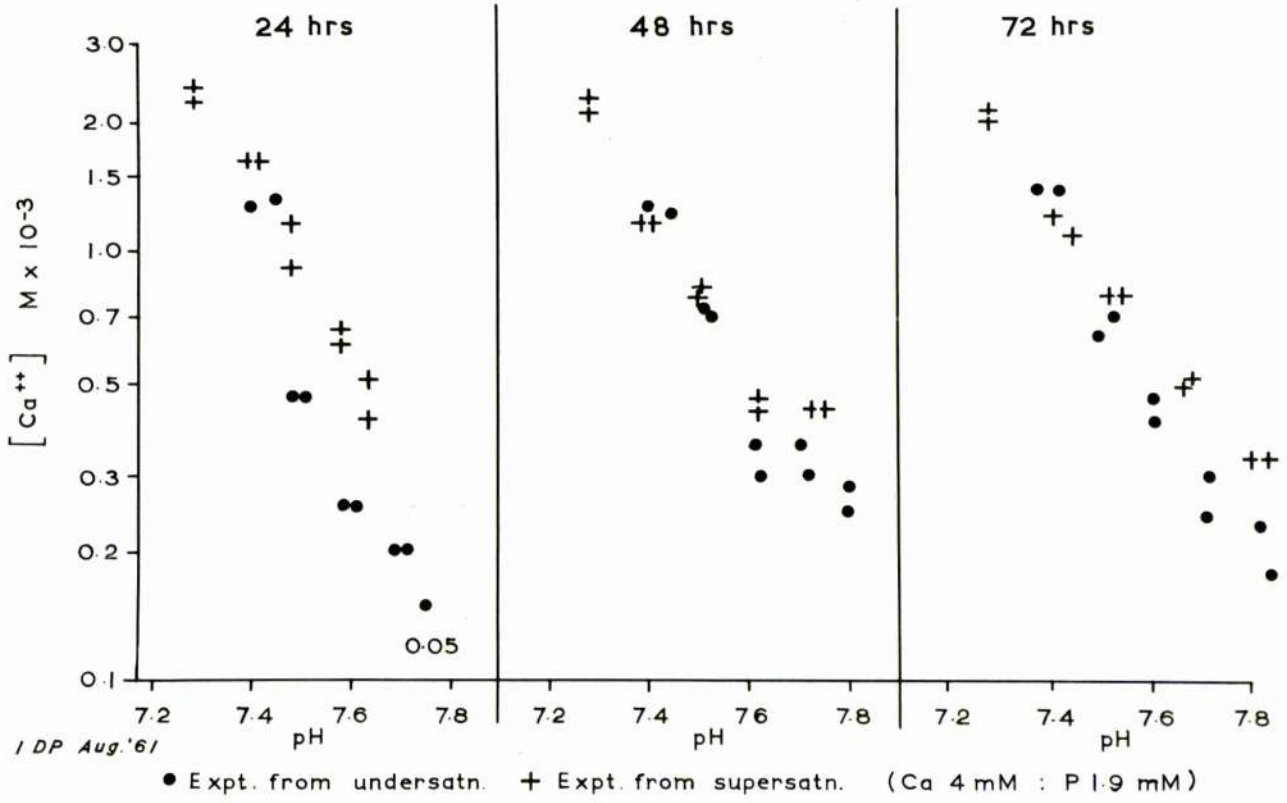


Figure 40. $[Ca^{++}]$ plotted against pH after 24, 48 and 72 hours equilibration in experiments in tris buffer with added bicarbonate at constant ionic strength (μ = 0.15).

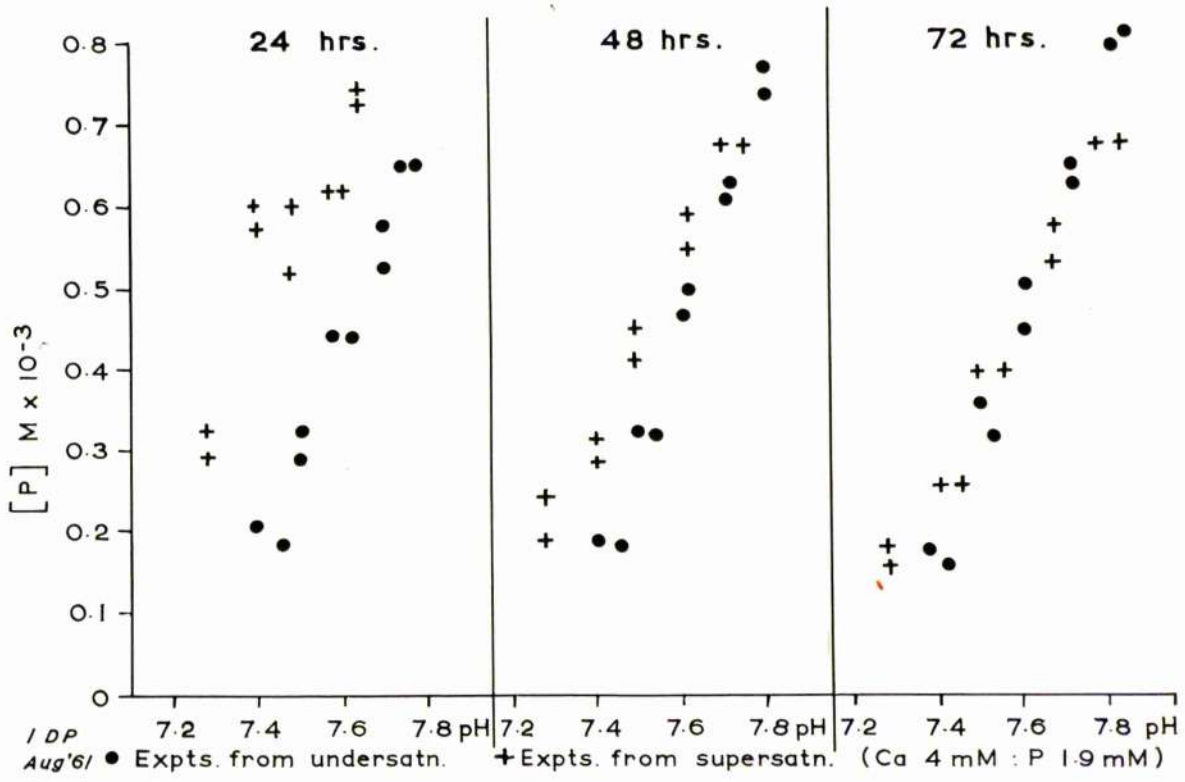


Figure 41. [P] plotted against pH after 24, 48 and 72 hours equilibration in experiments in tris buffer with added bicarbonate at constant ionic strength (μ = 0.15).

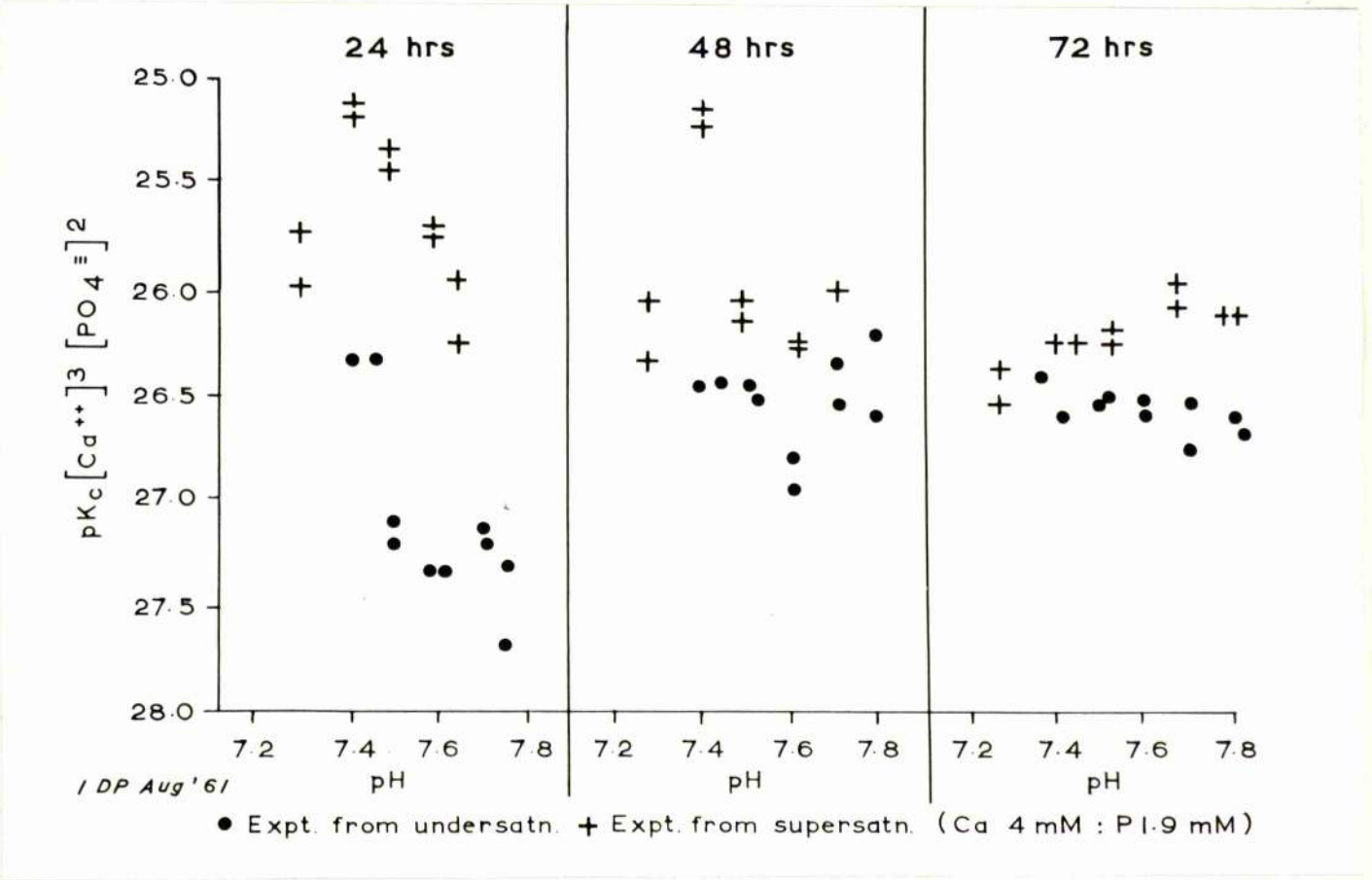


Figure 42. **$pK_c [Ca^{++}]^3 [PO_4^{\equiv}]^2$ values plotted against pH after 24, 48 and 72 hours equilibration in experiments in tris buffer with added bicarbonate at constant ionic strength (μ = 0.15).**

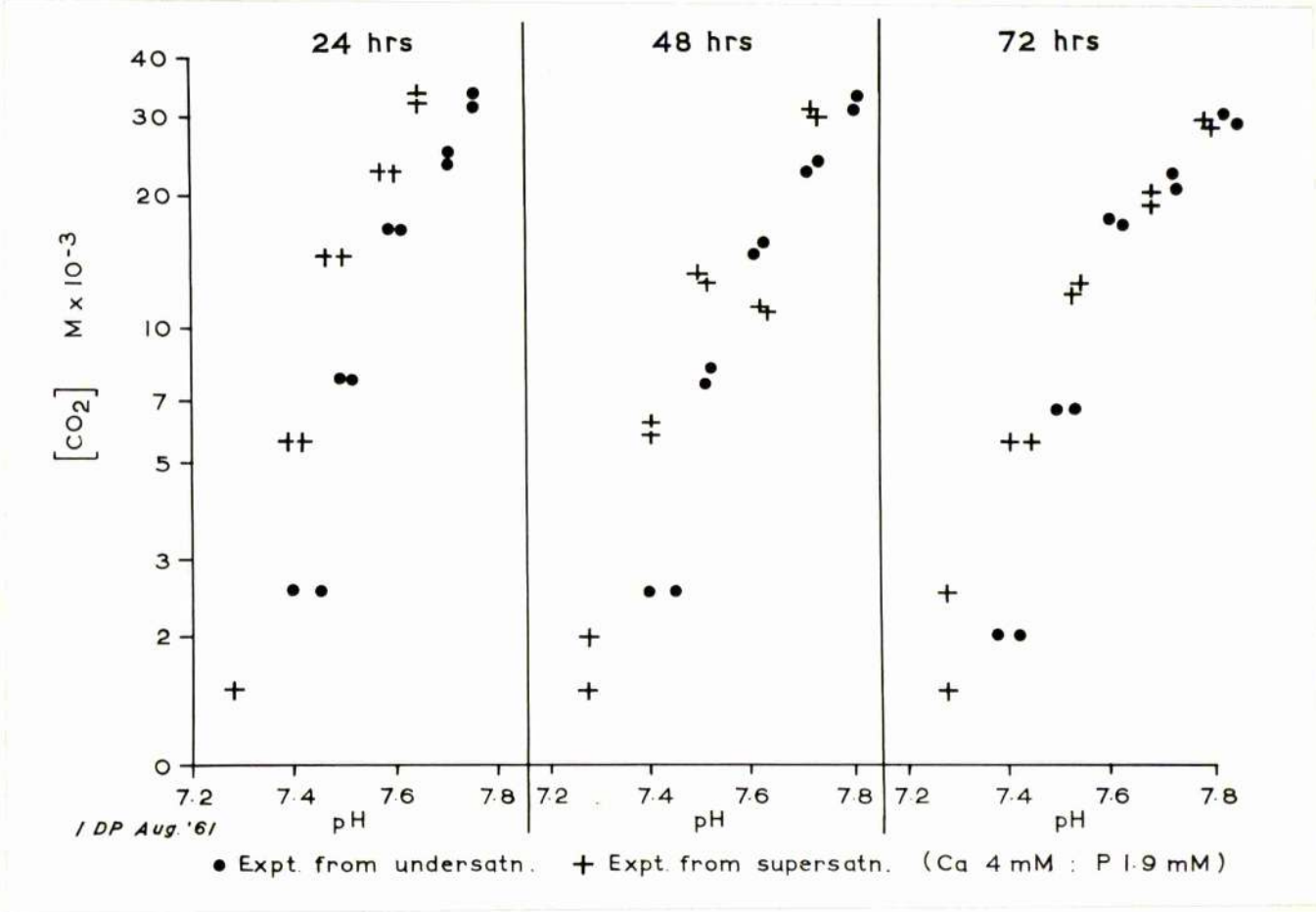


Figure 43. $[CO_2]$ plotted against pH after 24, 48 and 72 hours equilibration in experiments in tris buffer with added bicarbonate at constant ionic strength ($\mu = 0.15$).

Fig. 44 shows the ion product $pK_c[Ca^{++}][CO_3^{=}]$ plotted against pH. At 72 hours, it ranged from 8.48 at pH 7.28 to 7.46 at pH 7.80. It does not appear to be a constant, nor to have a linear relationship with pH. The distribution is similar to that of the total $[CO_2]$ at 72 hours (Fig. 43).

Discussion.

It is clear from the results shown in Fig. 42 that bone powder yields the same ion product $pK_c[Ca^{++}]^3[PO_4^{=}]^2$ in buffers whether or not bicarbonate is present. In these twenty experiments, an ion product has been observed which is independent of pH and is relatively constant whether the systems are initially undersaturated or supersaturated with respect to calcium and phosphate. Compared to the previous definitive equilibrations (2:2) it took rather longer for this product to be established (72 rather than 24 hours), but the mean value of 26.30 is not significantly different from that of 26.39 found previously. This suggests that we are observing a very reproducible phenomenon.

Although the $pK_c[Ca^{++}]^3[PO_4^{=}]^2$ values were identical to those found previously, the absolute values for calcium concentration and particularly inorganic phosphate concentration were quite different. In previous experiments in bicarbonate-free buffers the total $[P]$ was about 0.2mM over the pH range 6.6 to 7.8. In the presence of

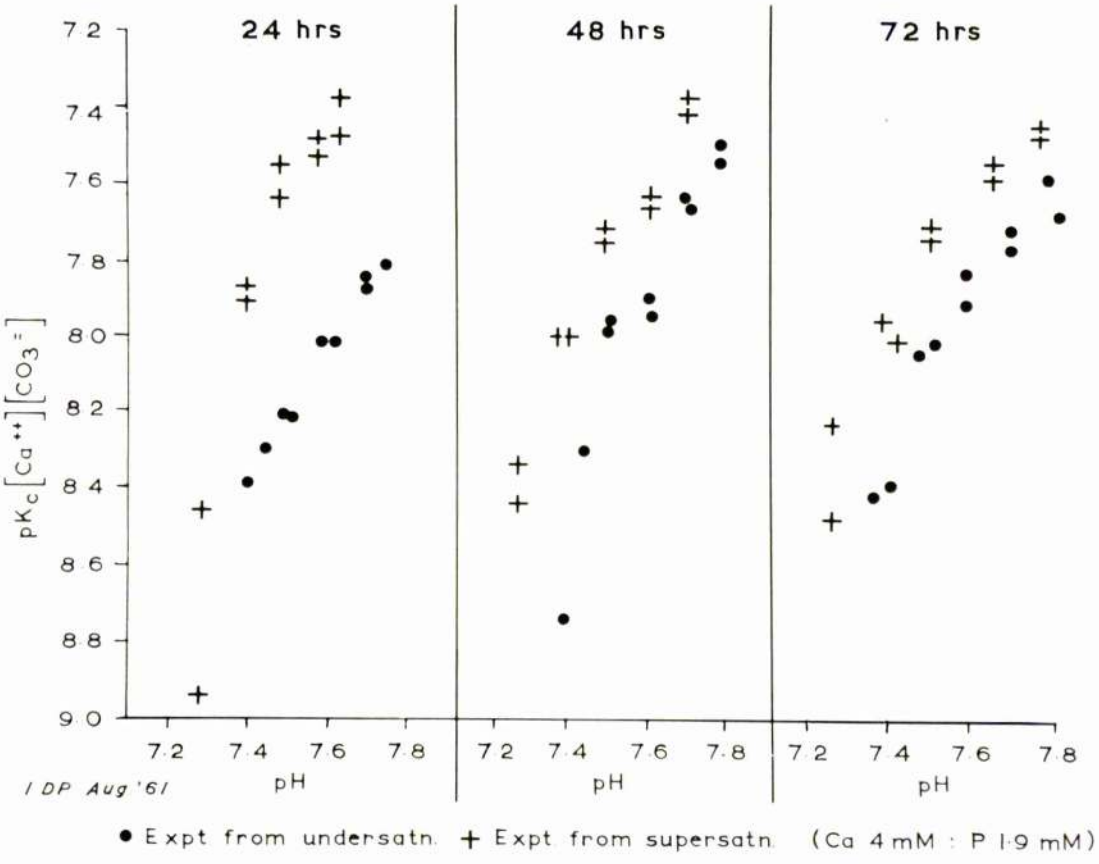


Figure 44. pK_c $[Ca^{++}][CO_3^{=}]$ values plotted against pH after 24, 48 and 72 hours equilibration in experiments in tris buffer with added bicarbonate at constant ionic strength (μ = 0.15).

bicarbonate, [P] ranges from about 0.2mM at pH 7.3 with a $[CO_2]$ of about 2mM, to 0.8mM at pH 7.85 where $[CO_2]$ is about 20mM, and the total phosphate concentration appears to be related either to pH or to carbonate concentration. The correlation between [P] and $[CO_2]$ at 72 hours can be seen in Fig. 45 to be very high and [P] has already been shown to be independent of pH in bicarbonate buffers (3:1 & 3:2).

Although the range of $pK_c[Ca^{++}][CO_3^{=}]$ at 72 hours is somewhat narrower than at 24 hours, there is little to suggest that a physical constant is being established. The distribution of the calculated values suggests a dependence more on the total CO_2 concentration than on anything else.

Nordin (110) has suggested that the differences between ion products in published experiments from undersaturation and from supersaturation might be due to the relatively small amount of solid phase present in the system employed. Consequently, there might well be a limit to the amount of inorganic material which can be taken up or given up. This possibility might be valid in the case of the $[Ca^{++}][CO_3^{=}]$ ion product in the current experiments since the amount of calcium carbonate in the bone powder employed would only be of the order of 40 mg. This possibility was tested in a further series of 5 experiments in which the solid:solution ratio was raised by a factor of 10.

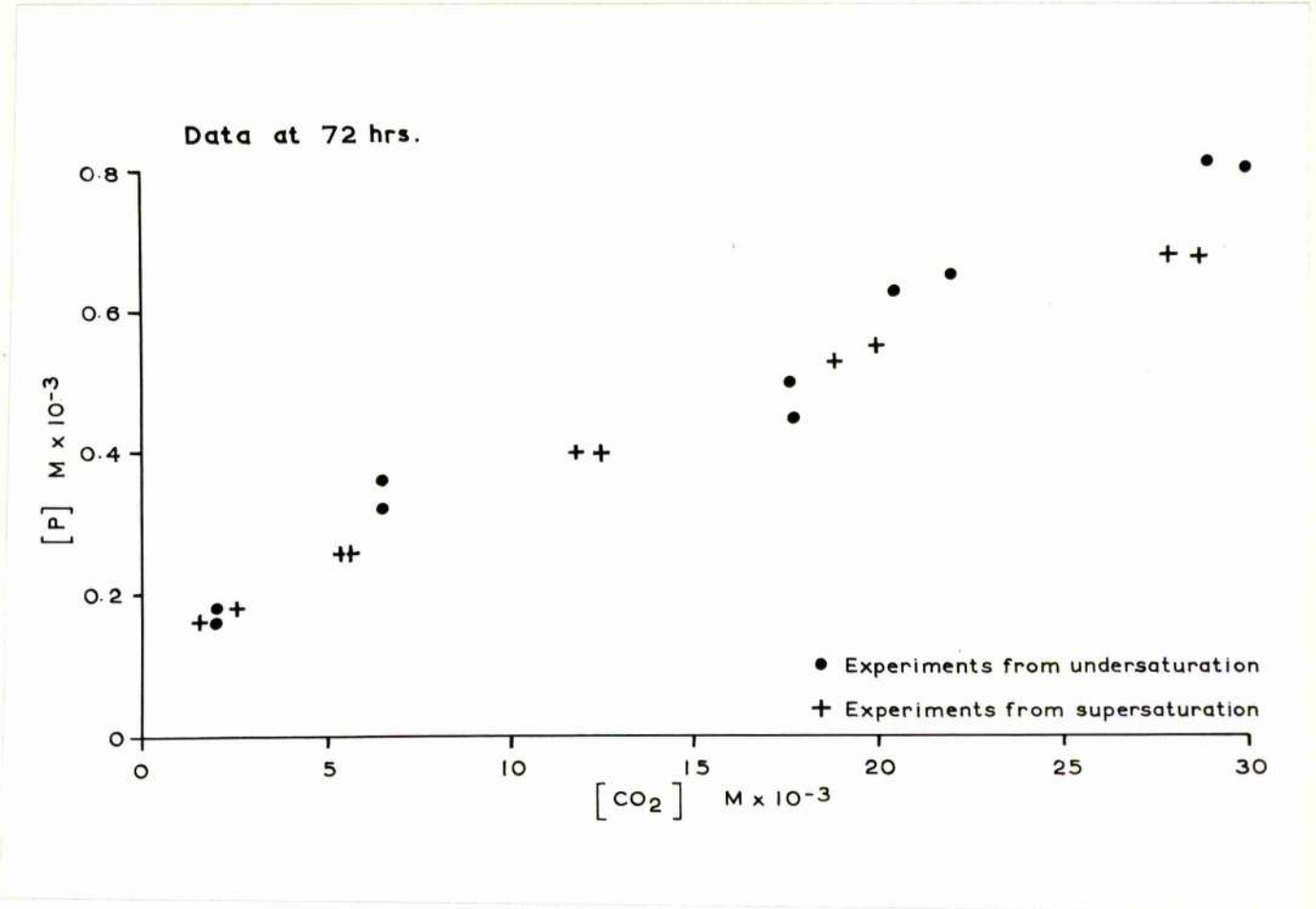


Figure 45. The concentration of total inorganic phosphate plotted against the total carbonate concentration at 72 hours in equilibration studies in tris buffer with added bicarbonate at constant ionic strength (μ = 0.15).

