CCXIII. NON-DIFFUSIBLE SERUM-CALCIUM AT VARIOUS CALCIUM, PHOSPHATE AND HYDROGEN ION CONCENTRATIONS.

By RALPH GRAFTON SMITH.

*From the Department of Materia Medica and Therapeutics, University of
Michigan Medical School, Ann Arbor, Michigan.*

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THE study of the diffusibility of blood serum-calcium has been an interesting development in the investigation of calcium metabolism. The finding of Rona and Takahashi [1911], that only a part of the serum-calcium was diffusible through membranes impermeable to protein, has been corroborated by many observers and by various methods. The figures for diffusibility recorded in the literature cover a wide range, but most of them lie between 45 and 70 % of the total serum-calcium. It is generally assumed that the non-diffusible fraction is bound to protein, this being suggested by the removal of a certain fraction of calcium in serum-protein precipitation [Csapo and Faubl, 1924], by the parallelism between the non-diffusible calcium and protein contents of various body fluids [Salvesen and Linder, 1923-24; Liu, 1927], by an apparent binding of a fraction of the calcium when CaCl_2 is added to protein solutions, as shown by diffusion experiments [Loeb, 1928; Marrack and Thacker, 1926], and by conductivity titrations when calcium is added to serum solutions [Shear and Offner, 1931]. Furthermore, when the total serum-calcium concentration is increased the non-diffusible fraction also increases [Loeb and Nichols, 1927; Brull, 1930; Smith and Sternberger, 1932; and Benjamin and Hess, 1933]. It is natural to assume that at least a part of this process is due to a further combination of calcium with serum-protein to preserve the equilibrium between protein-bound calcium and calcium ions.

In contrast to the state of serum-calcium, the inorganic phosphate of mammalian serum is generally agreed to be entirely diffusible or almost so [Rona and Takahashi, 1913; Cushny, 1919-20; Neuhausen and Pincus, 1923; Grollman, 1927; Brull, 1930; Greene and Power, 1931; Benjamin and Hess, 1933]. However, the interesting observation was made by Grollman [1927] by ultrafiltration of pig and dog sera that inorganic phosphate gradually becomes non-diffusible as the serum-calcium is raised above the normal level. He suggested the formation of a colloidal complex of $\text{Ca}_3(\text{PO}_4)_2$ and protein. This finding has since been corroborated by other observers [Brull, 1930; Benjamin and Hess, 1933; Laskowski, 1933].

Thus when calcium is added to serum a part of the added calcium becomes non-diffusible. As Laskowski [1933] has stated, this may be accounted for by two different processes—firstly by a combination of calcium with protein (as occurs in phosphate-free protein solutions [Loeb, 1928]), and secondly by the formation of a calcium phosphate complex with protein (as suggested by the formation of non-diffusible phosphate along with the increase in non-diffusible

calcium). The question naturally arises: Do the two processes occur simultaneously, or is the newly formed non-diffusible calcium at first the result of calcium phosphate formation only until all the free phosphate has been combined in this manner, with a secondary binding of calcium by protein if the calcium concentration is sufficiently increased?

The present paper reports a series of *in vitro* ultrafiltration experiments which indicates the simultaneous occurrence of both processes. Also more direct evidence is presented for the formation of $\text{Ca}_3(\text{PO}_4)_2$. Furthermore, by carrying out experiments in which the effect of changes in hydrogen ion concentration on the diffusibility of serum-calcium was studied, the importance of the phosphate factor in such variations is indicated.

EXPERIMENTAL METHODS.

Blood was obtained from normal dogs by exsanguination under light ether anaesthesia and allowed to clot at room temperature. The serum was separated by centrifuging and was stored in stoppered tubes in the refrigerator, the experiments being carried out within 24 hours after obtaining the blood. The calcium and phosphate concentrations were increased as desired by the addition of small volumes of 10 % CaCl_2 and Na_2HPO_4 solutions. The latter was brought to p_{H} 7.4 with HCl. Variations in hydrogen ion concentration were made by adding 0.25 ml. of NaOH or HCl of suitable strengths to 10 ml. samples of serum. An equivalent volume of saline was added to the control sample in each experiment. Such additions increased the volume of the normal serum by from 1 to 5 %.

5 ml. samples of serum were ultrafiltered through collodion sacs impermeable to protein at 150 mm. Hg pressure by the method of Greenberg and Gunther [1929-30] with minor modifications as previously described [Smith and Sternberger, 1932]. Calcium was determined by the Clark-Collip [1925] modification of the Kramer-Tisdall method, allowing at least 2 hours for the precipitation of calcium oxalate, inorganic phosphate by the method of Fiske and Subbarow [1925], and p_{H} by the colorimetric method of Hastings and Sendroy [1924].

Hydrogen ion concentration experiments were unsatisfactory to some degree in that the p_{H} of the residue and usually of the ultrafiltrate rose above that of the original serum during ultrafiltration, presumably owing to a loss of CO_2 . This was not avoided by connecting the collodion sacs to flasks containing NaHCO_3 in buffer solutions of the desired p_{H} as suggested by Greenberg and Gunther [1929-30]. The p_{H} readings recorded are those of the serum at the beginning of ultrafiltration.

RESULTS.

Calcium and inorganic phosphate concentrations are expressed in mg. per 100 ml. of serum. The diffusible calcium and phosphate were calculated from the ultrafiltrate concentration by making a 5 % correction for protein volume in all experiments as previously described [Smith and Sternberger, 1932].

Variations in calcium and phosphate concentrations.

In Table I are recorded the results of experiments in which the calcium concentration has been gradually increased in separate samples of the same serum. The "increase in non-diffusible Ca and P" (columns (8) and (9)) refers to the increases of these fractions above the level present in the normal serum which is recorded in the first line in each experiment. The Ca : P ratio of these values

Table I. *Effect of increased calcium concentration on diffusibility of serum-calcium and inorganic phosphate.*

Exp. No.	Serum-Ca			Serum-inorganic PO ₄ (P)			Increase in non-diffusible			p _H
	Total	Diffu-	Non-	Total	Diffu-	Non-	Ca	P	Ca:P	
	mg.	sible	diffu-	mg.	sible	diffu-	mg.	mg.		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
I	10.0	6.2	3.8	5.2	5.4	—	—	—	—	7.82
	22.4	12.2	10.2	5.2	3.4	1.8	6.4	1.8	3.6	7.85
	35.0	18.7	16.3	5.2	1.7	3.5	12.5	3.5	3.6	7.85
II	9.7	5.5	4.2	5.6	5.7	—	—	—	—	7.75
	20.9	12.0	8.9	5.6	4.2	1.4	4.7	1.4	3.4	7.85
	31.0	17.6	13.4	5.6	3.0	2.6	9.2	2.6	3.5	7.80
III	11.2	6.5	4.7	11.6	11.6	—	—	—	—	—
	22.8	10.5	12.3	11.6	7.6	4.0	7.6	4.0	1.9	—
IV	9.7	5.6	4.1	5.2	5.2	—	—	—	—	—
	20.8	10.1	10.7	5.2	3.4	2.1	6.6	2.1	3.1	—
	31.8	16.9	14.9	5.2	2.3	2.9	10.8	2.9	3.7	—
V	10.1	6.5	3.6	7.0	7.1	—	—	—	—	7.57
	22.3	13.9	8.4	7.0	5.5	1.5	4.8	1.5	3.2	7.53
	33.0	20.7	12.3	7.0	4.1	2.9	8.7	2.9	3.0	7.63
VI	10.7	5.9	4.8	5.7	5.8	—	—	—	—	7.77
	16.2	7.9	8.3	5.7	4.6	1.1	3.5	1.1	3.2	7.77
	24.0	12.6	11.4	5.7	3.6	2.1	6.6	2.1	3.1	7.80
	29.6	14.3	15.3	5.7	2.2	3.5	10.5	3.5	3.0	7.82
	35.4	17.1	18.3	5.7	1.9	3.8	13.5	3.8	3.6	7.85
VII	10.8	6.9	3.9	9.5	8.8	0.7	—	—	—	—
	69.0	49.0	20.0	9.5	1.6	7.9	16.1	7.2	2.2	—
	143.0	117.0	26.0	9.5	0.7	8.8	22.1	8.1	2.7	—
VIII	10.4	6.0	4.4	7.3	7.7	—	—	—	—	7.77
	46.0	23.5	22.5	7.3	2.1	5.2	18.1	5.2	3.5	7.77
	118.0	90.0	28.0	7.3	0.9	6.4	23.6	6.4	3.7	7.77
IX	9.0	6.0	3.0	5.7	6.3	—	—	—	—	7.72
	26.5	15.2	11.3	5.7	3.1	2.6	8.3	2.6	3.2	7.77
	43.5	29.7	13.8	5.7	2.1	3.6	10.8	3.6	3.0	7.80
	79.5	58.6	20.9	5.7	0.8	4.9	17.9	4.9	3.6	7.80
	127.0	102.0	25.0	5.7	0.4	5.3	22.0	5.3	4.1	7.75

(with one exception which will be mentioned later) is higher than the ratio in such calcium phosphates as CaHPO_4 (Ca: P=1.3) or $\text{Ca}_3(\text{PO}_4)_2$ (Ca: P=1.9). This of course is to be expected, if a combination with protein is responsible for a part of the increase in non-diffusible calcium. The results show further that the two processes occur simultaneously, *i.e.* the phosphate gradually becomes non-diffusible as the calcium concentration is increased, suggesting a combination of calcium and phosphate, yet the Ca: P ratio of the increase in non-diffusible compounds remains between 3 and 4 throughout, indicating, both with small and large increases in calcium concentration, some other combination—presumably with protein. In most cases there is some increase in the ratio with higher calcium concentrations, but even above 100 mg. per 100 ml. as in Exps. VII, VIII and IX this increase is not marked. Thus it is evident that when the greater part of the phosphate has become combined, the calcium-combining power of the protein is also approaching exhaustion.