4:5 Equilibrations in tris-bicarbonate buffers at very high solid:solution ratios.

Experimental.

Five experiments were set up with 20 gm bone powder and 10 ml buffer inside the dialysis bag and 30 ml buffer soutside. The solid:solution ratio was therefore about 500 gm/litre.

The buffer employed was 0.15 M to 0.11 M tris at pH 7.0 with 0, 10mM, 20mM, 30mM and 40mM $KHCO_3$ added respectively. The final ion strength was 0.15 μ. Samples for analysis were removed at 24, 48 and 72 hours.

Results.

The results can be seen in Table 10 and Fig. 46.

The $pK_c [Ca^{++}][CO_3^{=}]$ at 72 hours ranged from 8.12 at pH 7.55 to 7.82 at pH 7.71. This ion product was not a constant, and the range of values observed was no different from that shown in the previous experiments (4:3).

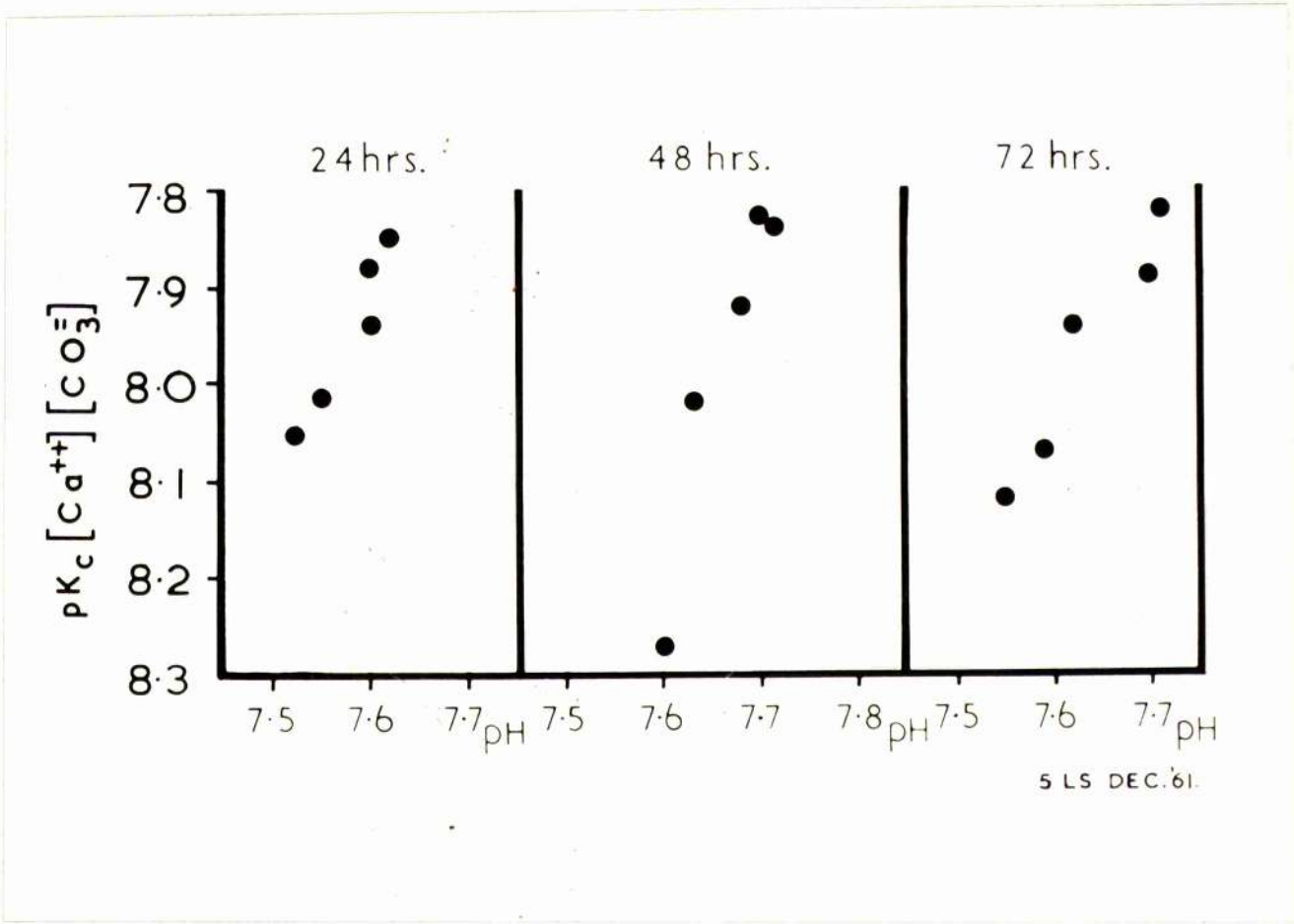


Figure 46. $pK_c\,[Ca^{++}][CO_3^{=}]$ values plotted against pH after 24, 48 and 72 hours equilibration in tris buffer with added bicarbonate in studies with very high solid:solution ratios of 500 gm bone powder per litre of buffer.

Summary.

Bone powder was equilibrated with buffers containing bicarbonate both from undersaturation and supersaturation with respect to calcium and phosphate. The arithmetic mean product expressed as $pK_c [Ca^{++}]^3 [PO_4^{\equiv}]^2$ was 26.30 compared to 26.39 in bicarbonate-free buffers, an insignificant difference.

It was clearly shown that the equilibrium phosphate concentration is dependent on the concentration of bicarbonate in the buffer and is directly related to it.

It has not been possible to demonstrate a $[Ca^{++}][CO_3^{=}]$ ion product even at solid:solution ratio as high as 500 gm bone/litre of solution. The absence of such a 'constant' is compatible with the view that there is no separate calcium carbonate phase in bone mineral salt.

The biological implications of these observations are interesting. The view has been expressed that the constancy of the ion product in solutions bathing bone was biologically valid, and the higher absolute phosphate concentrations in tissue fluid were explained by postulating that phosphate from extra-skeletal sources, e.g. diet, was continually being mobilised into the tissue fluids to raise the phosphate concentration, and lower that of

calcium. In the present experiments the concentration of total inorganic phosphate was about 0.7mM when the $[CO_2]$ was about 25mM (Fig. 43). Thus at biological concentrations of bicarbonate the equilibrium phosphate concentration was about 0.7mM as compared to about 1.0mM in normal human tissue fluids.

Walser (157, 158) has suggested recently that normal plasma inorganic phosphate is only about 50-60% free or unassociated, and if this is so, then the addition of bicarbonate to the in vitro system yields ionic phosphate concentrations virtually identical with those found in extravascular, extracellular tissue fluid.

These results reinforce the previous conclusion that the ion products obtained in vitro are compatible with those found in normal human tissue fluids, provided one assumes that the environment of the bone salt crystal is at a lower pH than extracellular fluid.

EQUILIBRATION STUDIES WITH ADULT HUMAN BONE IN NORMAL SERUM

5:1 The ion product $\left[Ca^{++}\right]^3 \left[PO_4^{\equiv}\right]^2$ in normal adult human serum.

5:2 Equilibration studies with human bone powder in serum.

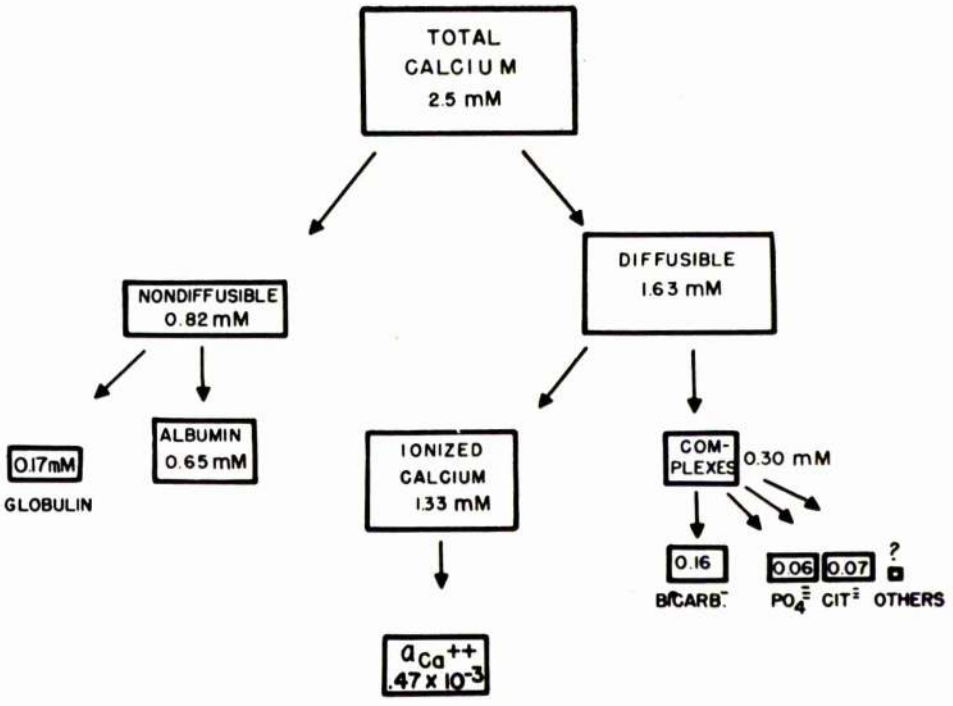
Discussion.

5:1. The ion product $\left[Ca^{++}\right]^3 \left[PO_4^{\equiv}\right]^2$ in normal adult human serum.

In section 2:2 of this thesis, we accounted for the 'high plasma ion product found <u>in vivo</u>' by postulating that the pH of bone extracellular fluid was at the intracellular pH of about 6.8 rather than at the extracellular pH of 7.4. In order to examine the implications of this hypothesis, it is necessary to consider the various forms of calcium and phosphate normally present in body fluids, and in particular in plasma or serum.

<u>Serum calcium</u>.

Normal adult human serum contains about 10 mg calcium/100 ml (i.e. 2.5 mM). The distribution of the various forms of calcium has been summarised by Neuman (126). He used an ultracentifugation technique and employed known formation constants in order to determine the distribution of calcium as shown in Fig. 47. The fraction in which we are interested, the ionised calcium amounts to 1.3mM or 5.3 mg/100 ml. This figure agrees with that of McLean and Hastings (93), who used the classical frog-heart method (94), and also that of Yendt et al (163) who used <u>in vitro</u> mineralisation of rachitic rat cartilage as a bio-assay technique. More recently, Walser (157,158), found on ultrafiltration, that 1.2mM free calcium was present in a serum containing 2.5mM total calcium. This order of



The state of calcium in normal serum as calculated from ultrafiltration data and formation constants. In ignoring possible ion competition—with Mg^{++}, for example—the amount of calcium estimated to be complexed may be in error (too high). This error could hardly affect the estimated ionized calcium by more than 2 or 3 per cent, however. *mM* designates mM/l.

Figure 47. The distribution of the various forms of calcium in normal human plasma or serum. Reproduced from Neuman (106).

free calcium concentration is generally accepted as representative of normal sera and is not in dispute.

Serum inorganic phosphate.

Many papers have been written to show that serum phosphate is wholly ultrafiltrable (9,61,62,112,116,132,131,161,156), but more recent attempts to explain discrepancies in the specific activity of P^{32} in blood and urine have resulted in the suggestion that there is a non-filtrable phosphate complex in serum (40,42,43,44,113,114,137). The latter findings were not confirmed in several other laboratories (55,67,78), and Neuman (106) has stated ...

> "that the ultrafiltrable phosphorus is almost exclusively inorganic phosphate and that all the inorganic phosphorus in normal serum is ultrafiltrable".

The most recent work is that of Walser (157, 158), who has suggested that in normal individuals about 12% of serum inorganic phosphate is protein bound. Further, he has postulated that 29% of serum phosphate exists as $NaHPO_4^-$ and 6% as $CaHPO_4$ and $MgHPO_4$. The free ionic phosphate according to Walser is thus about 50% of total serum inorganic phosphate.

Ion product in serum.

In view of the conflict between the statements of Neuman and

Walser, we will calculate the ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ assuming first of all that 100% and then that 53% (158) of total inorganic phosphate is unassociated or free, and, that the normal plasma inorganic phosphate concentration is 1mM. The ionic calcium concentration in plasma will be assumed to be 1.3mM.

Assuming 100% free phosphate.

	$[Ca^{++}]$ =	1.3×10^{-3}M
and	$[P]$ =	1.0×10^{-3}M
At pH 7.4 the fraction of P as $PO_4^{\equiv}$ =		7.2×10^{-6}M
so $[PO_4^{\equiv}]$	=	7.2×10^{-9}M
Then $[Ca^{++}]^3[PO_4^{\equiv}]^2$	=	1.14×10^{-25}M
and $pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$	=	24.94

Assuming 53% free phosphate.

	$[P]$ =	0.53×10^{-3}M
and	$[PO_4^{\equiv}]$ =	3.8×10^{-9}M
so $[Ca^{++}]^3 . [PO_4^{\equiv}]^2$	=	3.21×10^{-26}M
and $pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$	=	25.49

Both ion products are greater than that obtained empirically in equilibrium with bone powder which we have found to be 4.1×10^{-27} corresponding to a pK_c of 26.4. If we assume that the difference is due, primarily, to a reduced pH at the mineral surface resulting in a reduced proportion of trivalent phosphate ions, then:-

Observed $[Ca^{++}]^3[PO_4^{\equiv}]^2$	=	4.1×10^{-27}
Since $[Ca^{++}]$	=	1.3×10^{-3}
Then $[PO_4^{\equiv}]$	=	1.37×10^{-9}

Assuming 100% free phosphate.

$[P]$	=	1.0×10^{-3}
Fraction of P as $PO_4^{\equiv}$	=	1.37×10^{-6}

Which corresponds to pH 6.90.

Assuming 53% free phosphate.

$[P]$	=	0.53×10^{-3}
Fraction of P as $PO_4^{\equiv}$	=	2.59×10^{-6}

Which corresponds to pH 7.07.

Thus if Walser's statement is valid a pH of about 7.1 at the tissue fluid:bone mineral interface would explain the ion product found in normal serum. If Neuman is right, the pH would have to be as low as 6.9. Both findings are dependent on the concentrations of normal serum Ca^{++} and P assumed for the purposes of calculation. The calculations also ignore theoretical objections such as the redistribution of the ionic species along the bone tissue fluid to circulating body fluid pH gradient.

5:2. Equilibration studies with bone powder in serum.

Two groups of experiments are reported. In the first, 8 experiments were set up with 0.5 g bone powder and 2 ml of normal human serum inside a dialysis bag, and a further 18 ml serum outside the bag. The pH of the serum was previously adjusted by bubbling through it a mixture of CO_2 and N_2, the pCO_2 being directly controlled by rotameters. Four pairs of experiments were run at pH 6.8, 7.0, 7.2 and 7.4.

The second group of 4 experiments was similar except that 1 gm bone powder was placed in the dialysis bag with 10 ml serum, and a further 30 ml serum placed outside the bag. In this case the pH was titrated to 6.8, 7.0, 7.2 and 7.4 respectively with a 10% solution of lactic acid.

Chloroform, 6 ml/litre, was added as bacterial inhibitor in all experiments and samples removed for analysis at 24, 48 and 72 hours. The results of the two series were virtually identical and have been pooled for presentation.

Results.

The results are shown in Tables 11 and 12, and Figs. 48 to 51.

Fig. 48 shows the calcium concentration at equilibrium plotted against pH. It ranges from 1.69mM at pH 7.20 to 1.22mM at pH 7.72.

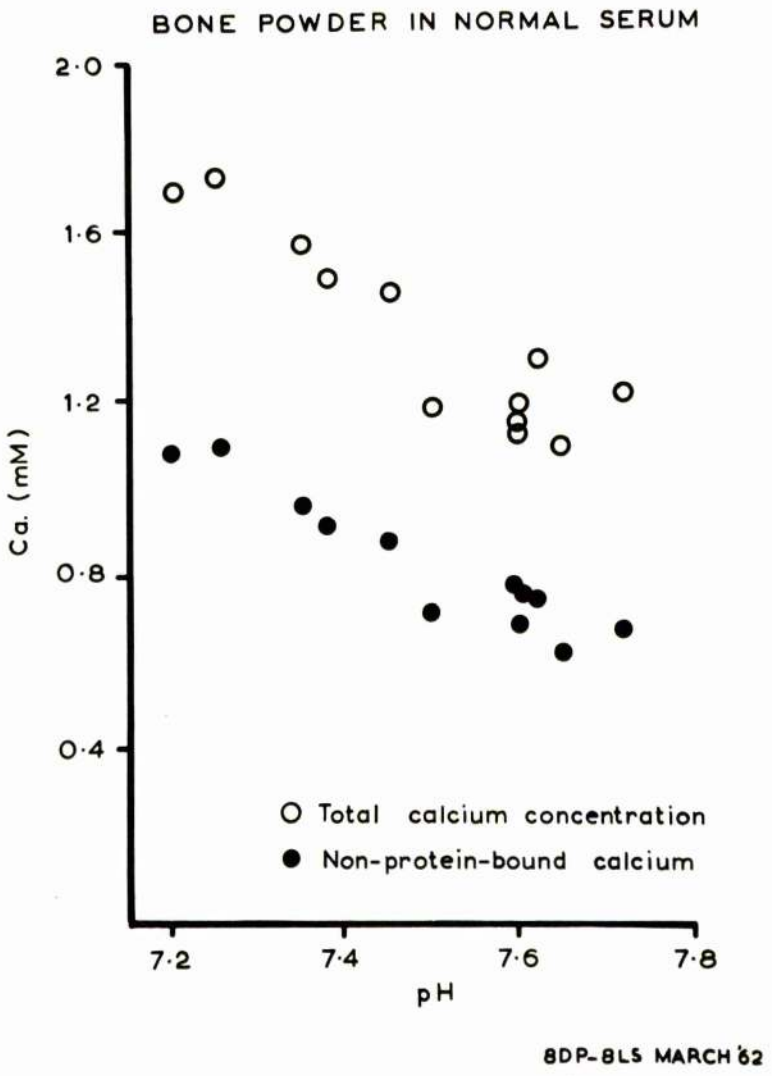


Figure 48. **Total and non-protein bound calcium concentrations plotted against pH in equilibration studies with human bone powder in normal serum.**

The proportion of calcium bound to protein has been calculated using the data published by Loken et al (47) and the 'free' calcium (see Table) plotted against pH. In these experiments the 'free' or ionic calcium ranges from 1.08mM at pH 7.20 to 0.65mM at pH 7.72.

Fig. 49 shows the calculated trivalent phosphate concentration plotted against pH. It ranges from 2.77×10^{-9}M at pH 7.20 to 1.17×10^{-8}M at pH 7.72. Also shown are the concentrations of non-associated trivalent phosphate calculated by assuming Walser's observation that only 53% of ultrafiltrable phosphate is free and un-associated (157, 158). In this case, $[PO_4^{\equiv}]$ ranges from 1.47×10^{-9}M at pH 7.20 to 6.20×10^{-9}M at pH 7.72.

Fig. 50 shows the pK of the ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ plotted against pH, firstly where $[Ca^{++}]$ is the non-protein-bound calcium and $[PO_4^{\equiv}]$ the total calculated trivalent phosphate concentration, and secondly, where $[Ca^{++}]$ is also the non-protein-bound calcium, but where $[PO_4^{\equiv}]$ is the calculated free $[PO_4^{\equiv}]$ assuming that only 53% of ultrafiltrable phosphate is free and non-associated. In the first case the values range from 26.02 at pH 7.20 to 25.39 at pH 7.72 and in the second from 26.59 at pH 7.20 to 25.95 at pH 7.72. The mean ion products have pK values of 25.61 and 26.17 respectively.

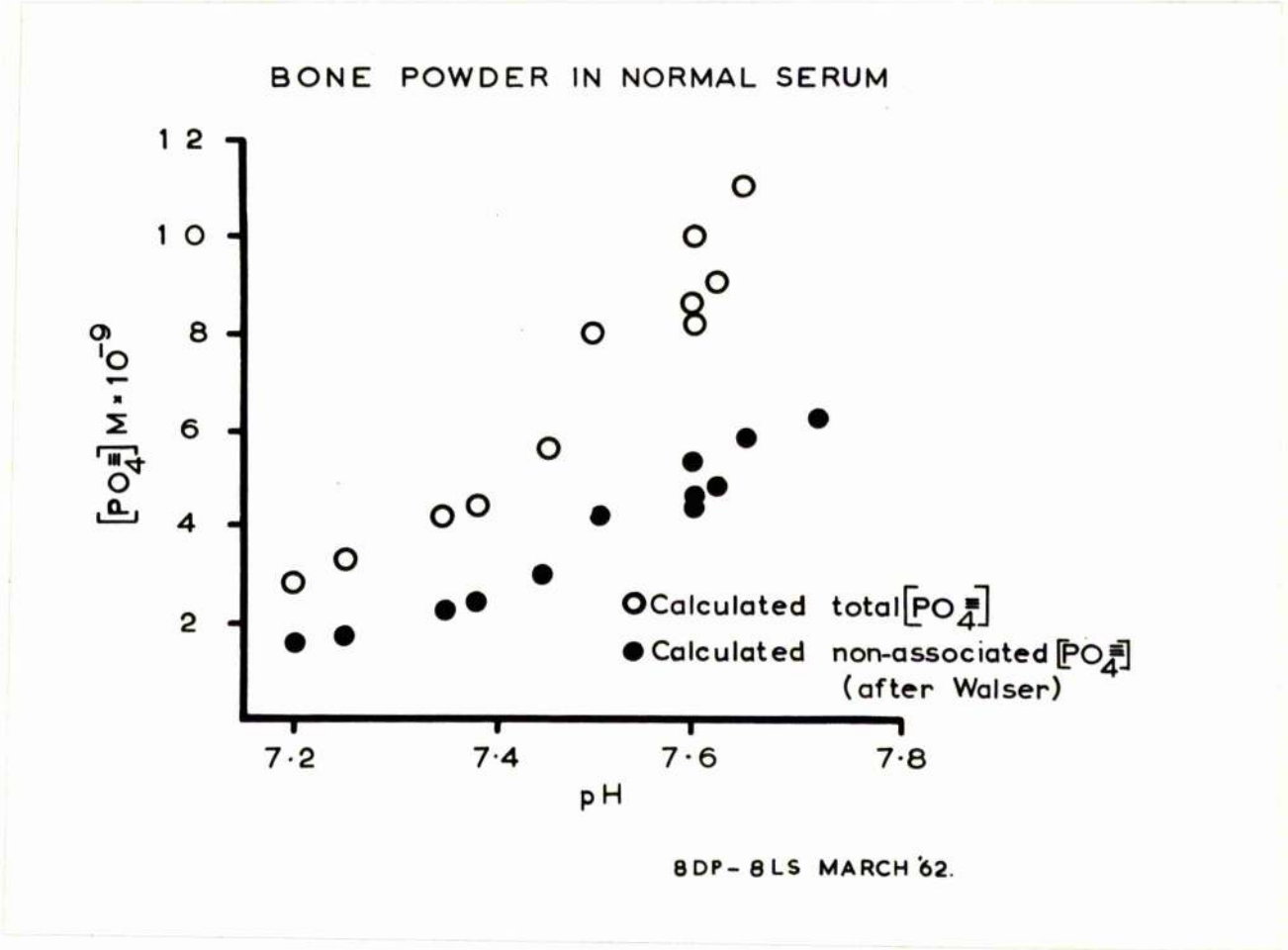


Figure 49. The trivalent phosphate ion concentrations plotted against pH in equilibration studies in normal serum.
Also shown are the concentrations of 'free' trivalent phosphate after applying Walser's correction for association of plasma inorganic phosphate with other normal serum constituents.

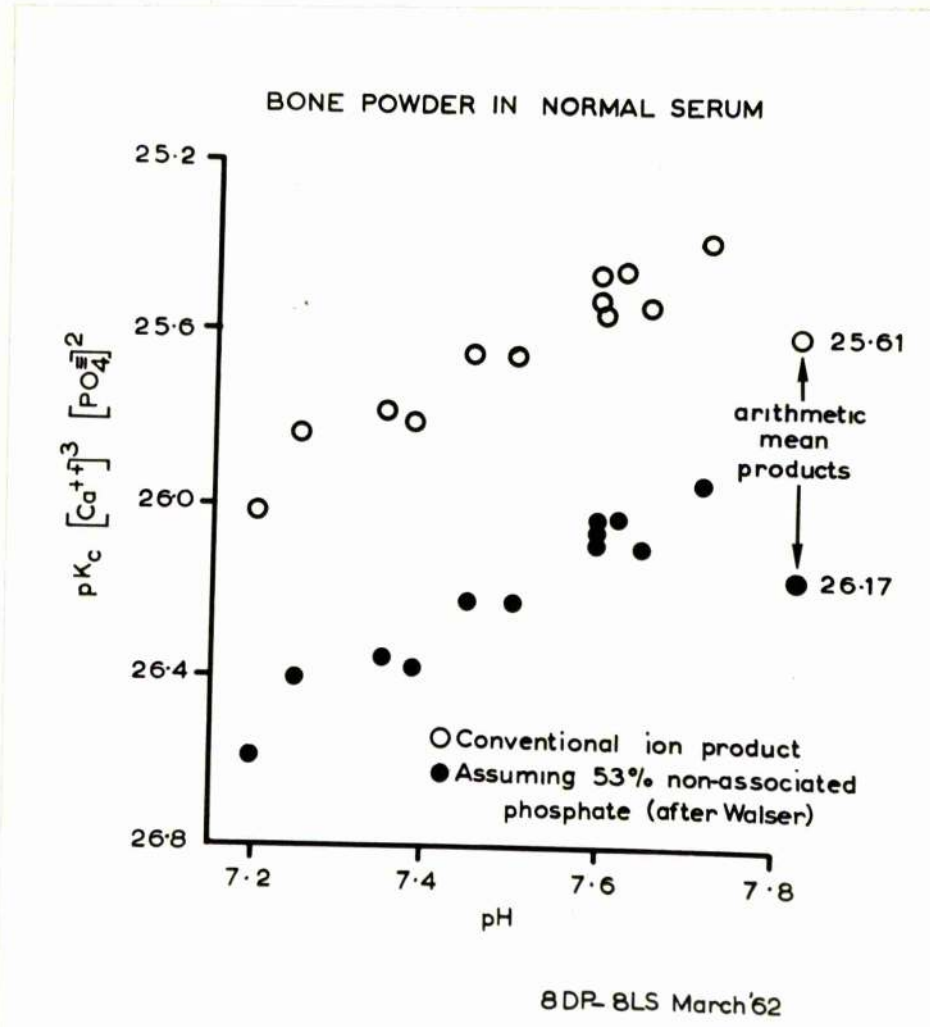


Figure 50. The pK of the ion product of $[\text{non-protein-bound calcium}]^3 \cdot [PO_4^{\equiv}]^2$ plotted against pH in equilibration studies in serum. The pK of the ion product $[\text{non-protein-bound calcium}]^3 \cdot [53\% \text{ trivalent phosphate}]^2$ (after Walser) is also shown plotted against pH. In both instances, the pK of the arithmetic means of the products are indicated.

Discussion.

To calculate an ion product in normal serum it is necessary to determine the concentrations of free ions present. All previous ion products have been determined bearing in mind that they are, in a sense, empirical, and reflect only the behaviour of bone salt under the specified experimental conditions. Normal human serum was employed at room temperature in the same empirical manner. The ion strength will be about 0.15, but in this case specific complexes will form and so reduce the proportion of the total calcium and phosphate in the free, ionised form. The total concentration of calcium and phosphate observed in solution will therefore be greater than expected from previous experience in synthetic media. In fact, the total calcium concentrations were rather less than those observed in bicarbonate-free tris and cacodylate, but the total phosphate (Table 12) was correspondingly higher.

In a recent paper (47) it was shown that the proportion of serum calcium which is ultrafiltrable is reasonably predictable, as seen in Fig. 51, and so the proportion of calcium bound to protein has been interpolated for room temperature (20°C) and used in calculating the free calcium concentrations in these experiments.

Walser (157,158) maintains that about half the total ultrafiltrable phosphate exists as complex ions, e.g. $NaHPO_4$, which do not

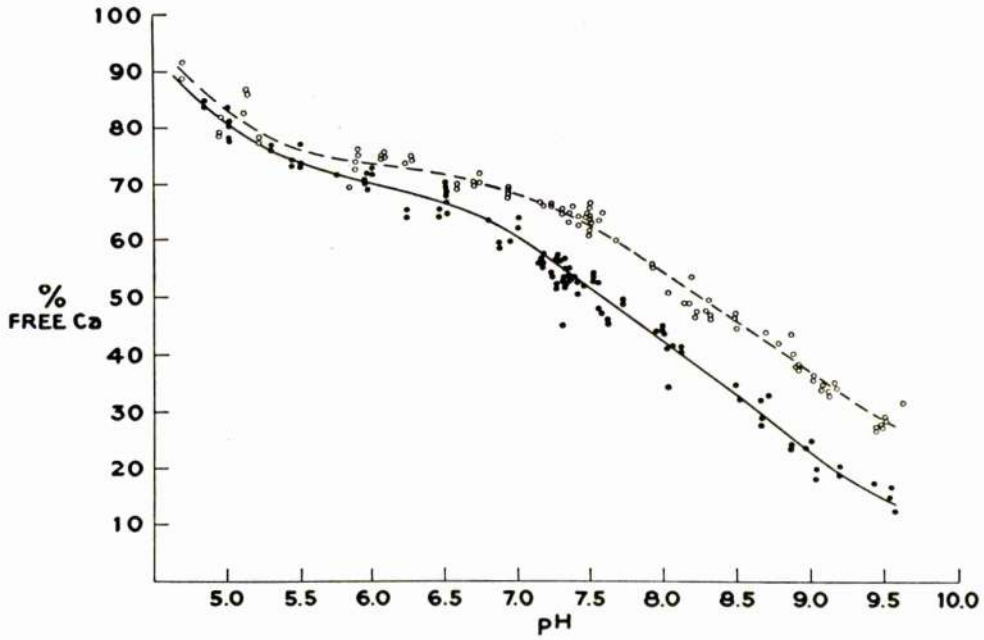


FIG. 1. Effect of pH on the percentage of free calcium in serum. Upper curve at 12°; lower curve at 37°.

Figure 51. The proportion of serum calcium ultrafiltrable plotted against pH. Reproduced from Loken et al (47).

enter into the ion equilibrium we have been studying. This conviction is not shared by Neuman. I have therefore calculated the free trivalent phosphate concentration with and without 'the Walser correction' and used both values to calculate pK ion products.

It can be seen that there appears to be a relationship between both $[Ca^{++}]^3[PO_4^{\equiv}]^2$ products and pH. The significance of this observation is quite unknown, but it might suggest that in calculating the ion product in serum, the phosphate concentration has been 'overweighted' relative to the calcium. As Neuman has said, a $[Ca]^{10}[P]^6$ product is thermodynamically meaningless, but a re-weighting of the concentration indices in this direction might make for an ion product more independent of pH.

An important observation is demonstrated in Fig. 52. Here are shown diagrammatically the means and ranges of the pKs of the ion products in several series of experiments to date. It is readily apparent that the use of the 'Walser correction' leads to a pK ion product range in serum which is not significantly different from those found in bicarbonate-free buffer (2:2) and tris - $KHCO_3$ buffer (4:3).

There are two important implications. Firstly, the Walser calculation is justified by an indirect, but rational, experimental observation. Secondly, bone salt behaves similarly in serum and in synthetic buffers and yields the same ion product provided the

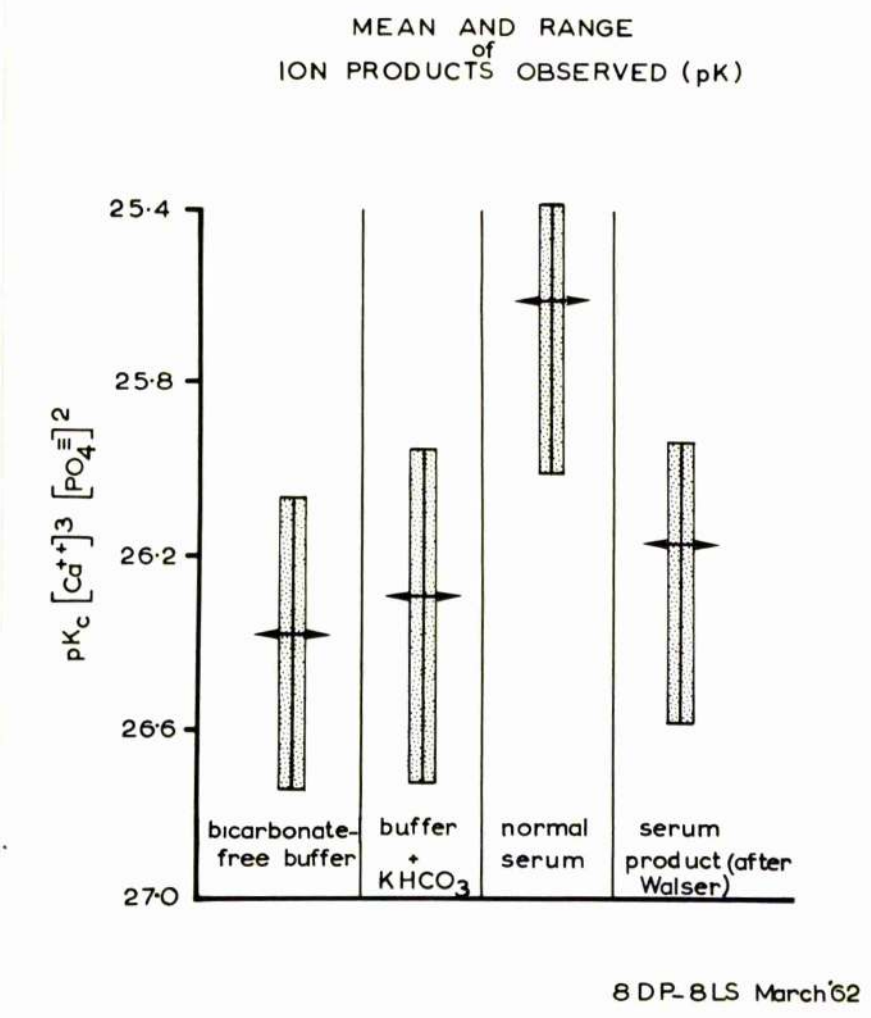


Figure 52. Means and ranges of ion products $[Ca^{++}]^3[PO_4^{\equiv}]^2$ observed in bicarbonate-free buffers, in tris buffer with added bicarbonate, in normal serum, assuming all inorganic phosphate is free, and in normal serum assuming that only 53% of the inorganic phosphate is non-associated.

necessary corrections are made for complex formation.

Accepting the Walser correction as proven, it follows from the relevant calculation in 5:1, that the bone salt can reproduce the concentrations of free calcium and free inorganic phosphate found in normal serum by a purely physical process provided the pH at the interface is about 7.1. This pH is almost midway between intracellular pH of 6.8 and the extracellular pH of 7.4 and is quite acceptable physiologically.

EQUILIBRATION STUDIES WITH CHILD AND RAT BONE POWDER.....

6:1 Studies with child bone

6:2 Studies with rat bone

General discussion.

We have seen that the equilibrium between human bone mineral and its ions in solution in tissue fluids can be expressed in terms of the ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$, and that one of the factors governing the relationship between $[Ca^{++}]$ and $[PO_4^{\equiv}]$ is the carbonate concentration in the fluids of the system. If the ion product has physiological significance, then a number of pertinent observations require explanation.

Studies with calf bone (110) resulted in a similar mean ion product to that observed in the present work. One implication of this fact is that all bone, irrespective of species, is composed of similar mineral salts. However, if we propose that the skeleton is in equilibrium with serum concentrations of calcium and phosphate, and this equilibrium is in part a reflection of a purely physico-chemical relationship between the ions in the skeleton and those in solution, then we must account for the wide range of serum inorganic phosphate concentrations found in different animal species. Similarly, we must examine the relationship in the case of the human infant, where a normal adult serum calcium concentration is allied to a significantly greater inorganic phosphate concentration. High serum phosphate concentrations are a feature of all young mammals. Clearly, the equilibrium product we have postulated will be exceeded in these instances unless the bone environment is at a lower pH than in adult man. Alternatively, a

greater proportion of the calcium and/or phosphate may be associated or bound, either to protein or to some other normal constituent of body fluids or plasma.

With these problems in mind, it was decided to investigate briefly the equilibrium ion products of child bone and rat bone.

6:1 Equilibration studies with child bone.

The material employed was obtained from a few cases of acute fatal illness where no longstanding metabolic abnormality was suspected. The small samples of femoral shaft obtained were pooled, and ground to a powder as previously described.

Twenty-four hour equilibrations (40-hour in 4 experiments) were performed from undersaturation in tris and cacodylate buffers over the pH range 6.6 to 7.4.

Results.

Twenty-five observations are reported on 21 experiments, and shown in Table 13 and Fig. 53.

It can be seen that the calcium concentration varies with pH ranging from about 4mM at pH 6.6 to about 1mM at pH 7.4.

The inorganic phosphate concentration varies considerably between 0.25mM and 0.70mM but above pH 6.8 the values lie between 0.3mM and 0.4mM.

The values for $pK_c [Ca^{++}]^3 [PO_4^{\equiv}]^2$ lie between 27.67 and 25.86 but above pH 6.8 the range is much less, the limits being 26.8 and 25.86.

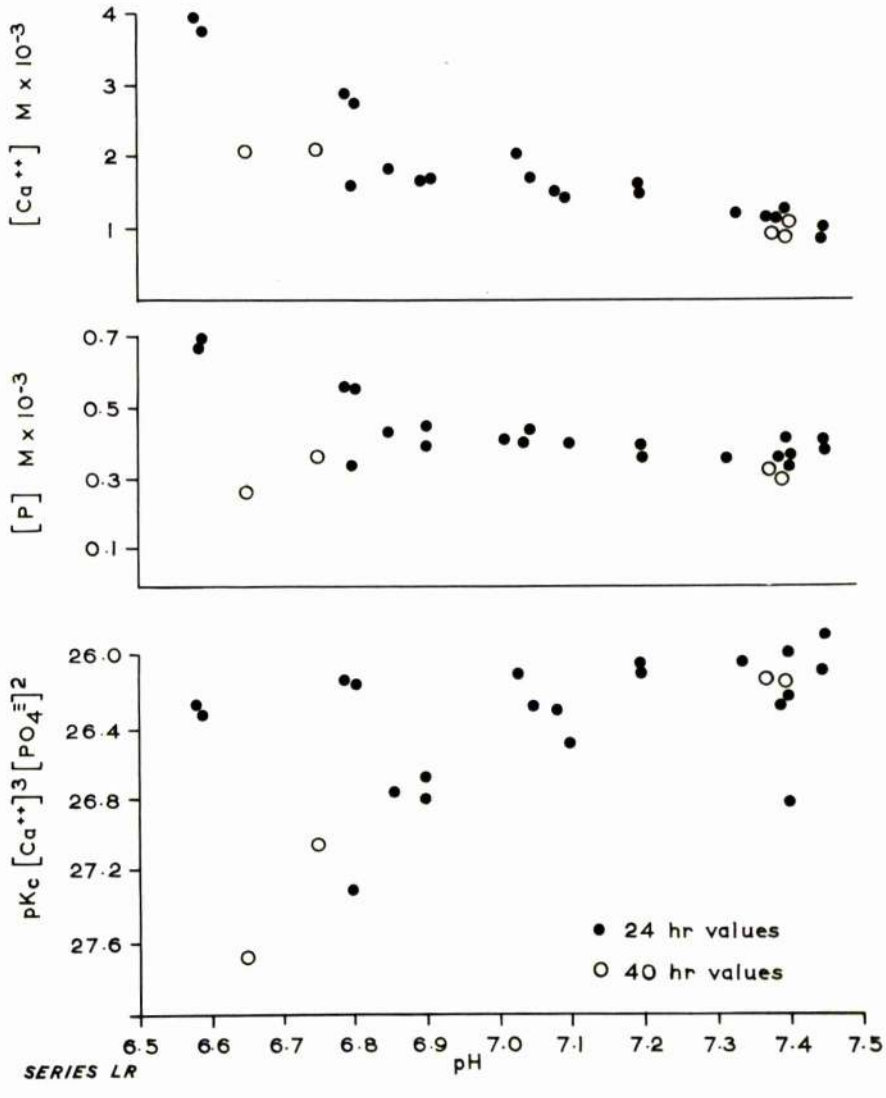


Figure 53. $[Ca^{++}]$, $[P]$ and $pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$ values plotted against pH in equilibration studies with child bone powder in tris buffer at $\mu = 0.15$.

Discussion.

It is immediately apparent that the reproducibility in this series of experiments is inferior to that demonstrated previously. In retrospect it was appreciated that the bone samples were from subjects ranging in age from 4 years to 12 years. The material was prepared, pooled and used as it became available, and it is quite possible that non-homogeneity of the solid phase may account for the scatter. Several important deductions can, however, be drawn from this data.

The calcium concentrations range from about 4mM at pH 6.6 to about 1.0mM at pH 7.4. These values are not substantially different from those found with adult bone powder. On the other hand, the phosphate concentrations tend to be higher than those found in equilibrations with adult bone powder in bicarbonate-free buffers at the same pH. The range was about 0.3mM to 0.4mM at pHs above 6.8 compared to 0.1mM to 0.3mM for adult bone.

The $[Ca^{++}]^3[PO_4^{\equiv}]^2$ ion products observed were of the same order as those found in adult bone, particularly in those experiments at a pH greater than 6.8. There is no evidence to suggest the 'calcium phosphate' of child bone is any different from that of adult bone.

The high values for the equilibrium phosphate concentration, associated with high normal plasma phosphate concentration with high normal plasma phosphate concentration in children suggested that further studies should be performed with bone of species whose normal plasma phosphate concentrations are known to be widely different.

6:2. Equilibration studies with rat bone.

Bone powder was prepared from pooled samples of the diaphyses of rat humeri and femora. Wister albino rats weighing 250 to 450 g were used, no distinction being made between sexes.

Twenty, 25-hour equilibrations from undersaturation and four from supersaturation (Ca 4mM:P1.94mM) were performed in tris and cacodylate buffers over the pH range 6.6 to 7.4. The data of a number of experimental groups were pooled and are shown in Table 13 and Fig. 54.

Results.

The results are shown in Table 13 and Fig. 54.

The equilibrium calcium concentrations varied from 4mM at pH 6.6 to about 1mM at pH 7.4.

The inorganic phosphate concentrations showed considerable scatter. They ranged from about 0.4mM to about 3.5mM.

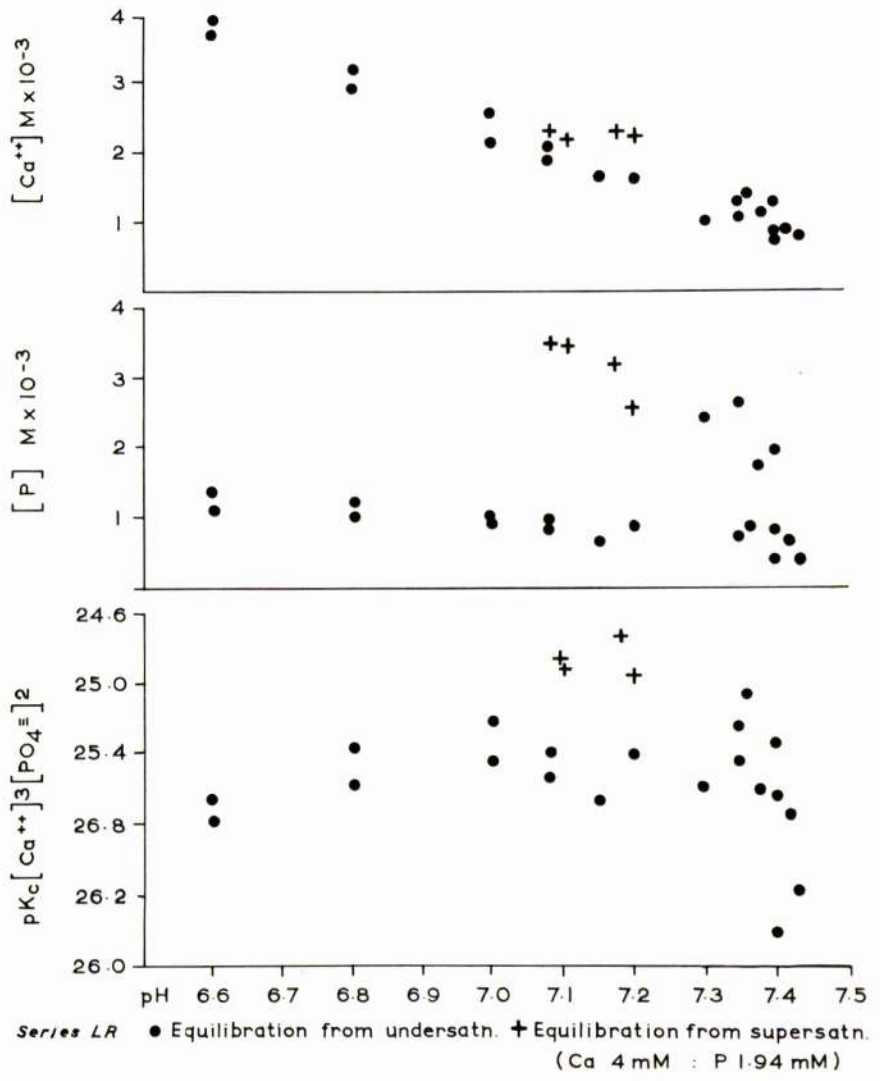


Figure 54. $[Ca^{++}]$, $[P]$ and $pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$ values plotted against pH in equilibration studies with normal rat bone powder in tris buffer at $\mu = 0.15$.

The $pK_c [Ca^{++}]^3 [PO_4^{\equiv}]^2$ values also demonstrated considerable scatter and ranged from 26.38 to 24.74 over the pH range 6.60 to 7.43. There was no apparent correlation with pH.

Discussion.

It is apparent that the consistency previously observed in these systems is lacking in this group of experiments with rat bone. The equilibrations reported here were performed over the same period of time as those with child bone, and it is now felt that more stringent selection of the bone by age and sex, might have reduced the variability.

Three important observations can, however, be made. Firstly, the calcium concentration at any particular pH is little different from that obtained with adult human bone. Secondly, the equilibrium phosphate concentration, despite the variation, is much higher than that observed in any previous equilibration. Thirdly, the ion products appear to be significantly greater than those found with human adult bone.

General Discussion.

These experiments raise a number of fundamental problems. We have now seen that in buffers containing no added bicarbonate, human adult bone, child bone and adult rat bone yield equilibrium phosphate concentrations of about 0.1 to 0.3mM, 0.3 to 0.4mM and 0.4 to 3.5mM respectively at pHs above 6.8. The normal serum phosphate concentration of adults, children and rats are about 0.7 to 1.4mM, 1.3 to 2.0mM and 2.0 to 3.0mM respectively. Thus it would appear plausible that there is a relationship between them.

It is known that the bones of children and young animals have a lower carbonate content than adults, the Ca:P ratio of such bone being significantly reduced. It has also been shown by Sobel (133), that the carbonate content of bone is related to the composition of blood and diet. In particular, rats on low phosphate intake had a low serum phosphate and a relatively high CO_3:P ratio in the skeleton.

In view of these observations, the possibility has to be considered that the equilibrium phosphate concentration *in vitro* is a function of serum phosphate concentration *in vivo*. It would therefore be desirable to study *in vitro* the ion equilibria of bone of different species known normally to have widely differing serum phosphate concentrations.

COMPARITIVE ASPECTS OF THE BLOOD:BONE EQUILIBRIUM

7.1 Equilibration studies with bone powder of laboratory species

7.2 Anomalous studies with ox, sheep and pig bone

7.3 Studies with cancellous bone, rachitic bone and puppy bone

Three series of experiments are presented. In most instances the results raise more problems than they solve, but they are presented here as an indication of the possible scope of future investigations.

Firstly, a group of observations on normal laboratory animals are presented, followed by some anomalous data. Finally, several observations are reported to demonstrate the possibilities of investigating bone from different sites within the one individual, from individuals with pathological skeletons, and from individuals of different age.

The three groups of experiments were performed simultaneously, but are presented separately for reasons of clarity.

7.1 Equilibration studies with bone powder of laboratory species.

Samples of bone powder were prepared from the long bones of a number of laboratory species, and 24-hour equilibrations with 0.15 M tris at pH 7.2 were conducted both from undersaturation and supersaturation (Ca 4.0mM: P 1.94mM) with respect to calcium and inorganic phosphate. The species employed were the domestic fowl, guinea pig, rabbit, cat, mouse and rat, and the bone powder was prepared immediately after sacrifice.

When possible, 4 experiments were performed on bone powder from each species but in some cases two had to suffice. Human adult bone powder was run simultaneously (2 experiments each from undersaturation and supersaturation) as a 'control'.

Results.

These are shown in Table 14 and Figs. 55 and 56.

Fig. 55 shows the equilibrium inorganic phosphate concentrations for the various species. They range from about 0.2mM for man and the fowl, to about 1.0mM for the mouse and the rat. The equilibrium phosphate concentration for the rabbit is about 0.3mM and that of the guinea pig and cat about 0.5mM. Also shown on the Figure are the mean and normal range of serum inorganic phosphate concentration in these species. The serum phosphate data are taken from a text-book of biological data (2) and do not represent the

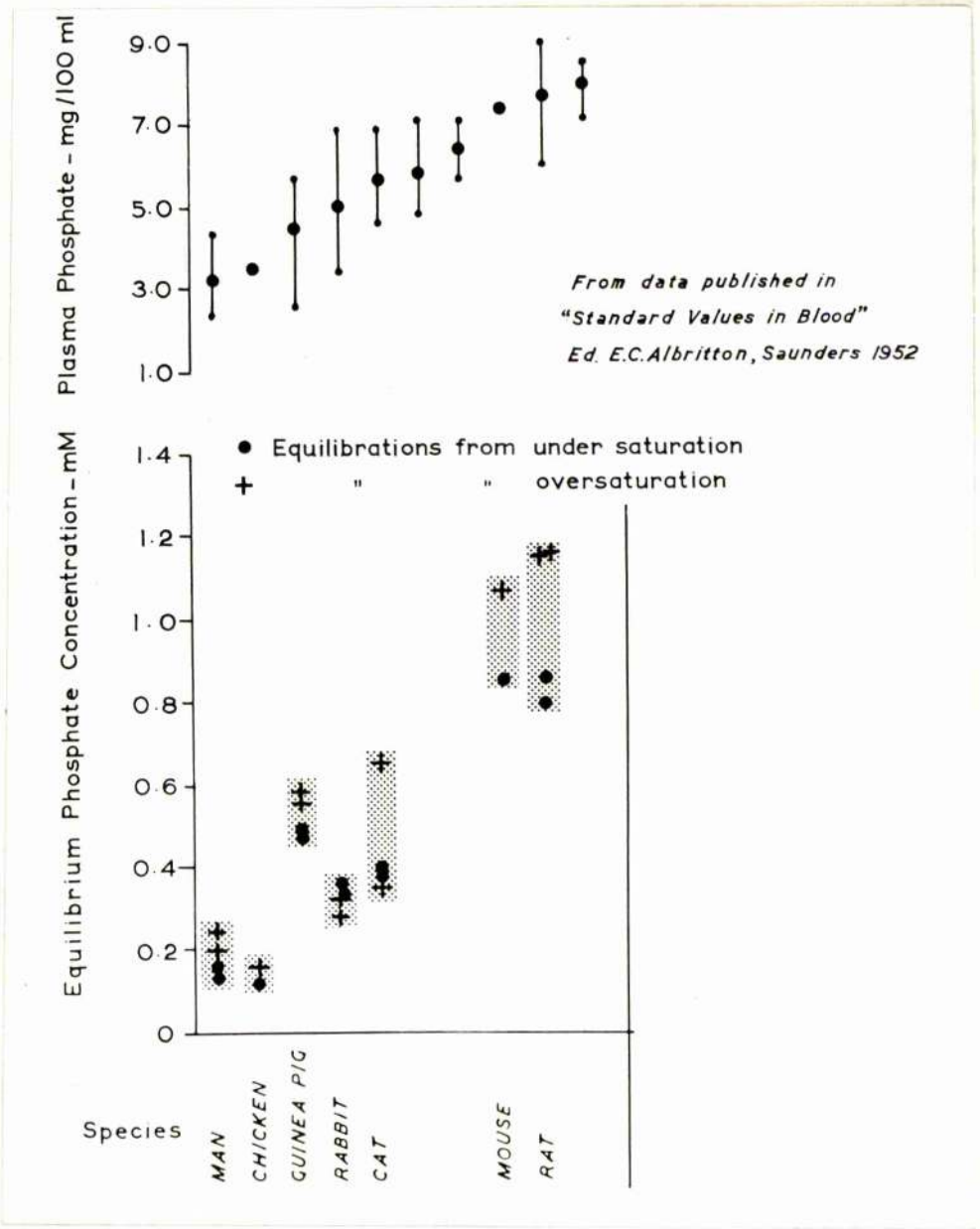


Figure 55. **Values of [P] at 24 hours in equilibration studies with cortical bone powder of several laboratory species.**
Also shown are the quoted mean and range of normal plasma inorganic phosphate concentration in these species.

blood levels in the particular animals used in these experiments.

It can be seen that the two phosphate concentrations appear to be related to one another.

Fig. 56 shows the values for $pK_c[Ca^{++}]^3[PO_4]^2$ found in these experiments. They range from about 27.0 to 25.0 and do not appear to demonstrate any apparent relationship with plasma phosphate concentration. Human and rat data are similar to those found previously in 2:2 and 6:2.

Discussion.

At first sight our postulate of a relationship between plasma and equilibrium phosphate concentrations appears substantiated, although certain anomolous data remain to be presented.

The ion product results present a very different picture. It is encouraging to see confirmation of the products for man and the rat, but unfortunately, for any general relationship, the ion product values for guinea pig and rabbit are undoubtedly above the range that would suggest a direct dependence of ion product on plasma phosphate concentration.

There is also little doubt that for guinea-pig, rabbit, mouse and rat bone, the ion product observed is significantly

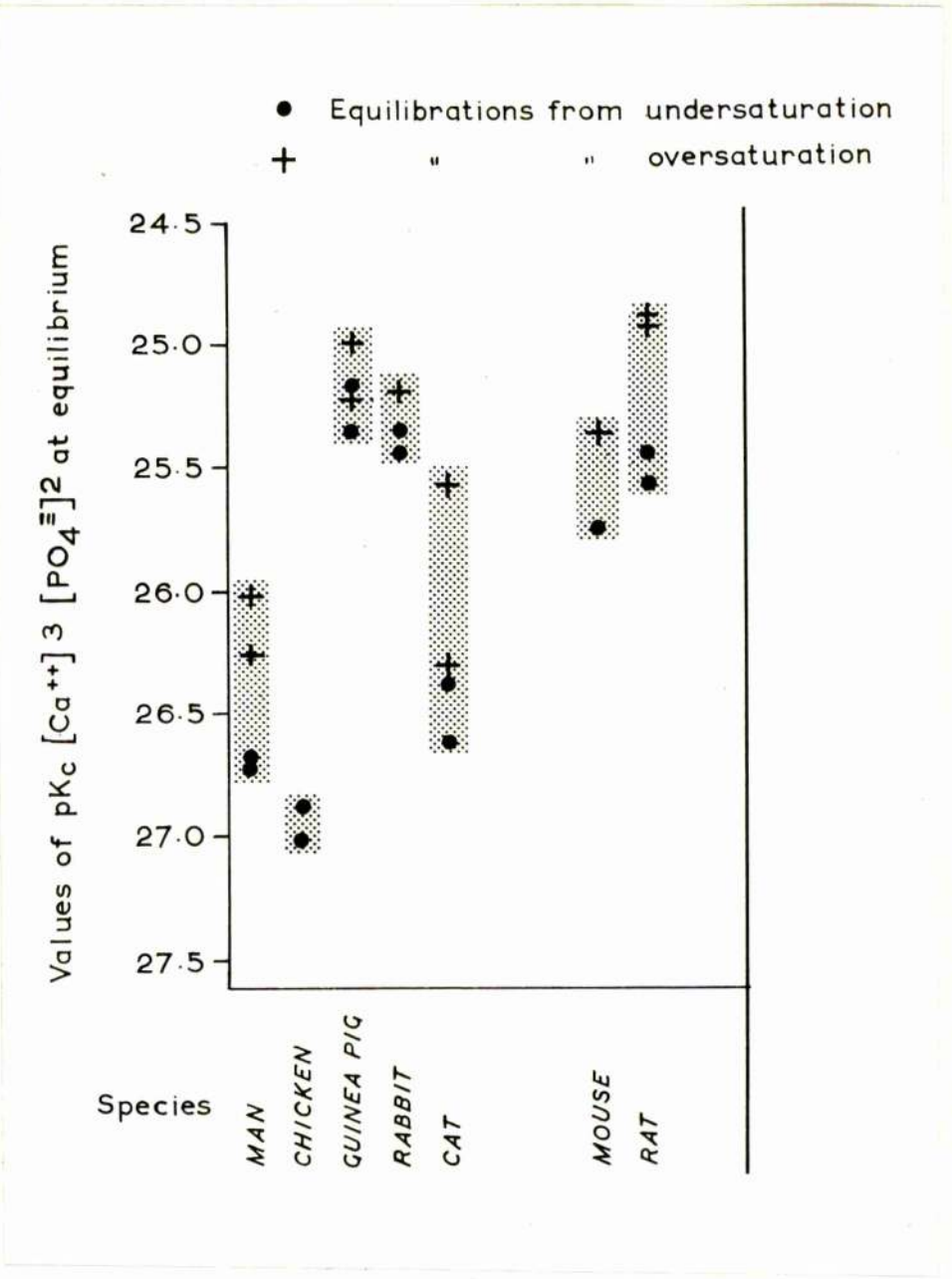


Figure 56. Values of $pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$ at 24 hours in equilibration studies with bone powder of several laboratory species.

greater than the accepted range for adult human bone.

There are at least two possible explanations - either bone from different species differs in its physico-chemical characteristics i.e. is qualitatively different, or our empirical ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$, although consistent within limits, becomes unrepresentative over greater ranges of bone salt composition.

7.2 Anomalous studies with ox, sheep and pig bone.

For economic reasons these bone samples were obtained from commercial sources, and no information was readily available as regards the past history of the material. In the three cases, it is likely that the material was imported, frozen, from the Argentine, New Zealand and Denmark, respectively.

Although the data have since been isolated, the experiments were designed to cover the known range of serum phosphate concentrations and were executed simultaneously with those presented above (7.1).

Results.

These are shown in Table 14 and Fig. 57.

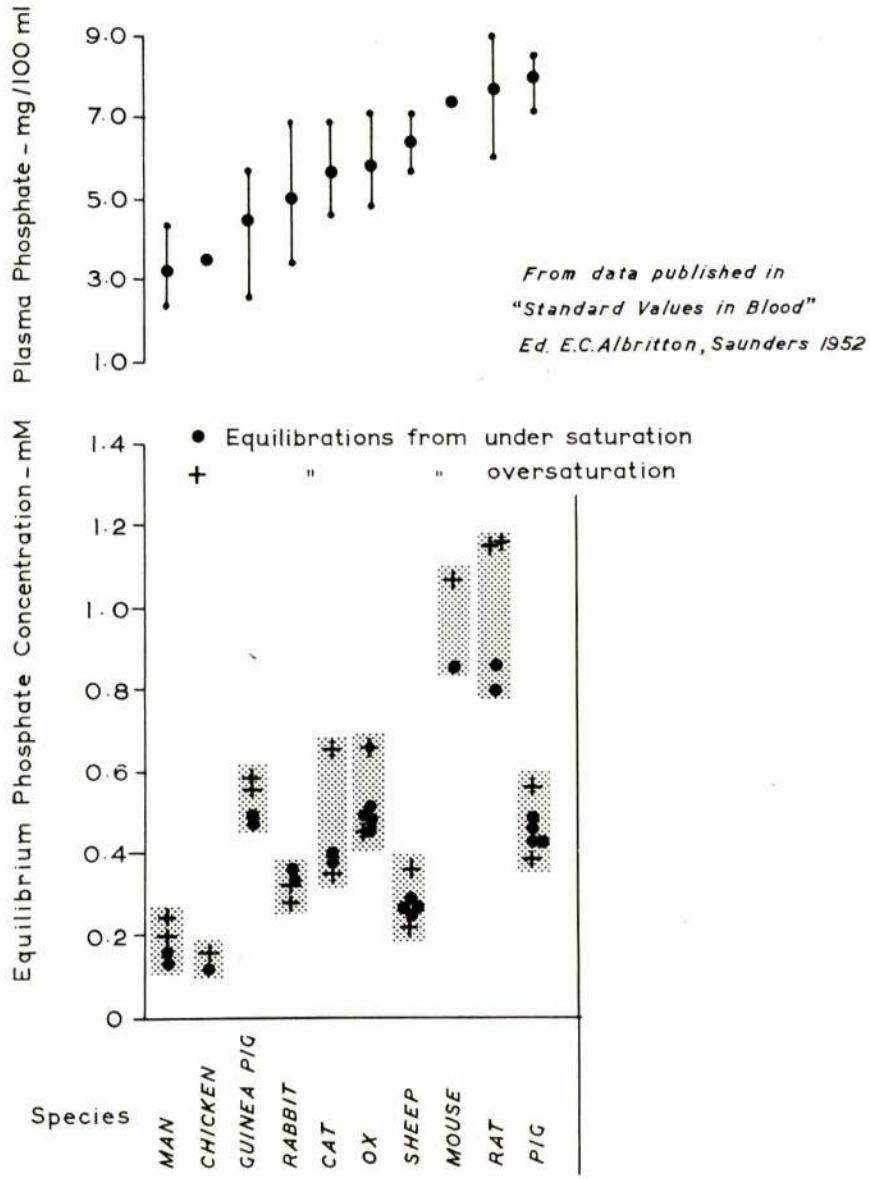


Figure 57. **Values of [P] at 24 hours in equilibration studies with cortical bone powder from several species.**
Also shown are the quoted mean and range of normal plasma inorganic phosphate concentration in these species.

Discussion.

The reason for the subsequent separation of the data is now apparent. There now appears to be a much less apparent relationship between the normal plasma inorganic phosphate concentrations and the *in vitro* equilibrium phosphate concentrations (Fig. 57).

It is felt that little can be said of this evidence, except that the distinction between the two sources of material is a real one; the bone in the 7.1 experiments was swiftly removed from the carcase in the laboratory, that in the 7.2 experiments, was prepared at an unknown, but considerable time after death. It is possible that during transport and storage, the physical nature of the bone has been changed, but this suggestion is pure speculation.

7.3 Studies with cancellous fowl bone, rachitic bone, and cortical puppy bone.

The cancellous bone of the fowl was employed in order to investigate the possibility that different parts of the same skeleton might have different chemical compositions resulting in different behaviour during equilibration.

Reproducible changes in bone composition in rachitic rats have been reported (64,73), and the serum phosphate concentration in rickets is known to be low. It was felt therefore that rachitic bone might yield results of interest. The animals were the untreated

controls of a vitamin D bio-assay, and were known to have rickets by 'line-test' criteria.

It has already been mentioned that young animals have higher serum phosphate concentrations than adults of the same species. The puppy bone used here was obtained at the termination of an experimental study of high phosphate intakes on normal pups. The plasma phosphate concentration of these animals was of the order of 10 to 12 mg/100 ml. Cortical bone samples were pooled for the equilibration studies.

A further three experiments with child bone were also performed as part of this series.

Results.

These are shown in Table 14, and the cumulative data of 7.1, 7.2 and 7.3 in Figs. 58 and 59.

Discussion.

It can be seen in Fig. 58 that child bone once again has a higher equilibrium phosphate concentration than adult bone, confirming previous findings. The ion product can be seen in this instance to be significantly higher (Fig. 59).

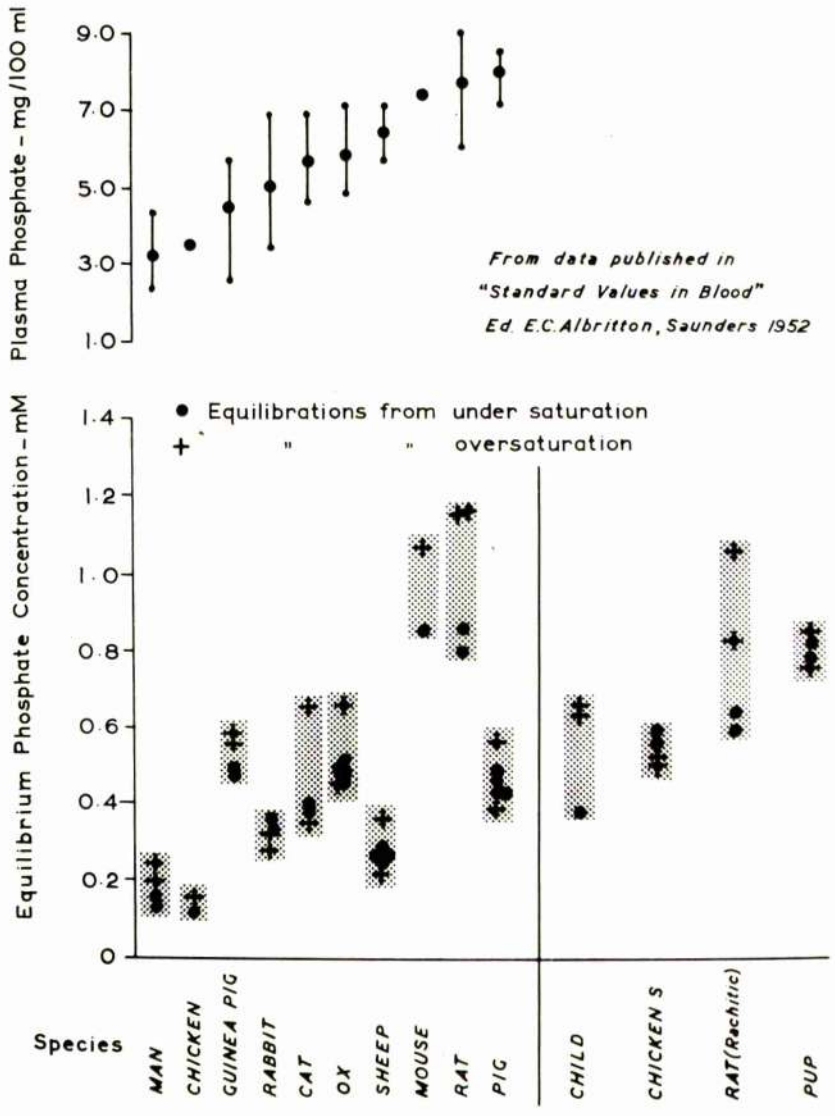


Figure 58. **Values of [P] at 24 hours in equilibration studies with cortical bone powder from normal animals, young animals and rachitic rats, and cancellous bone from the fowl.**

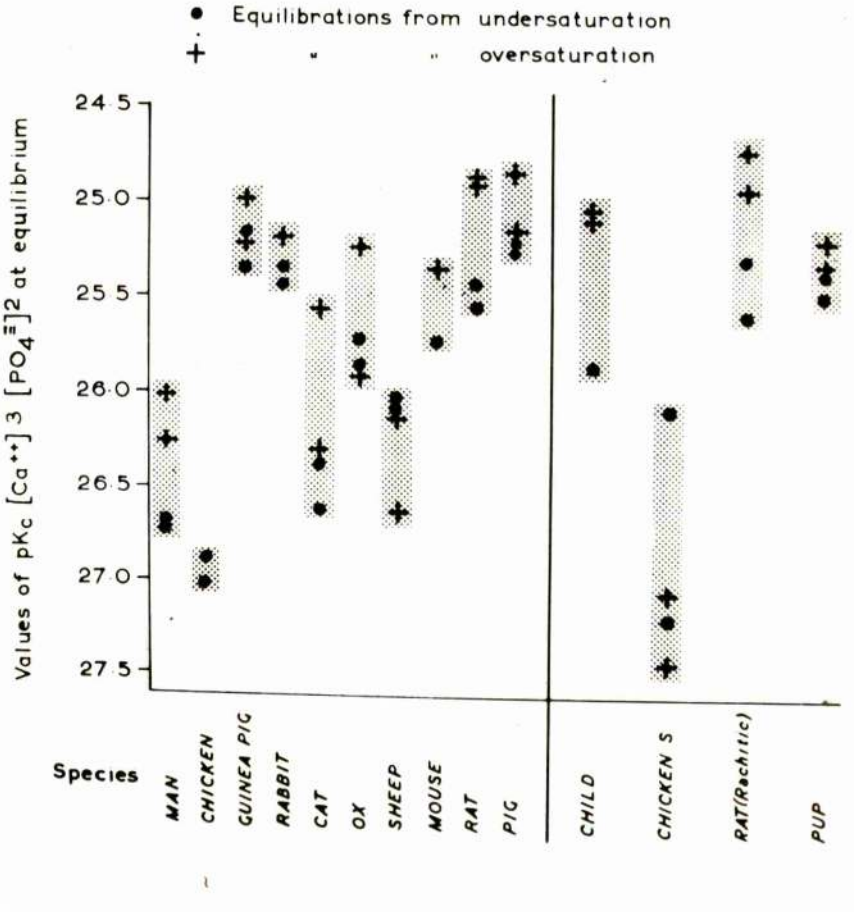


Figure 59. Values of $[Ca^{++}]^3[PO_4^{\equiv}]^2$ at 24 hours in equilibration studies with animal bone powder.

The cancellous bone of the fowl (skull) appears to yield a higher equilibrium phosphate concentration of 0.5mM to 0.6mM compared to the cortical bone (humeri and femora) figure of less than 0.2mM. In this case, the range of ion product is considerable and there is no apparent difference between the two types of bone from the same individual.

The rachitic rat bone yielded equilibrium phosphate concentrations and ion products that were no different from those of normal rats. A possible explanation is that our normal rat bone was from relatively young adults and was in fact 'low carbonate' bone yielding results similar to those of the rachitic material.

In the case of the puppy bone, the equilibrium phosphate concentration was not as high as might have been expected in view of the plasma phosphate concentration. However, no information is available on the ultrafiltrable portion of the total inorganic phosphate concentration in these animals. At concentrations as high as 10 mg/100 ml the formation of phosphate complexes is a distinct possibility. Urist (149) has postulated the existence of a calcium-protein-phosphate complex in shark serum to explain the observed concentrations of calcium and inorganic phosphate whose ion product certainly exceeds the 'solubility ceiling' of calcium phosphate.

Conclusion.

The results of these experiments are indicative of the possibilities inherent in this investigative technique. Much more exploratory work remains to be done in order to explain satisfactorily the various complex aspects of blood:bone equilibria.

THE ROLE OF BONE CARBONATE IN BLOOD:BONE EQUILIBRIA

Discussion

Summary

A series of equilibrations were performed with bone powder kindly supplied by Dr. W.G. Taylor, University Department of Physiological Chemistry, Reading. These bone samples were the subject of papers on 'variations in mineral composition of individual bones of the skeleton of the domestic fowl' (139, 140).

Dr. Taylor's carbonate analyses yielded values for the percentage of mineral matter as 'CO_2' in his various samples from 3.97% to 5.18% (S.D. $\pm$ 0.056). The intention in these experiments was to study the relationship between the equilibrium phosphate concentration <u>in vitro</u> and the bone carbonate content of different bone samples from the same individual.

Equilibration studies with bone powder prepared from different bones of the domestic fowl.

Samples of bone powder prepared from various bones of two birds were equilibrated for 24 hours in 0.15 M tris buffer at an initial pH of 7.1. As there was only sufficient material for one equilibration with each sample, all experiments were run from undersaturation.

The bone powder was prepared elsewhere (139, 140), but the technique employed was little different from that described in 1:5. The resulting particle size was similar to that of powder prepared in the Prolabo mill.

In all there were eight samples of powder from seven different bones of two birds, and the carbonate composition is shown in Table 15.

Results.

The data is presented in Table 15 and Figs. 60 to 64.

In these experiments the pH was held reasonably constant, and the independent variable was considered to be the carbonate content of the bone.

Fig. 60 shows the buffer $[Ca^{++}]$ plotted against bone carbonate content. There is considerable scatter but a possible correlation

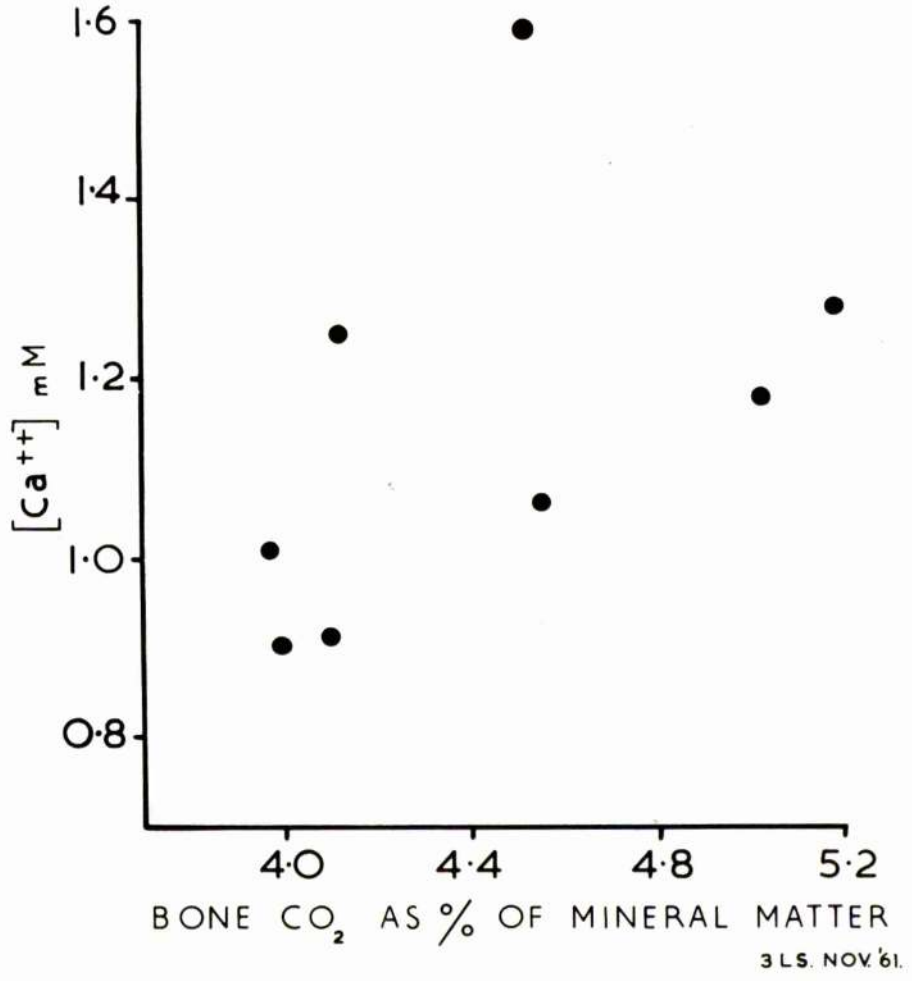


Figure 60. Buffer $[Ca^{++}]$ plotted against bone CO_2 content in equilibration studies with fowl bone powder in tris buffer.

is evident. The range of $[Ca^{++}]$ is from 0.90mM at pH 7.15 to 1.59mM at pH 7.20 and the respective bone carbonate contents are 3.99% and 4.52% of mineral matter by weight.

Fig. 61 shows the relationship between buffer $[P]$ and bone carbonate content. In this case, despite the scatter, an inverse correlation is apparent. The extremes of the data are $[P]$ 0.42mM and 0.16mM at carbonate contents of 3.97% and 5.18% respectively.

Fig. 62 shows buffer $[CO_2]$ plotted against bone carbonate content. Not surprisingly, bone of greatest carbonate content tends to produce the highest $[CO_2]$ in the buffer solution. Again there appears to be considerable scatter, the $[CO_2]$ values varying from 0.25mM at 3.99% bone CO_2 to 0.5mM at 5.18% bone CO_2.

Fig. 63 shows the ion products $pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$ and $pK_c[Ca^{++}][CO_3^{=}]$ plotted against bone carbonate content. Neither show any evidence of correlation with bone CO_2 as % of mineral matter; the former ranges from 27.26 to 26.52 and the latter from 9.72 to 9.19.

Fig. 64 shows the concentrations of trivalent phosphate ion in the buffers plotted against those of divalent carbonate ion. There is a suggestion of an inverse relationship, despite considerable scatter, and high $PO_4^{\equiv}$ concentrations appear to be associated with

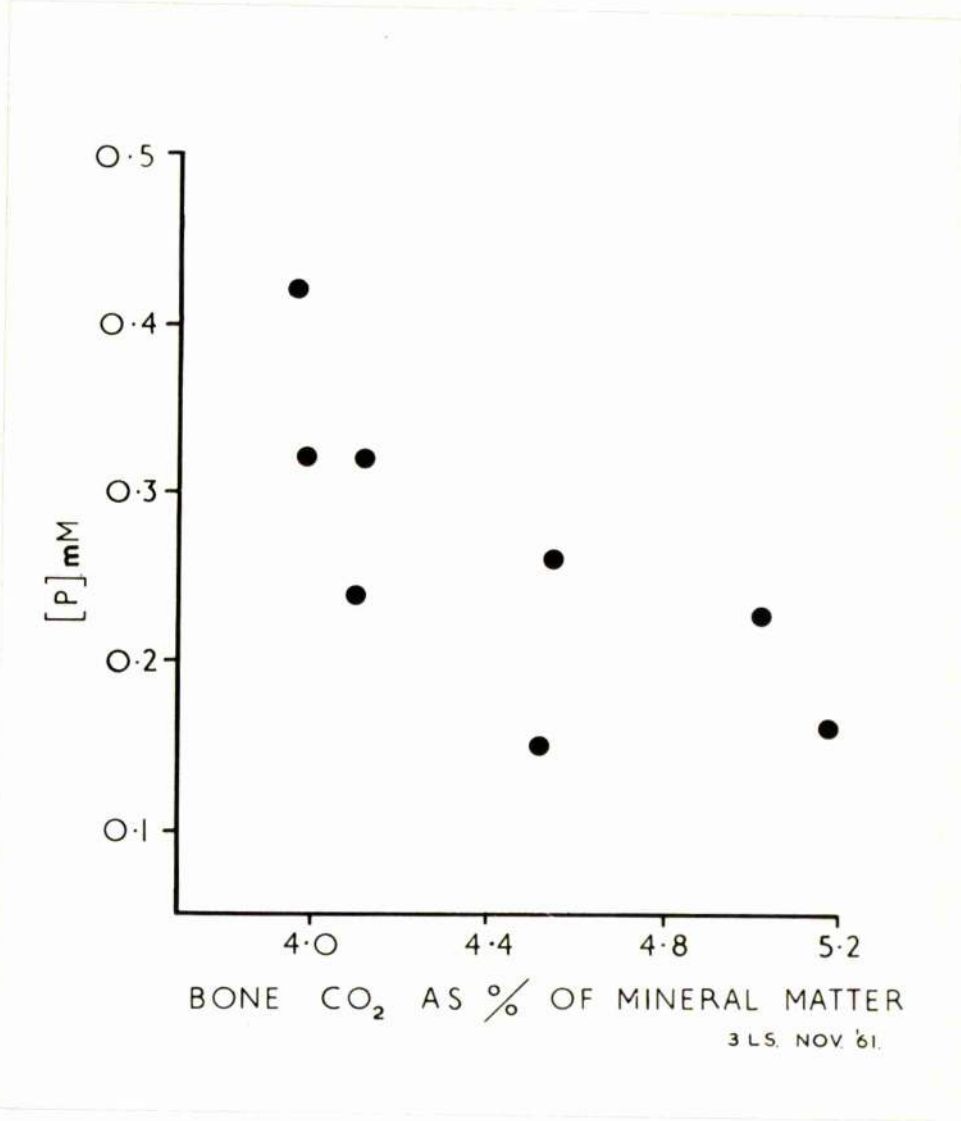


Figure 61. **Buffer [P] plotted against bone CO_2 content in equilibration studies with fowl bone powder in tris buffer.**

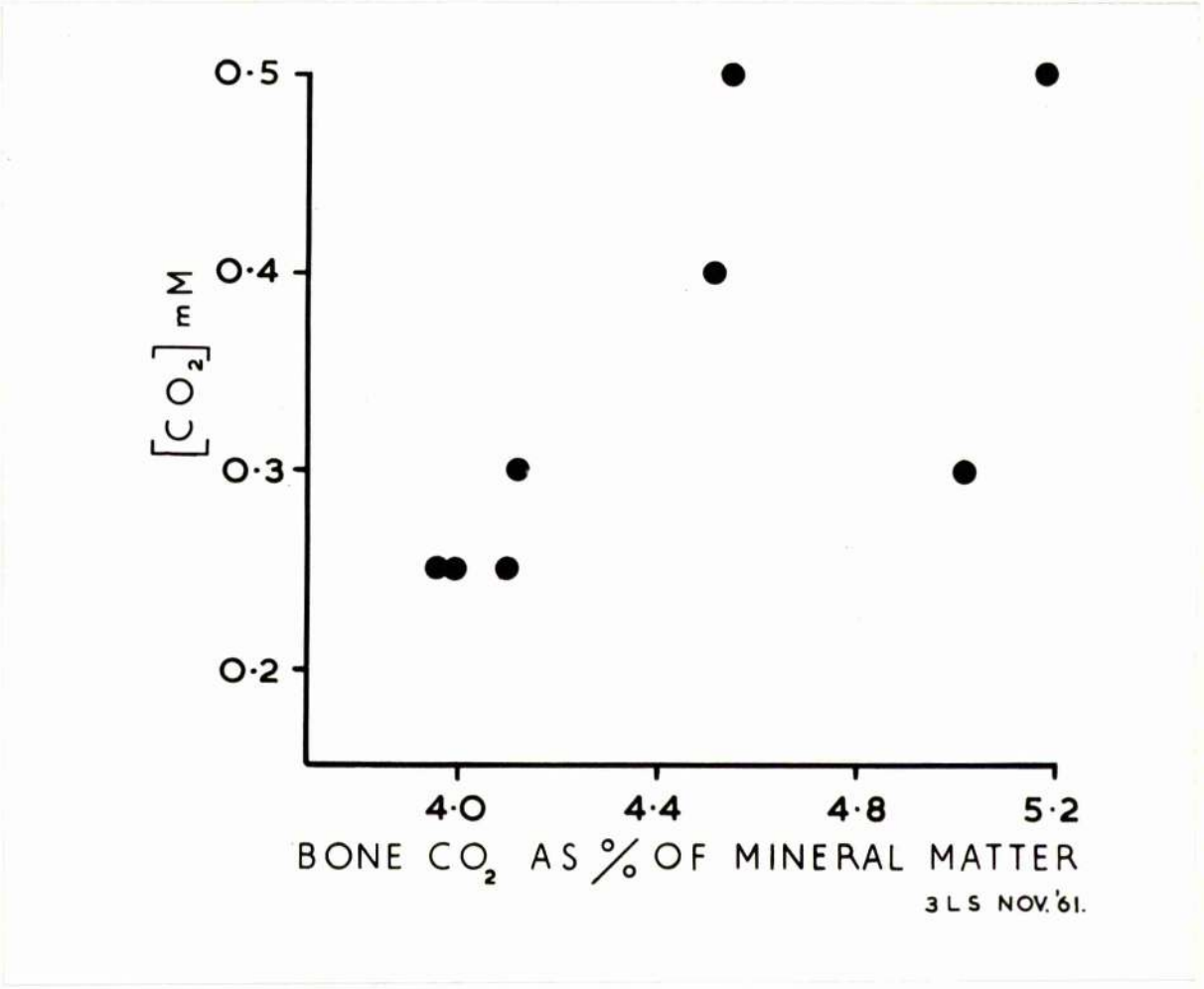


Figure 62. Buffer $[CO_2]$ plotted against bone CO_2 content in equilibration studies with fowl bone powder in tris buffer.

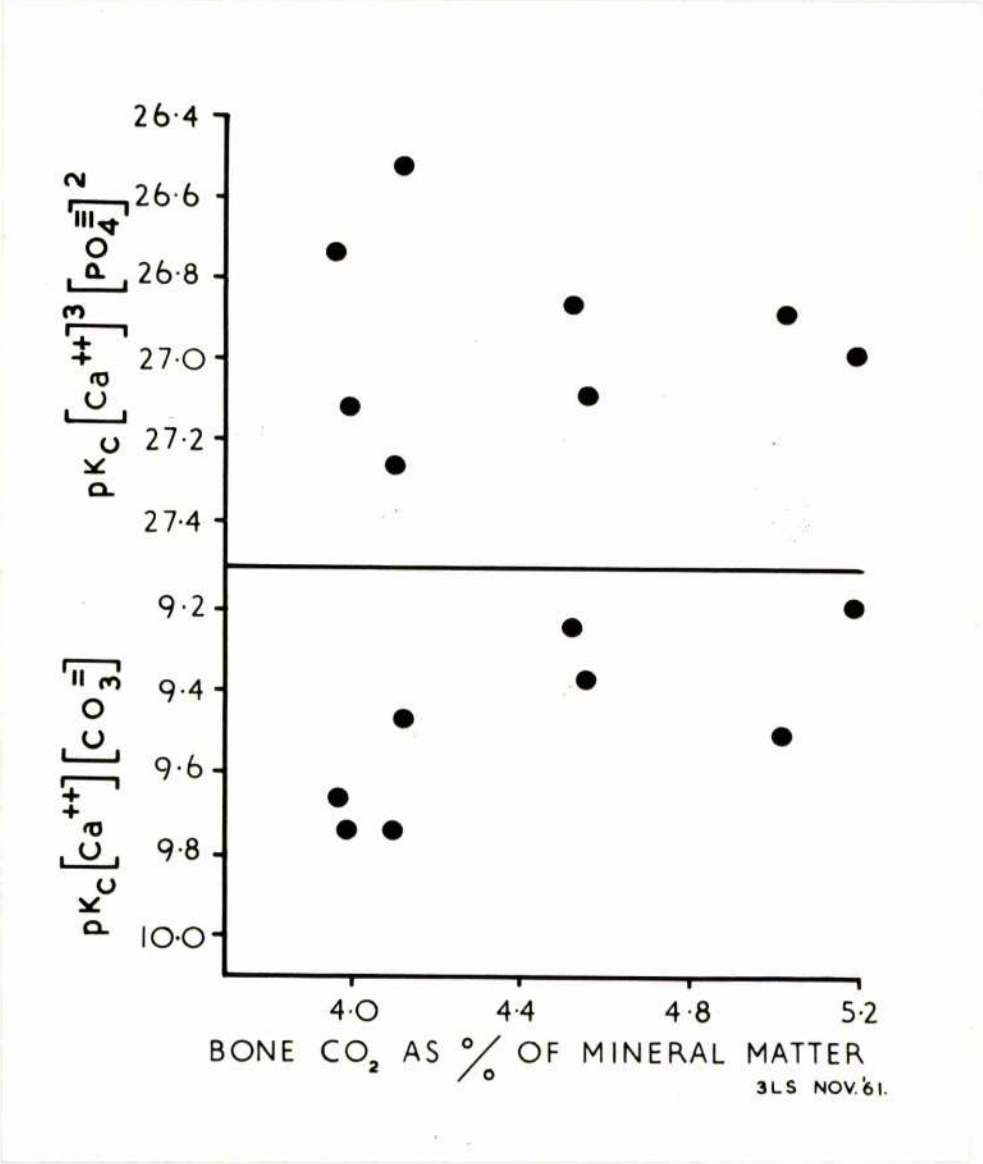


Figure 63. Values of $pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$ and $pK_c[Ca^{++}][CO_3^{=}]$ in equilibration fluid plotted against bone CO_2 content in studies with fowl bone powder in tris buffer.

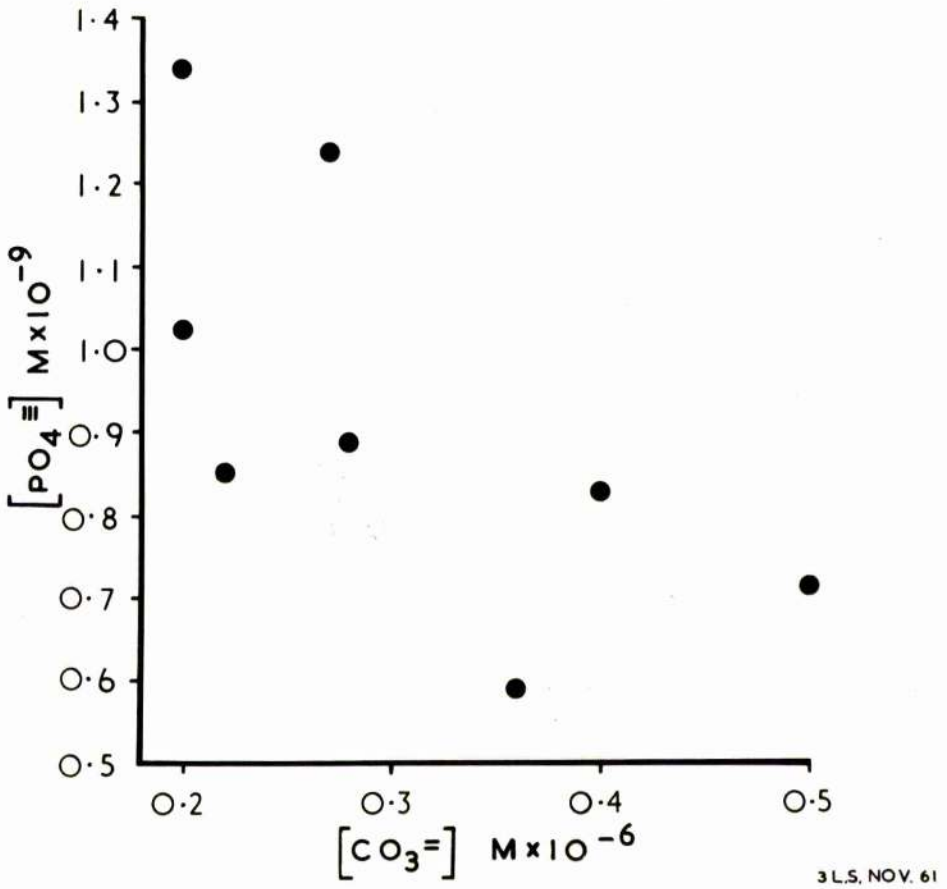


Figure 64. Buffer $[PO_4^{\equiv}]$ plotted against buffer $[CO_3^{=}]$ in equilibration studies with fowl bone powder in tris.

low $CO_3^=$ concentrations and vice versa.

Discussion.

The results reported above are of considerable theoretical importance, as they throw some light on aspects of the equilibrium mechanism which we were quite unable to resolve in the experiments performed so far.

It is apparent that bone powder of high carbonate content releases more carbonate into solution than bone of lower carbonate content, and in so doing raises the total CO_2 concentration of the buffer. The unexpected observation is that solutions with high $[CO_2]$ have low $[P]$ and vice versa (cf. Fig. 45 and 64). This can be seen directly in Fig. 64 where the concentrations of the two ion species $[PO_4^{\equiv}]$ and $[CO_3^=]$ can be seen to be inversely related.

The previous studies showed that when carbonate is supplied 'exogenously' by adding carbonate to the buffer, then the equilibrium phosphate concentration is also found to be raised (sections 3 and 4). These earlier observations were capable of at least two explanations. On the one hand, it was possible that an increased carbonate concentration, operating through a 'calcium carbonate solubility product', depressed the free calcium concentration which in turn, operating

through a 'calcium phosphate solubility product', permitted more inorganic phosphate to come into solution. Alternatively, one could postulate heteroionic exchange of carbonate for phosphate at the surface of the bone crystals. Thus raising the carbonate concentration would 'displace' phosphate from the lattice. The excess phosphate coming into solution would then, by means of a 'calcium phosphate equilibrium ion product' depress the free calcium concentration.

The present data, however, in which the carbonate in the solution has been given up by the bone mineral is compatible with only one of the two explanations.

When bone with a high CO_2 content is placed in buffer, relatively high carbonate concentrations are observed. In terms of the dual solubility theory, one would expect a lower $[Ca^{++}]$, which <u>is</u> true, and a raised $[PO_4^{\equiv}]$, which is <u>not</u> true. In terms of the heteroionic exchange theory, the more mineral carbonate that comes into solution the less phosphate one would expect on a stoichiometric basis and therefore a low equilibrium $[PO_4^{\equiv}]$ would result, which <u>is</u> true, which in turn would lead to a raised $[Ca^{++}]$ because of the 'calcium phosphate ion product' which is <u>also</u> true.

These deductions are dependent, of course, on a fair and accurate interpretation of results which do have considerable

scatter. However, until further experiments have been performed to extend these observations, it seems fairly likely that this last explanation for the behaviour of the carbonate and phosphate ions in the solution is plausible and in no way conflicts with current informed opinion.

Summary.

We have seen that the phosphate:carbonate relationship in fluids bathing bone powder is probably determined by a heteroionic exchange mechanism.

We have also seen that several bones of one individual have different composition and that this results in specific modification of the equilibrium between the bone and the bathing fluids dependent on the CO_2 content. Thus in the intact individual, the overall dynamic ionic equilibrium as observed in extracellular fluid, represents the total action of processes at an infinite number of skeletal sites. Each site presumably behaves according to its age, carbonate composition, degree of hydration, etc..

A dual mechanism of exchange solubility has been demonstrated which governs the relationship between inorganic phosphate concentration and bone composition, particularly with regard to bone carbonate. Variations in bone composition have been reported to occur with age (73,101) in rickets, (73,64) in acidosis, (48,19,66,135,69,97), in alkalosis (97), with variation in diet (73,66,97,160), and in osteopetrosis (74). It was Howland et al (64), and Kramer et al (74) who first suggested that variations in bone composition might be due to variations in the composition of the blood. It now appears that different parts of the same skeleton with different

compositions have different ionic equilibria. Therefore, children for example, have a high plasma phosphate concentration because their skeleton has a low carbonate content on average and not vice versa. It appears that variation in bone composition might lead to rather than be due to variations in blood electrolyte concentrations.

CONCLUSION

The experiments reported in this thesis were initiated at a time when the relationship between bone salt and its ions in tissue fluid was the subject of controversy. Authoritative sources (96,106), maintained that bone salt could not have a thermodynamic solubility product because of its complex nature and variable composition. Further, it was generally believed that the properties of bone salt were similar to those of its synthetic analogue, hydroxyapatite (77). The 'pure' compound calcium hydroxyapatite was described as a crystal lattice within which isomorphic substitutions produced variable composition between different portions of the same crystal. In a physical chemistry sense ...

> "the solubility of such a crystal cannot be expressed as a solubility product" (56).

Between the calcium and phosphate ions of bone and tissue fluid, however ...

> "It is unimaginable that any but an equilibrium condition exists. There must be a moment-to-moment interchange of ions from extracellular fluid on to the crystal surfaces" (56)....

and this equilibrium state must be determined by physico-chemical processes similar in many respects to those governing solubility.

The intention therefore was to study the equilibration of human bone with buffers on a purely empirical basis, ignoring initially the maze of theoretical conflict by defining any equilibrium in terms

of a product of ion concentrations at specified temperature, ion strength, buffer composition etc..

The simplest of experimental designs was employed. Bone was allowed to dissolve in buffer at constant ionic strength, and the material in solution given time to migrate through a dialysis membrane until apparent concentration equilibrium was achieved. In similar experiments in the past, it is fairly certain that protein debris of the bone matrix, complexed with calcium and perhaps even phosphate, has contributed substantially to the extreme variability in bone solubility data. The use of the dialysis membrane in the current experiments ensured that all particulate matter was excluded from samples submitted to analysis.

It was found that when the equilibrium was expressed in terms of the ion products $[Ca^{++}][HPO_4^{=}]$ and $[Ca^{++}]^3[PO_4^{\equiv}]^2$, bone mineral behaved in a characteristic manner, the former ion product being pH - dependent over the pH range 6.6 to 7.8, whereas the latter was not. Thus, bone salt does not behave like calcium acid phosphate, which is not surprising since all the available evidence suggests that $CaHPO_4$ is unstable at a pH above about 6.2. On the other hand, one cannot conclude that bone mineral is tri-calcium phosphate even if the equilibrium is best expressed by the $[Ca^{++}]^3[PO_4^{\equiv}]^2$ ion product.

The arithmetic mean value for the pK of the ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ for bone salt was found to be 26.39 in bicarbonate-free tris and cacodylate buffers, and 26.30 in tris buffer with added bicarbonate. The results of many studies indicated that the constancy of the ion product was predictable, reproducible and appeared to be a fundamental 'property' of bone mineral.

In the earlier equilibration experiments described in this thesis, there was a direct relation between the calcium and hydrogen ion concentrations, and a relatively constant absolute concentration of inorganic phosphorous in the buffers at equilibrium. It was thought at one stage that this relation could be explained by a heteroionic exchange between calcium and hydrogen ion (56). Such a possibility was finally disposed of when it was found that variations in total carbonate concentration resulted in changes in the calcium ion concentration independent of pH.

It was also established that the ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ was independent of solid:solution ratio. Logan and Taylor (81,82,83,80,79) first postulated an association between solid:solution ratio and ion product such that the smaller the ratio, the greater the product. The present data refute these authors' interpretation of their own findings.

The relationship between the bone powder ion product and the calcium phosphate ion product in serum was studied by equilibrating

bone powder in normal human serum. This showed that at a pH of about 7.4 there was a fall in both calcium and phosphate concentrations in the serum. On the other hand, when the non-protein-bound calcium and trivalent phosphate concentrations were calculated and the $[Ca^{++}]^3[PO_4^{\equiv}]^2$ ion products determined, they were found to be greater than those found in synthetic media, the pK of the mean being 25.61. When the total trivalent phosphate ion concentration was corrected to free trivalent phosphate ion concentration in accordance with Walser's data (157) (which suggests that only about 53% of total serum inorganic phosphate is non-associated) then the resulting ion products (pK of mean 26.17) were not substantially different from those found in synthetic media containing bicarbonate (pK of mean 26.30). No account was taken of citrated calcium in these experiments with normal human serum and any correction would tend to reduce the product and so bring the pK even closer to 26.30.

The data suggest not only that Walser's hypothesis is valid, but also that bone salt demonstrates a constant ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ at equilibrium, both in synthetic media and in normal human serum.

It is difficult to compare these findings with those of previous workers since the empirical approach precludes anything but comparison with experiments under identical conditions. Much of the early work did not allow for specific binding of calcium e.g. to protein, and none of it considered the formation of association complexes of phosphate. The dialysis feature of the present technique reduces

these problems to a minimum and when one considers the advances made in control of infection in these ideal culture media, and also the recent remarkable progress in the reliability of electrometric pH determination, then it is hardly surprising that there are great discrepancies in the published data, and yet reasonable consistency in the present results.

However, one group of workers (129,136) has reported a pK $[Ca^{++}]^3[PO_4^{\equiv}]^2$ for whole bone of 26.36, and Neuman's data of more recent date (103) yielded one value of 26.11 when recalculated.

The absolute value obtained for the pK of the ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ is probably the best 'empirical product' currently available for defining the equilibrium state between bone mineral and its ions in physiological media at physiological pH, ion strength, etc.. This value, between 26.39 and 26.30, is the first important finding of this work.

The second important observation concerns the controversy over the nature of the bone salt. The principal inorganic components of the crystal are calcium, phosphate and carbonate. The question at issue is simply which of the three principal structural hypotheses best fits the observed data.

When bone powder was placed in buffer containing carbonate

ions, the calcium and phosphate concentrations were reciprocally related and the trivalent phosphate concentration was directly proportional to the divalent carbonate concentration. Secondly, when different samples of bone powder containing different amounts of carbonate were allowed to dissolve in carbonate-free buffers, the phosphate concentration in the buffers at equilibrium was an inverse function of the carbonate concentration.

The carbonate-apatite theory (96,86,85,90,89), carried to its logical conclusion, would presumably require that the concentrations of the three ions $[Ca^{++}]$ $[PO_4^{\equiv}]$ and $[CO_3^{=}]$ be thermodynamically related at equilibrium, and that some ion product would appear to be a constant. We have demonstrated, however, that for bone powder, the product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ is a constant, and that in bicarbonate buffers, elevation of $[CO_3^{=}]$ raises $[PO_4^{\equiv}]$ and lowers $[Ca^{++}]$ in such a way that this ion product remains unchanged. It follows that within the limits of error of the method, the carbonate ion cannot itself be included in the product and therefore there does not appear to be any evidence in support of the postulate of a stoichiometric carbonate-apatite.

The two-phase hypothesis (25,26,30,34) would presumably require the demonstration of a calcium phosphate ion product and a calcium carbonate ion product, both independent of pH, but linked in operation through the common ion, calcium. Thus a rise in carbonate concentration

would depress the calcium concentration and thus permit the phosphate concentration also to rise. However, it has not been possible to demonstrate a calcium carbonate ion product and in fact, in the experiments in 8.1 higher carbonate concentrations in solution appear to be associated with lower phosphate concentrations. There is no evidence therefore to support the theory that the bone material employed in these experiments is a two-phase system of calcium phosphate and calcium carbonate.

There remains the third and final theory which considers that the bone salt is essentially a hydroxyapatite (106) or a 'defect' or 'calcium-deficient' apatite (119) which is able to exchange phosphate ions for carbonate ions at the surface of the crystal lattice. In this case one would expect a calcium phosphate ion product to operate in such a way that there was a reciprocal relationship between calcium and phosphate concentrations in solution. Further, one would expect that carbonate ions added to the solution would displace phosphate ions from the lattice. Also, if bone samples of different carbonate content were dissolving into a bicarbonate-free buffer solution, one would expect from stoichiometric considerations that the more carbonate ion that was released from the crystal surface into solution, the less phosphate ion would be released. These expectations have all been fulfilled, although the degree of certainty is limited by the experimental variation implicit in the method. Certainly it is felt that this

interpretation of the behaviour of bone salt in solution is at the moment the most plausible of the three, and must stand until further evidence is forthcoming.

Having demonstrated that bone mineral behaves as an 'apatite', the question remains - is bone mineral simply calcium hydroxyapatite? In other words, does whole bone behave similarly to pure synthetic calcium hydroxyapatite in solution as has been suggested in the past (77).

No experimental evidence has been presented on this point, but in fact a number of equilibration experiments have been performed with synthetic products. In brief, the ion product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ for a highly characterised calcium hydroxyapatite is about <u>ten</u> times that of whole bone. The physiological significance of this finding is that it suggests that the skeleton will take up calcium and phosphate from tissue fluids well below the solubility ceiling for inorganic calcium hydroxyapatite and so prevent systemic precipitation.

The physical mechanism by which an ion product lower than that of the synthetic analogue of bone is achieved, is quite unknown. Is it a functional property of the matrix portion of bone? Explanation of this enigma will probably require much more intensive research into the physico-chemical aspects of whole bone equilibria at the microscopic level.

The biological implications of these studies concern primarily calcium homeostasis and the control of plasma calcium concentration.

At an early stage in the investigation the suggestion was advanced that if the bone crystal environment was at pH 6.8 then the concentrations of calcium and phosphate ions found in normal plasma could be completely explained by the physico-chemical equilibrium mechanism. Later, because of our validation of Walser's belief that only about 53% of plasma phosphate is in the ionised form, it was necessary to reconsider. Recalculation showed that the pH necessary to explain tissue fluid concentrations of calcium and phosphate under these conditions was about 7.1, i.e. between intracellular and extracellular pH.

The concept that bone pH might be less than that of circulating body fluids was first proposed by Nordin (110), and has since been accepted by other workers who have produced confirmatory, though circumstantial evidence (15,16,17,68,76,98).

The second concept that the equilibrium between the skeleton and the calcium and phosphate of body fluids could be maintained by a physico-chemical process is original to Nordin (110). An inevitable corollary of this second idea, whose plausibility I have substantiated, is that relatively small changes in the pH of the bone environment produce specific changes in free calcium concentration in body fluids.

We have seen that total phosphate concentration is a function of total carbonate concentration in solutions in contact with bone mineral, and so a fall in pH at the bone crystal surface results primarily in a rise in free calcium concentration due to a reduction in the proportion of the total phosphate as the trivalent species.

It has been shown (164), that parathyroid hormone can inhibit enzyme-dependent, oxidative phosphorylation in tissue homogenates *in vitro* with the result that anaerobic respiration and increased lactic acid production is favoured. At the same time, Neuman and Dowse (104) and Tereptka et al (141) have postulated an effect of parathyroid hormone on the transport of inorganic phosphate into and across cells with consequent changes in ATP synthesis and lactic acid production. In brief, PTH appears to reverse the Pasteur effect in surviving bone preparations *in vitro*, and there is an increase in lactic acid production even in the presence of adequate partial pressures of oxygen.

Thus it is possible that increased organic acid production in the bone environment is the end-result of parathyroid gland activity *in vivo*. The properties of the physico-chemical equilibrium established above are therefore of considerable importance in

providing a dynamic concept for the link between metabolic processes changing in response to hormone release and variations in free calcium concentration in the body fluids.

It is clear that many problems remain to be studied. The properties of bone mineral of different species and of bone mineral of different ages has been lightly touched. It is felt that the characteristically high plasma inorganic phosphate concentration of children is probably a function of the composition of child bone in that young bone tends to have low carbonate content. In the laboratory, calcium phosphate salts undergo slow, spontaneous re-organisation, and it may be that as the child matures into the adult, a somewhat similar phenomenon occurs <u>in vivo</u> in an environment whose partial pressure of CO_2 is rather greater.

It is also apparent that there are many possible avenues of exploration into the pathology of bone. Osteoporosis, osteomalacia, osteopetrosis and ectopic pathological calcification all require investigation. The calcium phosphate ionic equilibria governing calcification in nephrocalcinosis and renal lithiasis are also worthy of study.

In closing, we must consider the validity, value and limitations of the empirical approach that has been forced upon us. It is true that bone is variable in composition. It is true that

we cannot derive a thermodynamic solubility product for bone; physico-chemical theory is incapable of coping with such complexities. It is <u>not</u> true that we must therefore reject a quantitative approach altogether. Even although the ion product $[Ca^{++}]^3[PO_4^{=}]^2$ is meaningless in a strict sense, it has enabled us, in this case, to consolidate conflicting data into a simple, if theoretically inadequate, concept.

When faced with a similar physical problem in biology, Neuman wrote (106) ...

> "One can obtain interpretable results only with simplified systems (that's physical chemistry!) and with purified components (that's common sense!) but, if one cannot generalise from the experiment, what earthly good can come of it all."

One might apply the inverse of his reasoning. Since bone salt cannot be adequately described in a physico-chemical sense, let us set aside the theoretical complexities, express experimental observations with whole bone as simple ion products, and generalise in terms of this approximation to the thermodynamic behaviour of bone mineral. That has been done, and the passage of time will assess whether our understanding of bone physiology is the greater.

Acknowledgements

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T A B L E S

Potentiometric titration curves for 0.15 M Cacodylic Acid & Tris

Vol. of alkali added	5 ml 0.15 M Cacodylic Acid		5 ml 0.15 M Tris	
	0.0253N NaOH	0.0257N $Ca(OH)_2$	0.0253N NaOH	0.0257N $Ca(OH)_2$
1 ml	pH 4.73	pH 4.66	pH 6.86	pH 6.89
2	5.02	4.94	7.01	7.07
3	5.21	5.20	7.19	7.19
4	5.35	5.38	7.28	7.30
5	5.47	5.48	7.38	7.39
6	5.58	5.58	7.45	7.45
7	5.62	5.66	7.52	7.52
8	5.74	5.72	7.58	7.59
9	5.80	5.80	7.64	7.65
10	5.89	5.88	7.70	7.70
11	5.91	5.92	7.74	7.75
12	6.00	6.00	7.79	7.80
13	6.06	6.08	7.82	7.83
14	6.13	6.11	7.87	7.89
15	6.19	6.20	7.91	7.92
16	6.26	6.25	7.95	7.97
17	6.31	6.30	7.99	8.00
18	6.40	6.37	8.01	8.04
19	6.42	6.42	8.05	8.08
20	6.50	6.48	8.10	8.11
21	6.57	6.55	8.13	8.16
22	6.62	6.62	8.16	8.20
23	6.70	6.72	8.20	8.22
24	6.82	6.80	8.24	8.27
25	6.90	6.90	8.29	8.31
26	7.00	7.02	8.31	8.35
27	7.21	7.15	8.35	8.39
28	7.32	7.35	8.38	8.42
29	7.68	7.89	8.42	8.48
30	8.50	8.90	8.46	8.50
31			8.49	8.55
32			8.52	8.60
33			8.56	8.65
34			8.60	8.70
35			8.65	8.75
36			8.70	8.80
37			8.75	8.85
38			8.80	8.91
39			8.85	8.99
40			8.90	9.60

Table 1. Titration data for tris and cacodylate buffers with NaOH and $CaOH_2$.

Expt. No.	Duration (hrs.)	Initial Concentration $[Ca^{++}]$ $X10^{-3}$	Initial Concentration $[P]$ $X10^{-3}$	Final Concentrations $[Ca^{++}]$ $X10^{-3}$	Final Concentrations $[P]$ $X10^{-3}$	Final Concentrations $[HPO_4^{=}]$ $X10^{-4}$	Final Concentrations $[PO_4^{\equiv}]$ $X10^{-11}$	$pK_c[Ca^{++}][HPO_4^{=}]$	$pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$	pH
13DP1	24	-	-	1.25	0.27	2.00	154.0	6.60	26.35	7.32
2	"	-	-	1.23	0.27	2.00	154.0	6.61	26.36	7.32
3	"	1.5	0.16	2.04	0.24	1.87	88.5	6.41	26.18	7.18
4	"	1.5	0.16	2.00	0.23	1.61	88.5	6.49	26.24	7.18
1	48	-	-	1.33	0.24	1.86	163.0	6.61	26.20	7.38
2	"	-	-	1.44	0.24	1.86	163.0	6.54	26.10	7.38
3	"	1.5	0.16	2.23	0.19	1.28	74.0	6.55	26.21	7.20
4	"	1.5	0.16	2.15	0.19	1.28	74.0	6.55	26.26	7.20
1	72	-	-	1.33	0.23	1.80	145.0	6.62	26.30	7.35
2	"	-	-	1.38	0.23	1.80	145.0	6.61	26.26	7.35
3	"	1.5	0.16	2.21	0.16	1.18	73.5	6.58	26.23	7.25
4	"	1.5	0.16	2.21	0.19	1.41	87.5	6.49	26.08	7.25

Table 2. **Typical behaviour of bone powder in buffer at pH 7.0. Concentrations in Moles.**

Expt. No.	Duration (hrs.)	Initial Concentration $[Ca^{++}]$ $\times 10^{-3}$	Initial Concentration $[P]$ $\times 10^{-3}$	Final Concentrations $[Ca^{++}]$ $\times 10^{-3}$	$[P]$ $\times 10^{-3}$	$[HPO_4^{=}]$ $\times 10^{-4}$	$[PO_4^{\equiv}]$ $\times 10^{-11}$	$pK_c[Ca^{++}][HPO_4^{=}]$	$pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$	pH
15.6	24	1.00	1.60	4.88	0.38	1.38	1.62	6.17	26.51	6.58
13.6	"	–	–	5.30	0.31	1.29	1.95	6.17	26.25	6.60
15.5	"	1.00	1.60	5.25	0.27	0.98	1.23	6.29	26.66	6.60
13.5	"	–	–	5.20	0.33	1.30	1.67	6.17	26.41	6.63
15.4	"	0.74	0.97	4.30	0.25	1.17	2.19	6.30	26.42	6.78
13.8	"	–	–	4.10	0.28	1.32	2.69	6.27	26.30	6.79
15.3	"	0.74	0.97	4.34	0.22	1.04	2.00	6.35	26.48	6.80
13.7	"	–	–	4.00	0.29	1.73	3.23	6.16	26.17	6.84
12.1	"	3.83	0.81	3.45	0.15	0.87	3.06	6.52	26.41	6.93
12.2	"	3.83	0.81	3.48	0.19	1.09	3.82	6.42	26.21	6.93
15.1	"	0.73	1.11	3.55	0.17	1.03	3.31	6.44	26.31	6.98
15.2	"	0.73	1.11	3.28	0.24	1.46	4.79	6.32	26.09	6.99
13.10	"	–	–	3.10	0.16	0.90	3.10	6.55	26.54	7.00
13.9	"	–	–	3.30	0.12	0.75	2.88	6.61	26.53	7.05
12.4	"	3.68	0.78	2.50	0.16	1.01	4.37	6.50	26.53	7.12
11.1	"	–	–	1.76	0.19	1.23	7.25	6.33	26.54	7.15
12.3	"	3.68	0.78	2.43	0.18	1.19	6.05	6.54	26.28	7.15
11.2	"	–	–	1.75	0.18	1.20	6.85	6.68	26.50	7.18
11.3	"	–	–	1.33	0.19	1.38	10.00	6.74	26.63	7.28
11.4	"	–	–	1.33	0.18	1.31	9.50	6.76	26.67	7.28
12.6	"	2.10	0.78	1.68	0.21	1.52	10.90	6.59	26.41	7.30
12.7	"	2.15	0.63	1.40	0.14	1.10	11.70	6.81	26.43	7.45
11.5	"	–	–	0.93	0.22	1.77	21.20	6.79	26.39	7.46
11.6	"	–	–	0.98	0.18	1.47	17.50	6.84	26.54	7.48
12.8	"	2.15	0.63	1.43	0.13	1.05	12.30	6.83	26.36	7.50
19.5	"	0.50	0.50	0.36	0.25	2.11	24.67	7.12	27.25	7.63
11.8	"	–	–	0.69	0.24	2.06	40.10	6.85	26.27	7.65
12.9	"	1.95	0.63	1.13	0.08	0.64	12.80	7.14	26.63	7.68
12.10	"	1.95	0.63	1.20	0.11	0.95	18.50	6.94	26.23	7.70
19.4	"	1.39	0.03	0.61	0.19	1.73	43.65	6.98	26.37	7.80
19.8	"	0.50	0.32	0.33	0.27	2.47	77.62	7.09	26.66	7.81
11.10	"	–	–	0.44	0.23	2.11	51.50	7.03	26.63	7.83
19.3	"	1.39	0.03	0.59	0.22	2.00	85.11	6.93	25.83	7.84
19.6	"	0.50	0.59	0.33	0.31	2.24	77.62	7.13	26.66	7.85
11.9	"	–	–	0.41	0.22	2.01	61.70	7.08	26.57	7.85
19.7	"	0.50	0.32	0.34	0.25	2.31	67.61	7.10	26.75	7.89
19.1	"	2.13	–	0.59	0.18	1.63	50.12	7.02	26.39	7.90
19.2	"	2.13	–	0.60	0.19	1.75	53.70	6.98	26.21	7.90

Table 3. **Equilibration studies in bicarbonate-free buffers. Concentrations in Moles.**

(see over)

Expt. No.	Duration (hrs.)	Initial Concentration $[Ca^{++}]$ X10^{-3}	Initial Concentration $[P]$ X10^{-3}	Final Concentrations $[Ca^{++}]$ X10^{-3}	Final Concentrations $[P]$ X10^{-3}	Final Concentrations $[HPO_4^{=}]$ X10^{-4}	Final Concentrations $[PO_4^{\equiv}]$ X10^{-11}	$pK_c[Ca^{++}][HPO_4^{=}]$	$pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$	pH
15.9	24	1.13	1.08	8.95	0.29	0.60	0.25	6.27	27.34	6.21
15.7	"	1.11	1.32	6.45	0.30	0.85	0.60	6.26	27.01	6.40
15.8	"	1.11	1.32	6.45	0.29	0.81	0.58	6.28	27.05	6.40
20.2	"	7.44	-	9.00	0.21	0.64	0.50	6.24	26.84	6.45
20.8	"	0.06	0.48	4.70	0.46	1.39	1.17	6.18	26.85	6.46
20.4	"	5.69	-	7.89	0.26	0.81	0.74	6.19	26.57	6.48
20.6	"	0.06	1.93	4.58	0.53	1.65	1.50	6.12	25.66	6.48
20.7	"	0.06	0.48	4.33	0.52	1.62	1.47	6.15	26.75	6.48
20.5	"	0.06	1.93	4.39	0.53	1.69	1.56	6.13	25.69	6.49
20.3	"	5.69	-	7.75	0.27	0.89	0.83	6.16	26.50	6.50
21.1	48	-	-	7.69	0.71	1.49	0.63	5.94	26.74	6.22
21.2	"	-	-	7.89	0.61	1.37	0.72	5.97	26.60	6.27
21.3	"	-	-	7.89	0.53	1.11	0.47	6.06	26.96	6.22
21.1	75	-	-	7.83	0.65	1.40	0.61	5.96	26.75	6.23
21.2	"	-	-	8.33	0.61	1.49	0.86	5.08	26.37	6.32
21.3	"	-	-	8.33	0.57	1.23	0.53	5.99	26.78	6. 3

Table 3 cont'd...

Expt. No.	Duration (hrs.)	Solid:Soln. ratio (gm/l)	Initial Concentrations	Final Concentrations $[Ca^{++}]$ x10^{-3}	$[P]$ x10^{-3}	$[PO_4^{=}]$ x10^{-10}	pK_c	$[Ca^{++}]^3$	$[PO_4^{=}]^2$	pH
5DP/1	24	25.0	-	1.00	0.23	16.6		26.56		7.42
2	"	25.0	-	0.89	0.23	19.3		26.58		7.45
3	"	12.5	-	0.75	0.24	17.3		26.90		7.40
4	"	12.5	-	0.83	0.29	20.4		26.60		7.40
5	"	6.25	-	0.60	0.24	17.3		27.19		7.39
6	"	6.25	-	0.70	0.29	20.9		26.83		7.40
7	"	2.50	-	0.56	0.19	13.7		27.49		7.40
8	"	2.50	-	0.59	0.23	16.6		27.24		7.40
9	"	0.25	-	0.51	0.23	16.6		27.44		7.39
10	"	0.41	-	0.41	0.16	11.5		28.04		7.38
1	48	25.0	-	1.05	0.19	10.7		26.93		7.30
2	"	25.0	-	0.98	0.20	14.4		26.71		7.40
3	"	12.5	-	0.89	0.23	16.6		26.71		7.40
4	"	12.5	-	0.83	0.19	13.7		26.97		7.40
5	"	6.25	-	0.73	0.20	14.4		27.10		7.38
6	"	6.25	-	0.75	0.19	13.7		27.10		7.40
7	"	2.50	-	0.65	0.23	16.6		27.12		7.37
8	"	2.50	-	0.63	0.18	13.0		27.37		7.40
9	"	0.25	-	0.53	0.16	11.5		27.71		7.38
10	"	0.25	-	0.53	0.19	10.1		27.82		7.38
1	96	25.0	-	1.18	0.16	8.5		26.93		7.30
2	"	25.0	-	1.03	0.18	13.0		26.74		7.40
3	"	12.5	-	0.93	0.19	13.7		26.82		7.38
4	"	12.5	-	0.93	0.23	9.0		27.19		7.20
5	"	6.25	-	0.73	0.20	14.4		27.10		7.38
6	"	6.25	-	0.98	0.26	5.2		27.60		7.02
7	"	2.50	-	0.63	0.19	10.1		27.59		7.32
8	"	2.50	-	0.65	0.19	10.1		27.56		7.30
9	"	0.25	-	0.55	0.19	10.1		27.77		7.28
10	"	0.25	-	0.53	0.19	10.1		27.82		7.30
7DP/1	24	25.0	-	0.96	0.18	14.4		26.74		7.44
2	"	25.0	-	0.96	0.18	13.7		26.78		7.42
3	"	12.5	-	0.85	0.19	14.4		26.90		7.42
4	"	12.5	-	0.83	0.19	13.7		26.97		7.40
5	"	6.25	-	0.73	0.19	14.4		27.10		7.42
6	"	6.25	-	0.71	0.23	16.6		27.01		7.40
7	"	2.50	-	0.51	0.21	16.0		27.33		7.42
8	"	2.50	-	0.57	0.19	14.4		27.42		7.42
9	"	0.25	-	0.33	0.11	7.9		28.64		7.40
10	"	0.25	-	0.35	0.11	7.9		28.58		7.40
11	24	25.0	*	1.70	0.32	23.0		25.58		7.40
12	"	25.0	*	1.74	0.32	23.0		25.55		7.40
13	"	12.5	*	1.89	0.39	28.1		25.27		7.40
14	"	12.5	*	1.88	0.45	32.4		25.16		7.40
15	"	6.25	*	1.94	0.55	39.6		24.94		7.40
16	"	6.25	*	1.94	0.53	38.2		24.97		7.40
17	"	2.50	*	2.24	0.77	55.4		24.46		7.40
18	"	2.50	*	2.19	0.79	56.9		24.47		7.40
19	"	0.25	*	2.58	1.05	75.6		24.01		7.40
20	"	0.25	*	2.55	1.10	78.2		23.98		7.40

Table 4. **The relationship of solid:solution ratio to ion product.**
(Initial concentrations: - Ca nil, P nil; * Ca 3mM, P 1.3mM) **(see over)**

20	"	0.25	+	2.55	1.10	78.2	23.98	7.40
1	48	25.0	-	1.16	0.19	11.8	26.60	7.35
2	"	25.0	-	1.18	0.19	11.8	26.40	7.35
3	"	12.5	-	0.96	0.19	12.5	26.86	7.37
4	"	12.5	-	0.98	0.19	18.8	26.88	7.35
5	"	6.25	-	0.89	0.23	12.2	26.98	7.30
6	"	6.25	-	0.89	0.26	13.8	26.87	7.30
8	"	2.50	-	0.68	0.23	14.3	27.27	7.25
7	"	2.50	-	0.75	0.26	16.1	26.96	7.35
9	"	0.25	-	0.40	0.16	9.9	28.20	7.35
10	"	0.25	-	0.40	0.18	11.2	28.10	7.32
11	"	25.0	+	1.61	0.21	14.3	26.07	7.38
12	"	25.0	+	1.72	0.23	15.2	25.03	7.35
13	"	12.5	+	1.67	0.21	19.2	25.97	7.35
14	"	12.5	+	1.67	0.35	20.0	25.93	7.32
15	"	6.25	+	1.79	0.37	22.9	25.52	7.35
16	"	6.25	+	1.81	0.40	23.6	25.48	7.33
17	"	2.50	+	2.10	0.38	21.7	25.36	7.32
18	"	2.50	+	2.07	0.38	20.1	25.45	7.30
19	"	0.25	+	2.46	1.00	62.0	24.28	7.35
20	"	0.25	+	2.46	1.10	79.0	24.03	7.40
1	72	25.0	-	1.20	0.16	13.3	26.52	7.45
2	"	25.0	-	1.20	0.18	16.0	26.34	7.47
3	"	12.5	-	1.01	0.19	13.7	26.72	7.40
4	"	12.5	-	0.95	0.19	16.9	26.62	7.47
5	"	6.25	-	0.88	0.21	16.0	26.76	7.42
6	"	6.25	-	0.87	0.23	20.7	26.55	7.48
7	"	2.50	-	0.69	0.24	19.9	26.89	7.45
8	"	2.50	-	0.66	0.23	18.4	27.01	7.44
9	"	0.25	-	0.50	0.19	14.4	27.59	7.42
10	"	0.25	-	0.44	0.19	15.2	27.72	7.44
11	72	25.0	+	1.56	0.16	12.2	26.25	7.42
12	"	25.0	+	1.78	0.23	19.1	25.69	7.45
13	"	12.5	+	1.61	0.23	17.5	25.90	7.42
14	"	12.5	+	1.67	0.24	19.9	25.74	7.45
15	"	6.25	+	1.68	0.35	29.1	25.40	7.45
16	"	6.25	+	1.66	0.32	24.3	25.57	7.42
17	"	2.50	+	1.93	0.52	37.4	24.98	7.40
18	"	2.50	+	2.01	0.52	37.4	24.95	7.40
19	"	0.25	+	2.48	0.95	72.2	24.10	7.42
20	"	0.25	+	2.48	1.03	78.3	24.03	7.42
1	96	25.0	-	1.20	0.18	10.3	26.74	7.32
2	"	25.0	-	1.20	0.16	11.5	26.65	7.40
3	"	12.5	-	1.01	0.19	13.7	26.72	7.40
4	"	12.5	-	1.00	0.19	13.7	26.73	7.40
5	"	6.25	-	0.83	0.21	16.4	26.89	7.40
6	"	6.25	-	0.85	0.23	16.6	26.78	7.40
7	"	2.50	-	0.71	0.29	20.9	26.81	7.40
8	"	2.50	-	0.71	0.24	17.3	26.89	7.40
9	"	0.25	-	0.38	0.19	13.7	27.00	7.40
10	"	0.25	-	0.50	0.19	13.7	27.63	7.40
11	"	25.0	+	1.61	0.16	9.2	26.40	7.35
12	"	25.0	+	1.58	0.15	10.8	26.34	7.40
13	"	12.5	+	1.56	0.19	14.4	26.31	7.42
14	"	12.5	+	1.60	0.23	16.6	25.95	7.40
15	"	6.25	+	1.60	0.32	24.3	25.46	7.42
16	"	6.25	+	1.58	0.29	21.5	25.78	7.41
17	"	2.50	+	1.79	0.45	22.4	25.23	7.40
18	"	2.50	+	1.78	0.47	33.8	25.19	7.40
19	"	0.25	+	2.45	0.94	69.7	24.74	7.40
20	"	0.25	+	2.43	1.03	74.2	24.11	7.40

Table 4 cont'd...

Expt. No.	Duration (hrs.)	Initial Concentrations $[Ca^{++}]$ mM	Initial Concentrations $[P]$ mM	Initial Concentrations $[CO_2]$ mM	Final Concentrations $[Ca^{++}]$ mM	Final Concentrations $[P]$ mM	Final Concentrations $[CO_2^-]$ mM	Final Concentrations $[PO_4^{=}]$ Mx10^{-10}	pK_c $[Ca^{++}]^3$ $[PO_4^{=}]^2$	pH
5SD1	4	-	-	16.6	1.55	0.39	32.0	3.82	27.27	6.78
2	4	-	-	"	1.50	0.38	38.0	3.72	27.33	6.79
3	4	-	-	"	1.45	0.39	25.0	3.82	27.35	6.78
1	6	-	-	"	1.73	0.46	26.9	4.51	26.98	6.75
2	6	-	-	"	1.58	0.46	33.2	4.51	26.06	6.80
3	6	-	-	"	1.55	0.50	26.6	4.90	27.05	6.80
6SD1	4	-	-	"	0.84	0.37	19.4	10.4	27.19	7.13
2	4	-	-	"	0.80	0.38	21.5	14.8	26.95	7.21
3	4	-	-	"	0.90	0.45	21.7	17.6	26.65	7.22
1	6	-	-	"	0.96	0.47	23.8	13.2	26.81	7.13
2	6	-	-	"	0.85	0.45	22.0	14.9	26.87	7.15
3	6	-	-	"	0.80	0.42	19.8	16.4	26.86	7.20
4	4	-	-	"	0.80	0.55	19.4	21.5	26.65	7.20
7SD1	4	-	-	"	0.65	0.33	16.2	12.9	27.34	7.22
2	4	-	-	"	0.58	0.36	21.1	19.1	27.14	7.33
3	4	-	-	"	0.55	0.36	19.4	25.9	26.95	7.37
1	6	-	-	"	0.95	0.36	16.7	10.1	27.06	7.09
2	6	-	-	"	0.70	0.36	20.4	14.0	27.18	7.20
3	6	-	-	"	0.70	0.38	17.9	20.1	26.86	7.28
9SD1	4	-	-	33.2	1.20	0.61	40.8	5.00	27.36	6.75
3	4	-	-	"	1.13	0.65	43.2	6.37	27.23	6.77
4	4	-	-	"	1.00	0.62	45.7	6.08	27.04	6.78
1	6	-	-	"	1.35	0.62	43.4	4.15	27.37	6.68
3	6	-	-	"	1.08	0.59	43.1	3.95	27.71	6.71
10SD1	4	-	-	8.4	1.30	0.31	17.6	2.08	28.02	6.70
2	4	-	-	"	1.16	0.31	17.0	2.08	28.17	6.72
3	4	-	-	"	1.18	0.30	18.4	2.01	28.18	6.70
1	6	-	-	"	1.71	0.39	18.1	2.61	27.47	6.70
2	6	-	-	"	1.40	0.36	17.8	2.41	27.80	6.70
3	6	-	-	"	1.16	0.34	16.6	2.28	28.09	6.70
12SD1	4	-	-	33.2	1.05	0.65	42.8	6.37	27.33	6.80
2	4	-	-	"	1.03	0.67	42.7	6.57	27.33	6.80
3	4	-	-	"	1.05	0.63	41.0	6.17	27.35	6.79
4	4	-	-	"	1.03	0.65	41.3	6.37	27.35	6.80
1	6	-	-	"	1.09	0.69	41.0	6.76	27.23	6.80
2	6	-	-	"	1.03	0.70	42.0	6.86	27.29	6.82
3	6	-	-	"	1.03	0.69	41.0	6.76	27.30	6.82
4	6	-	-	"	1.09	0.67	39.8	6.57	27.25	6.82
15SD1	4	-	-	8.3	1.23	0.35	14.5	3.43	27.66	6.80
2	4	-	-	"	1.08	0.29	17.2	2.84	27.99	6.80
1	6	-	-	"	1.08	0.34	16.6	3.33	27.85	6.80
2	6	-	-	"	1.19	0.29	15.2	4.06	27.55	6.80
16SD1	4	-	-	16.6	1.13	0.42	21.0	4.12	27.57	6.78
2	4	-	-	"	1.11	0.38	21.9	3.72	27.72	6.80
3	4	-	-	"	1.10	0.38	21.2	3.72	27.74	6.80
4	4	-	-	"	0.94	0.38	21.0	3.72	27.99	6.80
1	6	-	-	"	1.12	0.45	21.1	4.41	27.57	6.76
2	6	-	-	"	1.09	0.42	21.1	4.12	27.66	6.80
3	6	-	-	"	1.01	0.43	14.1	4.21	27.74	6.79
4	6	-	-	"	1.00	0.43	22.6	4.21	27.74	6.79
17SD1	4	-	-	"	0.70	0.40	22.1	15.6	27.08	7.16
2	4	-	-	"	0.61	0.36	19.8	12.2	27.47	7.15
3	4	-	-	"	0.60	0.34	22.3	13.3	27.42	7.16
4	4	-	-	"	0.68	0.32	22.1	9.0	27.60	7.13
1	6	-	-	"	0.70	0.45	25.4	12.9	27.24	7.10
2	6	-	-	"	0.70	0.45	22.1	15.6	27.08	7.15
3	6	-	-	"	0.71	0.44	21.8	12.3	27.27	7.13
4	6	-	-	"	0.70	0.41	20.5	13.9	27.18	7.15
18SD1	4	-	-	"	0.60	0.36	19.3	19.1	27.10	7.30
2	4	-	-	"	0.46	0.31	18.9	16.4	27.58	7.28
3	4	-	-	"	0.49	0.32	18.9	17.0	27.47	7.27
4	4	-	-	"	0.50	0.34	19.2	15.6	27.52	7.25
1	6	-	-	"	0.61	0.38	19.0	20.1	27.04	7.30
2	6	-	-	"	0.48	0.38	19.8	20.1	27.35	7.28
3	6	-	-	"	0.55	0.36	19.6	19.1	27.22	7.30
4	6	-	-	"	0.51	0.38	19.3	20.1	27.27	7.30
19SD1	4	-	-	14.5	1.38	0.39	18.0	3.82	27.42	6.78
2	4	-	-	"	1.20	0.35	17.7	3.43	27.69	6.80
3	4	-	-	"	1.25	0.37	18.5	3.63	27.59	6.80
4	4	-	-	"	1.23	0.34	19.3	3.33	27.69	6.80
1	6	-	-	"	1.66	0.46	21.3	4.51	27.03	6.78
2	6	-	-	"	1.35	0.41	21.1	4.02	27.40	6.80
3	6	-	-	"	1.36	0.41	20.8	4.02	27.39	6.80
4	6	-	-	"	1.44	0.41	22.1	4.02	27.32	6.80
20SD1	4	-	-	12.5	1.58	0.39	15.3	3.82	27.24	6.80
2	4	-	-	"	1.49	0.38	17.4	3.72	27.34	6.81
3	4	-	-	"	1.44	0.38	17.4	3.72	27.39	6.80
4	4	-	-	"	1.50	0.38	17.4	3.72	27.33	6.80
1	6	-	-	"	1.73	0.41	18.7	4.02	27.08	6.78
2	6	-	-	"	1.63	0.39	18.1	3.82	27.20	6.80
3	6	-	-	"	1.56	0.39	17.8	3.82	27.26	6.80
4	6	-	-	"	1.60	0.39	18.7	3.82	27.22	6.78

Table 5. **Equilibration studies with human adult bone in synthetic ultrafiltrate of plasma - 4 to 6 hour studies.**

Expt. No.	Duration hrs.)	Initial Concentrations $[Ca^{++}]$ mM	$[P]$ mM	$[CO_2]$ mM	Final Concentrations $[Ca^{++}]$ mM	$[P]$ mM	$[CO_2^{=}]$ mM.	$[PO_4^{\equiv}]$ $Mx10^{-10}$	pK_c $[Ca^{++}]^3$ $[PO_4^{\equiv}]^2$
1.1	24	-	-	14.5	1.06	0.25	28.10	18.0	26.41
1.2	24	-	-	14.5	0.60	0.34	28.60	24.5	26.89
2.1	24	-	-	14.5	1.38	0.42	28.76	54.6	25.11
2.2	24	-	-	14.5	1.21	0.56	31.65	72.8	25.03
4.1	26	0.45	0.29	16.6	1.84	0.41	25.40	53.3	24.75
4.2	26	0.45	0.29	16.6	1.10	0.35	19.60	18.6	26.34
4.1	30	0.45	0.29	16.6	1.17	0.49	29.00	63.7	24.70
4.2	30	0.45	0.29	16.6	1.30	0.40	19.00	15.9	26.27
5.1	24	0.45	0.29	16.6	2.25	0.45	26.40	4.4	26.75
5.2	24	0.45	0.29	16.6	3.08	0.46	25.80	3.3	26.51
6.1 *	24	2.50	0.81	16.6	2.00	0.35	27.10	25.2	25.29
6.2 *	24	2.50	0.81	16.6	2.03	0.31	27.70	25.2	25.38
7.1	24	0.50	0.29	16.6	1.95	0.43	26.21	4.2	26.88
7.2	24	0.50	0.29	16.6	1.94	0.41	25.48	4.04	26.93
8.1	24	0.50	0.29	16.6	2.48	0.40	25.00	3.5	26.76
8.2	24	0.50	0.29	16.6	3.55	0.49	25.20	3.3	26.71
8.1	28	0.50	0.29	16.6	2.20	0.62	25.31	17.4	25.49
8.2	28	0.50	0.29	16.6	2.18	0.65	25.30	13.4	25.75
9.1	24	0.50	0.29	16.6	1.84	0.45	26.45	4.2	26.95
9.2	24	0.50	0.29	16.6	1.79	0.32	27.14	3.1	27.29
10.1	31	-	-	16.6	1.50	0.48	26.20	13.4	26.22
10.2	31	-	-	16.6	1.45	0.44	26.00	12.3	26.34

Table 6. **Equilibration studies with human adult bone in synthetic ultrafiltrate of plasma - 24 to 31 hour studies.**
(* Equilibration from supersaturation).

Expt. No.	Ionic Strength	$[Ca^{++}]$ Mx10^{-3}	$[P]$ Mx10^{-3}	$[CO_2]$ Mx10^{-3}	$[PO_4^{\equiv}]$ Mx10^{-9}	$[CO_3^{=}]$ Mx10^{-5}	$pK_c[Ca^{++}]^3_{\cdot}[PO_4^{\equiv}]^2$	$pK_c[Ca^{++}]_{\cdot}[CO_3^{=}]$	$pK_{sp}[Ca^{++}]^3_{\cdot}[PO_4^{\equiv}]^2$	$pK_{sp}[Ca^{++}][CO_3^{=}]$	pH
12LR/1	0.16	0.96	0.34	9.0	2.11	1.17	26.41	7.95	30.19	8.97	7.35
2	"	0.35	0.35	9.0	2.52	1.32	26.32	7.92	30.10	8.94	7.40
3	"	0.89	0.35	8.5	2.52	1.24	26.36	7.96	30.14	8.98	7.40
4	"	0.91	0.34	8.5	2.45	1.24	26.34	7.95	30.12	8.97	7.40
5	0.17	0.70	0.47	17.5	4.47	3.29	26.16	7.64	30.01	8.67	7.50
6	"	0.68	0.47	18.0	4.47	3.39	26.20	7.64	30.05	8.67	7.50
7	"	0.68	0.47	18.0	4.47	3.39	26.20	7.64	30.05	8.67	7.50
8	"	0.70	0.45	18.0	4.28	3.39	26.20	7.62	30.05	8.65	7.50
9	0.18	0.56	0.58	25.5	6.38	5.36	26.15	7.52	30.05	8.57	7.55
10	"	0.55	0.58	26.5	6.38	5.57	26.17	7.51	30.07	8.56	7.55
11	"	0.60	0.55	27.0	6.05	5.66	26.10	7.47	30.00	8.52	7.55
12	"	0.58	0.55	27.0	6.05	5.66	26.14	7.48	30.04	8.53	7.55
13	0.19	0.41	0.65	39.5	8.45	9.35	26.30	7.42	30.28	8.48	7.60
14	"	0.46	0.69	39.5	8.97	9.35	26.11	7.37	30.29	8.43	7.60
15	"	0.50	0.68	39.5	8.84	9.35	26.01	7.33	29.99	8.39	7.60
16	"	0.46	0.68	39.5	8.84	9.35	26.12	7.37	29.10	8.43	7.60
16LR/1	0.15	1.18	0.32	8.5	1.71	1.02	26.43	7.96	30.18	8.96	7.32
2	"	1.10	0.13	8.0	2.20	1.17	26.19	7.89	29.94	8.89	7.40
3	"	1.06	0.29	9.5	1.90	0.98	26.37	7.98	30.12	8.98	7.35
4	"	1.10	0.27	7.5	1.78	0.98	26.37	7.97	30.12	8.97	7.35
5	"	1.05	0.29	7.5	1.89	0.98	26.38	7.99	30.13	8.99	7.35
6	"	0.73	0.38	15.5	3.42	2.56	26.34	7.73	30.09	8.73	7.45
7	"	0.59	0.45	16.5	3.98	2.64	26.48	7.81	30.23	8.81	7.45
8	"	0.58	0.45	15.5	3.98	2.56	26.51	7.83	30.26	8.83	7.45
9	"	0.75	0.44	16.0	2.82	2.64	26.40	7.70	30.21	8.70	7.45
10	"	0.73	0.40	16.0	3.55	2.64	26.29	7.71	30.04	8.71	7.45
11	"	0.74	0.45	23.5	3.97	4.43	26.19	7.48	29.94	8.48	7.50
12	"	0.58	0.48	24.5	4.25	4.61	26.45	7.57	30.21	8.57	7.50
13	"	0.68	0.55	24.0	4.82	4.52	26.14	7.51	29.89	8.51	7.50
14	"	0.61	0.48	24.5	4.25	4.61	26.39	7.55	30.14	8.55	7.50
15	"	0.65	0.52	24.5	4.55	4.61	26.25	7.52	30.00	8.52	7.50
16	"	0.49	0.61	31.5	7.35	6.93	26.36	7.47	30.11	8.07	7.57
17	"	0.49	0.58	33.0	6.95	6.93	26.25	7.47	30.00	8.47	7.55
18	"	0.46	0.61	31.5	7.40	6.61	26.27	7.52	30.02	8.52	7.55
19	"	0.45	0.78	32.0	9.31	7.04	26.10	7.50	29.85	8.50	7.57
20	"	0.38	0.84	32.0	10.10	7.20	26.25	7.56	30.00	8.56	7.58

Table 7. **Equilibration studies in tris buffer with added bicarbonate. Table of final concentrations and ion products.**

Expt. No.	Ionic Strength	Initial Concentrations	Final Concentrations $[Ca^{++}]$ $Mx10^{-3}$	$[P]$ $Mx10^{-3}$	$[PO_4^{\equiv}]$ $Mx10^{-10}$	$pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$	$pK_{sp}[Ca^{++}]^3[PO_4^{\equiv}]^2$	pH
9DP/1	0.01	–	0.40	0.10	17.00	27.73	29.07	7.70
2	"	–	0.40	0.11	24.20	27.43	28.77	7.80
3	"	*	1.39	0.06	4.32	27.30	28.64	7.40
4	"	*	1.34	0.06	4.32	27.25	28.59	7.40
5	0.05	–	0.74	0.13	12.40	27.20	29.80	7.50
6	"	–	0.73	0.11	10.50	27.37	29.97	7.50
7	"	*	1.67	0.06	4.32	27.06	29.66	7.40
8	"	*	1.57	0.08	5.44	26.73	29.33	7.38
9	0.10	–	1.01	0.16	13.10	26.75	30.07	7.45
10	"	–	0.88	0.15	12.30	26.99	30.31	7.45
11	"	*	1.74	0.11	7.48	26.53	29.85	7.38
12	"	*	1.78	0.08	5.44	26.78	30.10	7.38
13	0.20	–	1.17	0.19	12.90	26.70	30.92	7.38
14	"	–	1.12	0.19	13.70	26.58	30.60	7.40
15	"	*	2.07	0.11	5.83	26.52	30.54	7.30
16	"	*	2.07	0.13	6.89	26.38	30.40	7.30
17	0.30	–	1.33	0.23	11.50	26.51	30.92	7.28
18	"	–	1.40	0.24	12.70	26.36	30.77	7.30
19	"	*	2.07	0.18	9.54	26.09	30.50	7.30
20	"	*	2.16	0.16	8.48	26.54	30.55	7.30
21	0.45	–	1.40	0.27	12.20	25.89	30.56	7.25
22	"	–	1.53	0.29	15.40	26.07	30.74	7.30
23	"	*	2.23	0.23	11.30	25.85	30.52	7.28
24	"	*	2.30	0.21	10.50	25.87	30.54	7.57

Table 8. The effect of ionic strength on bone equilibration studies. (Initial concentrations: Ca nil, P nil; * Ca 2mM, P 0.26mM).

Expt. No.	Duration (hrs.)	Initial Concentrations $[Ca^{++}]$ mM	Initial Concentrations $[P]$ mM	Initial Concentrations $[CO_2]$ mM	Final Concentrations $[Ca^{++}]$ mM	Final Concentrations $[P]$ mM	Final Concentrations $[CO_2]$ mM	Final Concentrations $[PO_4^{\equiv}]$ Mx10^{-10}	Final Concentrations $[CO_3^{=}]$ Mx10^{-6}	pK_c $[Ca^{++}]^3$ $[PO_4^{\equiv}]^2$	pK_c $[Ca^{++}]$ $[CO_3^{=}]$	pH
1	24	–	–	–	1.25	0.21	2.50	15.2	3.6	26.34	8.38	7.40
2	"	–	–	–	1.23	0.19	2.50	15.8	4.1	26.33	8.29	7.45
3	"	–	–	10	0.44	0.29	7.50	27.9	14.1	27.19	8.21	7.50
4	"	–	–	10	0.44	0.32	7.50	30.8	14.1	27.10	8.21	7.50
5	"	–	–	20	0.25	0.44	16.50	57.2	39.6	27.31	8.01	7.60
6	"	–	–	20	0.25	0.44	16.50	57.2	39.6	27.31	8.01	7.60
7	"	–	–	30	0.20	0.53	23.50	90.0	68.0	27.19	7.87	7.70
8	"	–	–	30	0.20	0.58	24.50	98.5	71.5	27.14	7.85	7.70
9	"	–	–	40	0.15	0.65	32.00	130.0	103.0	27.29	7.81	7.75
0	"	–	–	40	0.05	0.65	33.00	130.0	106.0	27.68	8.28	7.75
1	"	4.0	1.94	–	2.15	0.29	1.50	10.9	1.7	25.93	8.45	7.28
2	"	"	"	–	2.30	0.32	0.50	12.5	0.5	25.72	8.94	7.28
3	"	"	"	10	1.16	0.60	5.50	43.3	8.0	25.11	7.89	7.40
4	"	"	"	10	1.16	0.58	5.50	41.8	8.0	25.15	7.89	7.40
5	"	"	"	20	1.15	0.60	14.50	57.6	24.5	25.42	7.55	7.48
6	"	"	"	20	0.90	0.52	14.50	50.0	25.4	25.32	7.64	7.48
7	"	"	"	30	0.65	0.62	22.50	80.5	50.6	25.75	7.48	7.58
8	"	"	"	30	0.60	0.62	22.50	80.5	50.6	25.70	7.52	7.58
9	"	"	"	40	0.50	0.74	32.00	96.0	83.2	25.94	7.38	7.64
0	"	"	"	40	0.40	0.73	32.30	95.0	83.8	26.24	7.47	7.64
1	48	–	–	–	1.25	0.19	2.50	13.7	3.7	26.44	8.34	7.40
2	"	–	–	–	1.20	0.18	2.50	14.9	4.1	26.42	8.30	7.45
3	"	–	–	10	0.73	0.32	7.50	30.7	14.3	26.44	7.98	7.51
4	"	–	–	10	0.70	0.32	8.00	30.7	15.6	26.49	7.96	7.52
5	"	–	–	20	0.35	0.47	15.00	61.1	36.0	26.80	7.90	7.61
6	"	–	–	20	0.30	0.50	15.50	65.0	38.0	26.93	7.94	7.62
7	"	–	–	30	0.35	0.61	22.50	104.0	67.5	26.34	7.63	7.71
8	"	–	–	30	0.30	0.63	23.50	107.0	72.7	26.51	7.66	7.72
9	"	–	–	40	0.28	0.77	31.00	169.0	112.0	26.19	7.50	7.80
0	"	–	–	40	0.25	0.74	32.00	163.0	115.0	26.60	7.50	7.80
1	"	4.0	1.94	–	2.08	0.19	2.00	7.4	2.2	26.31	8.34	7.28
2	"	"	"	–	2.20	0.24	1.50	9.4	1.7	26.03	8.44	7.28
3	"	"	"	10	1.15	0.31	6.00	22.3	8.8	25.12	8.00	7.40
4	"	"	"	10	1.15	0.29	6.00	20.9	8.8	25.18	8.00	7.40
5	"	"	"	20	0.80	0.45	13.00	43.2	24.4	26.02	7.75	7.50
6	"	"	"	20	0.78	0.42	13.00	40.3	24.4	26.11	7.72	7.50
7	"	"	"	30	0.45	0.55	21.00	71.5	51.5	26.33	7.64	7.62
8	"	"	"	30	0.43	0.59	21.00	76.7	51.5	26.34	7.66	7.62
9	"	"	"	40	0.43	0.68	29.50	116.0	91.5	25.98	7.41	7.82
0	"	"	"	40	0.43	0.68	31.00	116.0	96.0	25.98	7.38	7.72
1	72	–	–	–	1.35	0.18	2.00	13.0	2.8	26.38	8.42	7.38
2	"	–	–	–	1.35	0.16	2.00	11.5	3.0	26.51	8.40	7.42
3	"	–	–	10	0.63	0.36	6.50	34.6	12.2	26.32	8.11	7.50
4	"	–	–	10	0.70	0.32	6.50	30.7	12.7	26.49	8.05	7.53
5	"	–	–	20	0.45	0.45	17.75	58.50	33.0	26.51	7.83	7.61
6	"	–	–	20	0.40	0.50	17.75	65.0	33.0	26.57	7.91	7.61
7	"	–	–	30	0.30	0.63	25.00	107.0	63.6	26.51	7.72	7.72
8	"	–	–	30	0.25	0.65	22.00	111.0	68.2	26.73	7.77	7.72
9	"	–	–	40	0.18	0.81	29.00	203.0	116.0	26.65	7.68	7.84
0	"	–	–	40	0.23	0.80	30.00	117.0	114.0	26.45	7.58	7.82
1	"	4.0	1.94	–	2.08	0.18	2.50	7.0	2.8	26.35	8.24	7.28
2	"	"	"	–	2.00	0.16	1.50	6.2	1.7	26.51	8.48	7.28
3	"	"	"	10	1.20	0.26	5.50	18.7	9.1	26.22	7.96	7.41
4	"	"	"	10	1.08	0.26	5.50	21.6	9.1	26.23	8.01	7.45
5	"	"	"	20	0.78	0.40	12.00	38.4	24.0	26.17	7.23	7.53
6	"	"	"	20	0.78	0.40	12.50	38.4	25.0	26.19	7.71	7.53
7	"	"	"	30	0.48	0.53	19.00	90.0	53.2	26.05	7.59	7.68
8	"	"	"	30	0.50	0.55	20.00	93.5	96.0	25.96	7.55	7.68
9	"	"	"	40	0.33	0.68	28.00	150.0	101.0	26.09	7.48	7.80
0	"	"	"	40	0.33	0.68	28.75	150.0	104.0	26.09	7.46	7.80

Table 9. Equilibration studies with tris buffer and added bicarbonate.

Expt. No.	Duration (hrs.)	$[Ca^{++}]$ $Mx10^{-3}$	$[P]$ $Mx10^{-3}$	$[CO_2]$ $Mx10^{-3}$	$[PO_4^{\equiv}]$ $Mx10^{-9}$	$[CO_3^{=}]$ $Mx10^{-5}$	$pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$	$pK_c[Ca^{++}][CO_3^{=}]$	pH
5LS/1	24	0.66	0.39	6.50	3.90	1.34	26.36	8.05	7.52
2	"	0.64	0.40	7.50	4.40	1.50	26.29	8.02	7.55
3	"	0.46	0.53	11.00	6.36	2.53	26.41	7.94	7.60
4	"	0.46	0.60	12.50	7.20	2.88	26.30	7.88	7.60
5	"	0.38	0.60	15.50	8.11	3.72	26.44	7.85	7.62
1	48	0.58	0.35	5.25	4.20	1.16	26.32	8.27	7.60
2	"	0.55	0.42	7.00	5.88	1.72	26.24	8.02	7.63
3	"	0.46	0.52	9.50	8.32	2.61	26.17	7.92	7.68
4	"	0.43	0.58	11.75	9.86	3.41	26.11	7.83	7.70
5	"	0.34	0.65	14.25	11.10	4.28	26.32	7.84	7.71
1	72	0.58	0.32	6.50	3.52	1.30	26.62	8.12	7.55
2	"	0.48	0.39	8.00	4.68	1.76	26.61	8.07	7.59
3	"	0.45	0.47	10.25	6.35	2.56	26.44	7.94	7.62
4	"	0.38	0.55	11.75	9.35	3.41	26.34	7.89	7.70
5	"	0.35	0.65	14.50	11.10	4.35	26.28	7.82	7.71

Table 10. **Equilibration studies in tris bicarbonate buffer at high solid:solution ratios. Table of final concentrations and ion products.**

Expt. No.	Duration (hrs.)	$[Ca^{++}]$ $Mx10^{-3}$	$[P]$ $Mx10^{-3}$	$[CO_2]$ $Mx10^{-3}$	$[PO_4^{\equiv}]$ $Mx10^{-9}$	$[CO_3^{=}]$ $Mx10^{-5}$	$pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$	$pK_c[Ca^{++}][CO_3^{=}]$	pH
8DP/1	48	2.00	0.79	33.00	4.50	3.96	24.79	7.10	7.32
2	"	1.98	0.81	34.00	4.62	4.08	24.78	7.09	7.32
3	"	1.83	0.74	30.50	2.89	2.71	25.29	7.30	7.20
4	"	1.71	0.71	28.50	3.20	2.85	25.29	7.31	7.25
5	"	1.58	0.71	27.00	3.76	3.08	25.25	7.31	7.30
6	"	1.62	0.71	27.00	3.76	3.08	25.22	7.30	7.30
7	"	1.55	0.69	26.00	4.97	3.80	25.00	7.23	7.40
8	"	1.46	0.65	29.50	6.18	5.55	24.92	7.09	7.50
1	72	1.69	0.71	29.00	2.77	2.58	25.49	7.36	7.20
2	"	1.73	0.74	29.25	3.33	2.93	25.24	7.29	7.25
3	"	1.49	0.66	27.25	4.49	3.82	25.18	7.24	7.38
4	"	1.46	0.68	26.25	5.64	4.33	25.00	7.20	7.45
5	"	1.33	0.63	23.50	8.19	5.64	24.80	7.18	7.60
6	"	1.35	0.66	24.50	8.58	5.88	24.74	7.10	7.60
7	"	1.30	0.65	23.75	9.10	5.94	24.74	7.11	7.62
8	"	1.22	0.65	23.25	11.70	7.21	24.60	7.06	7.72
8LS/1	24	1.55	0.74	12.00	4.14	1.44	25.20	7.65	7.32
2	"	1.33	0.66	13.50	5.28	2.16	25.18	7.54	7.45
3	"	1.25	0.55	15.50	5.50	2.95	25.23	7.43	7.52
4	"	1.18	0.63	17.00	6.93	3.40	25.10	7.40	7.55
1	48	1.40	0.64	10.50	7.04	2.10	24.95	7.53	7.55
2	"	1.23	0.50	12.00	6.50	2.76	25.11	7.47	7.60
3	"	1.10	0.47	14.00	6.82	3.64	25.21	7.40	7.65
4	"	1.03	0.44	15.00	6.38	4.03	25.07	7.38	7.65
1	72	1.56	0.71	14.25	4.26	1.92	25.16	7.52	7.35
2	"	1.19	0.77	16.75	10.00	3.85	24.77	7.34	7.60
3	"	1.10	0.76	17.25	11.00	4.49	24.79	7.31	7.65
4	"	1.18	0.87	20.50	8.00	3.69	24.98	7.36	7.50

Table 11. **Equilibration studies in human serum. All concentrations and products refer to total measured Ca, P and CO_2 including any plasma bound fractions.**

Expt. No.	Total Ca $Mx10^{-3}$	% Ca^{++}	$[Ca^{++}]$ $Mx10^{-3}$	$[P]$ $Mx10^{-3}$	$[PO_4^{\equiv}]$ $Mx10^{-9}$	$pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$	$[PO_4^{\equiv}]'$ $Mx10^{-9}$	$pK_c[Ca^{++}]^3[PO_4^{\equiv}]'^2$	pH
8DP/1	1.69	64.0	1.08	0.71	2.77	26.02	1.47	26.59	7.20
8DP/2	1.73	63.2	1.09	0.74	3.33	25.84	1.77	26.41	7.25
8LS/1	1.56	61.5	0.96	0.71	4.26	25.79	2.26	26.36	7.35
8DP/3	1.49	61.0	0.91	0.66	4.49	25.81	2.38	26.39	7.38
8DP/4	1.46	60.0	0.88	0.68	5.64	25.66	2.99	26.23	7.45
8LS/4	1.18	59.0	0.70	0.87	8.00	25.66	4.25	26.23	7.50
8LS/2	1.19	57.0	0.68	0.77	10.00	25.50	5.30	26.07	7.60
8DP/5	1.33	57.0	0.76	0.63	8.19	25.53	4.34	26.10	7.60
8DP/6	1.35	57.0	0.77	0.66	8.58	25.47	4.55	26.04	7.60
8DP/7	1.30	56.6	0.74	0.65	9.10	25.47	4.82	26.04	7.62
8LS/3	1.10	56.0	0.62	0.76	11.00	25.54	5.82	26.43	7.65
8DP/8	1.22	54.7	0.67	0.65	11.70	25.35	6.20	25.95	7.72
						Arithmetic mean 25.61		Arithmetic mean 26.17	

Table 12. Equilibration studies in human serum. Ca^{++} represents calculated non-protein-bound calcium and $PO_4^{\equiv}$ the total trivalent phosphate assuming all measured P is non-associated. $PO_4^{\equiv}{}'$ represents the non-associated trivalent phosphate concentration assuming 47% association (after Walser).

Expt. No.	Final Concentrations $[Ca^{++}]$ $Mx10^{-3}$	$[P]$ $Mx10^{-3}$	$[PO_4^{\#}]$ $Mx10^{-10}$	$pK_c[Ca^{++}]^3[PO_4^{\#}]^2$	pH	
Equilibrations with child bone.						
4LR/4	0.95	0.38	31.90	26.06	7.45	
5	1.06	0.41	29.50	25.98	7.40	
6	1.05	0.41	34.40	25.86	7.45	
5LR/1	1.76	0.45	6.30	26.66	6.90	
2	1.85	0.44	5.28	26.75	6.85	
3	1.40	0.40	11.20	26.46	7.10	
4	1.56	0.42	11.80	26.28	7.08	
6LR/1	1.75	0.40	5.60	26.77	6.90	
2	1.63	0.34	3.40	27.30	6.80	
3	1.00	0.35	25.20	26.20	7.40	
4	0.93	0.35	25.20	26.22	7.40	
1	2.13	0.37	3.03	27.05	6.75	At 40 hours.
2	2.08	0.27	1.49	27.67	6.65	"
3	1.08	0.32	23.00	26.18	7.40	"
4	1.13	0.34	23.50	26.10	7.38	"
8LR/1	1.70	0.44	10.60	26.26	7.05	
2	2.11	0.42	9.24	26.14	7.03	
3	1.66	0.37	14.40	26.02	7.20	
4	1.54	0.40	15.60	26.05	7.20	
5	1.28	0.36	21.60	26.01	7.34	
6	1.31	0.37	26.60	26.80	7.40	
9LR/1	4.00	0.68	2.86	26.28	6.58	
2	3.80	0.69	2.97	26.31	6.59	
3	2.78	0.56	5.60	26.17	6.80	
4	2.95	0.56	5.83	26.13	6.79	
Equilibrations with rat bone.						
4LR/7	0.90	0.66	50.20	25.74	7.42	
8	0.90	0.79	56.90	25.63	7.40	
9	0.85	0.42	33.60	26.16	7.43	
10	0.75	0.44	31.70	26.38	7.40	
8LR/13	2.03	0.87	19.10	25.52	7.08	
14	1.95	0.89	23.10	25.40	7.08	
15	1.69	0.65	21.50	25.65	7.15	
16	1.65	0.88	29.90	25.40	7.20	
17	1.36	0.77	47.70	25.24	7.35	
18	1.40	0.87	51.80	25.13	7.36	
9LR/13	3.90	1.32	5.94	26.68	6.60	
14	3.75	1.16	5.67	25.77	6.60	
15	3.20	1.13	11.30	25.38	6.80	
16	2.95	1.03	10.30	25.57	6.80	
17	2.20	0.94	18.80	25.43	7.00	
18	2.53	0.97	19.40	25.22	7.00	
11LR/ 9	1.05	2.45	49.00	25.56	7.35	
10	1.10	2.65	52.70	25.43	7.35	
11	2.24	3.55	32.20	24.91	7.10	*
12	2.29	3.55	32.20	24.88	7.10	*
13	1.18	1.80	39.40	25.60	7.38	
14	1.33	1.95	45.40	25.31	7.40	
15	2.25	2.55	31.90	24.94	7.20	*
16	2.29	3.25	38.90	24.74	7.18	*

Table 13. **24-hour equilibration studies with child and rat bone powder. All experiments from undersaturation except where indicated.**

*** (Ca 4mM; P 1.94mM).**

Expt. No.	Final Concentrations $[Ca^{++}]$ $Mx10^{-3}$	$[P]$ $Mx10^{-3}$	$[PO_4^{\equiv}]$ $Mx10^{-10}$	$pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$	pH		
11LR/17	0.74	0.37	26.60	26.61	7.40	Cat.	
18	0.73	0.39	32.80	26.38	7.45	"	
19	1.56	0.35	11.60	26.29	7.15	"	*
20	1.81	0.65	21.50	25.56	7.15	"	*
21	0.93	0.85	47.60	25.74	7.32	Mouse	
22	2.00	1.06	23.30	25.36	7.02	"	*
23	1.58	1.35	33.60	25.33	7.50	Rabbit	
24	1.51	0.34	32.60	25.44	7.50	"	
25	2.31	0.32	23.00	25.19	7.40	"	*
26	2.16	0.29	20.90	25.36	7.40	"	*
27	1.58	0.47	33.80	25.35	7.40	Guinea pig	
28	1.61	0.48	40.30	25.17	7.45	" "	
29	2.25	0.55	23.10	25.22	7.22	" "	*
30	2.71	0.58	22.60	24.99	7.20	" "	*
31	0.60	0.55	21.50	26.00	7.20	Chicken skull	
32	0.56	0.50	19.50	27.19	7.20	" "	
33	1.22	0.52	5.20	27.31	6.80	" "	*
34	1.38	0.58	5.80	27.05	6.80	" "	*
35	1.16	0.11	7.92	27.01	7.40	Chicken humerus	
36	1.95	0.12	4.20	26.89	7.10	" "	*
37	1.01	0.77	55.40	25.50	7.40	Pup	
38	1.05	0.81	58.30	25.40	7.40	"	
39	1.79	0.74	28.90	25.33	7.20	"	*
40	1.78	0.84	32.80	25.21	7.10	"	*
13LR/ 1	0.90	0.48	44.32	25.87	7.48	Cow	
2	0.96	0.50	45.00	25.73	7.48	"	
3	1.68	0.65	34.50	25.24	7.30	"	*
4	1.50	0.45	23.90	25.91	7.30	"	*
5	1.30	0.48	52.50	25.23	7.55	Pig	
6	1.30	0.45	49.50	25.27	7.55	"	
7	2.30	0.55	34.10	24.85	7.35	"	*
8	2.30	0.38	23.60	25.17	7.35	"	*
9	1.19	0.26	21.80	26.09	7.45	Sheep	
10	1.24	0.26	21.80	26.03	7.45	"	
11	1.40	0.35	16.10	26.15	7.25	"	*
12	1.30	0.21	10.50	26.62	7.28	"	*

Table 14. **24-hour equilibration studies with animal bone powder. All experiments from undersaturation except where indicated. * (Ca 4mM; P 1.9mM).**

Expt. No.	Duration (hrs.)	$[Ca^{++}]$ $Mx10^{-3}$	$[P]$ $Mx10^{-3}$	$[CO_2]$ $Mx10^{-3}$	$[PO_4^{\equiv}]$ $Mx10^{-9}$	$[CO_3^{=}]$ $Mx10^{-5}$	$pK_c[Ca^{++}]^3[PO_4^{\equiv}]^2$	$pK_c[Ca^{++}][CO_3^{=}]$	pH	Bone CO_2	Bone Sample
3LS/1	24	1.06	0.26	0.50	0.83	0.040	27.09	9.37	7.15	4.55	Humerus 2
2	"	1.25	0.32	0.30	1.24	0.027	26.52	9.47	7.20	4.12	Sternum 2
3	"	0.90	0.32	0.25	1.02	0.020	27.12	9.74	7.15	3.99	Coracoid 2
4	"	1.01	0.42	0.25	1.34	0.020	26.73	9.66	7.15	3.97	Illium 2
5	"	0.91	0.24	0.25	0.85	0.022	27.26	9.74	7.18	4.10	Skull 3
6	"	1.28	0.16	0.50	0.72	0.050	26.98	9.19	7.25	5.18	Tibia 3
7	"	1.58	0.15	0.40	0.59	0.036	26.86	9.24	7.20	4.52	Sternum 3
8	"	1.18	0.23	0.30	0.89	0.027	26.88	9.50	7.20	5.02	Radius 3

Table 15. **24-hour equilibration studies with bone powder of the domestic fowl.**
Table of final concentrations and ion products from several bone samples of birds 2 and 3.

ABSTRACT

It is generally believed that the mineral portion of the skeleton cannot have a thermodynamic solubility product because of the complex and variable nature of the bone salt. This opinion is examined in the light of our present understanding of bone salt composition and of previous investigations into bone salt solubility.

A simple experimental technique is described for studying the relationship between powdered whole bone and the concentrations of the ions of bone salt in solution.

It is shown that in bicarbonate-free buffer, bone powder establishes an ionic equilibrium in terms of the product $[Ca^{++}]^3[PO_4^{\equiv}]^2$ within 24 hours. For human bone, the pK of the mean of this product is 26.39.

Studies with synthetic ultrafiltrate of plasma suggest that there is a direct relationship between the carbonate and phosphate concentrations in solutions in equilibrium with bone salt. In experiments using tris buffer with added bicarbonate, the bone salt yields constant calcium phosphate ion products within 72 hours, the pK of the mean being 26.30. It has not been possible to demonstrate any calcium carbonate ion product even when very high solid:solution ratios of about 500 gm bone per litre buffer are employed, but a direct relationship between

the carbonate and phosphate concentrations in solution is apparent.

Equilibration studies with bone powder in normal serum suggest that the equilibrium could be operative in vivo provided the recently proposed views of Walser are accepted. Walser maintains that only about 53% of the total serum inorganic phosphate is in the non-associated 'free' form. With this assumption our experiments indicate that the ion product $\left[Ca^{++}\right]^3\left[PO_4^{\equiv}\right]^2$ in serum is not significantly different from that in synthetic media, the pK of the mean being 26.17.

Accepting Walser's views, which are indirectly confirmed by our experiments, it is then shown that bone mineral, by a purely physico-chemical process, can maintain the concentrations of calcium and phosphate found in normal human extracellular fluids in vivo, provided the pH at the phase boundry is about 7.1. This value is roughly mid-way between intracellular and extracellular pH.

Brief studies with bone powder of children and several animal species suggest that there is a relationship between plasma phosphate levels in vivo, equilibrium phosphate concentrations with bone powder in vitro and the carbonate content of the bone. It is suggested that the high plasma phosphate concentrations in

children may be due in part to the low carbonate content of young immature bone mineral.

A series of equilibration studies with bone from different sites in the same skeletons has yielded evidence which is compatible with the view that bone mineral behaves essentially as a calcium phosphate salt with divalent carbonate ion substitution for trivalent phosphate ion at the surfaces of the apatitic crystals. The evidence obtained is in conflict with the two other principal theories, namely that bone salt is a stoichiometric carbonate-apatite, and alternatively that it is a two-phase system of calcium phosphate and calcium carbonate.

There are two principal biological implications of these studies. Firstly, calcium homeostasis can be explained in terms of a physico-chemical equilibrium state between the mineral of the skeleton and its ions in extracellular fluids. Secondly, slight variations in the pH of the bone environment lead primarily to changes in plasma calcium concentration, total inorganic phosphate concentration being a function of the bicarbonate concentration.

It is suggested that parathyroid hormone controls plasma calcium concentration by an effect on cellular metabolism which results in variations in the pH of the tissue fluids at the surface of the bone crystals.