In the results of Table I there is nothing to suggest the type of calcium phosphate formed apart from its non-diffusibility. However, in Table II which records similar experiments carried out in the presence of excess phosphate

Table II. *Effect of increased calcium concentration on diffusibility of serum-calcium and inorganic phosphate in the presence of excess phosphate.*

Exp. No.	Serum-Ca			Serum-inorganic PO_4 (P)			Increase in non-diffusible			p_{H}
	Total mg.	Diffu- sible mg.	Non- diffu- sible mg.	Total mg.	Diffu- sible mg.	Non- diffu- sible mg.	Ca mg.	P mg.	Ca:P	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
I	10.0	7.0	3.0	7.7	8.0	—	—	—	—	7.77
	33.6	7.2	26.4	30.8	19.1	11.7	23.4	11.7	2.0	7.92
II	9.8	5.8	4.0	7.6	7.9	—	—	—	—	7.73
	40.7	7.6	33.1	30.5	15.5	15.0	29.1	15.0	1.9	7.90
III	9.6	6.0	3.6	5.7	5.7	—	—	—	—	7.72
	38.0	8.7	29.3	26.0	12.6	13.4	25.7	13.4	1.9	7.88
IV	10.0	5.9	4.1	5.4	5.6	—	—	—	—	7.60
	22.0	7.2	14.8	15.4	10.2	5.2	10.7	5.2	2.1	7.65
	36.7	7.7	29.0	25.0	13.4	11.6	24.9	11.6	2.1	7.77
V	9.9	5.3	4.6	6.3	6.4	—	—	—	—	7.67
	35.1	5.1	30.0	38.0	24.1	13.9	25.4	13.9	1.8	7.67
	58.8	9.8	49.0	38.0	14.8	23.2	44.4	23.2	1.9	7.72
	85.0	21.5	63.5	38.0	7.3	30.7	58.9	30.7	1.9	7.77
VI	9.8	5.4	4.4	4.8	4.8	—	—	—	—	7.47
	22.8	9.0	13.8	19.4	14.4	5.0	9.4	5.0	1.9	7.50
	34.2	10.3	23.9	19.4	9.9	9.5	19.5	9.5	2.0	7.50
	57.4	21.1	36.3	19.4	4.7	14.7	31.9	14.7	2.2	7.50
	92.4	41.5	50.9	19.4	2.8	16.6	46.5	16.6	2.8	7.48
VII	9.1	5.7	3.4	4.6	4.6	—	—	—	—	7.52
	20.9	9.3	11.6	18.3	14.1	4.2	8.2	4.2	2.0	7.52
	45.8	16.8	29.0	18.3	7.5	10.8	25.6	10.8	2.4	7.50
	68.8	31.9	36.9	18.3	4.5	13.8	33.5	13.8	2.4	7.53
	114.0	71.2	42.8	18.3	2.1	16.1	39.4	16.1	2.4	7.55

the Ca: P ratio of the newly formed non-diffusible compounds is 1.9 or approximates to that value very closely, *i.e.* to the ratio of these elements in $\text{Ca}_3(\text{PO}_4)_2$. Some variation in this result may be expected in that it involves the combined errors of two calcium and two phosphate determinations and of the ultrafiltration process. In spite of this fact variations from 1.9, which occur in certain cases, are comparatively slight. This ratio is constant, irrespective of the amount of calcium and phosphate added, provided that the free phosphate remains above the level in the normal serum. However, as calcium is added in great excess, as in Exps. VI and VII, the diffusible phosphate falls below the level usually present in the normal serum and the ratio rises, in Exp. VI to 2.4, in Exp. VII to 2.8. Apparently in the presence of excess phosphate calcium combines only with phosphate to form $\text{Ca}_3(\text{PO}_4)_2$ which of course, in turn, may form a colloidal complex with protein as previously suggested. After the excess phosphate has been combined, further additions of calcium combine also with protein as indicated by the rise of the Ca: P ratio above 1.9. Of course such results do not prove that the normal serum-inorganic phosphate forms $\text{Ca}_3(\text{PO}_4)_2$ with added calcium but they suggest this possibility. Further evidence for it is found in Exp. III of Table I in which the serum-inorganic phosphate of this particular dog was definitely higher than that usually found. In this case the added calcium was sufficient to depress the diffusible phosphate only from 11.6 to 7.6 mg. per 100 ml., the latter figure still being a moderately high normal value. The Ca: P ratio of the increase in non-diffusible fractions was 1.9 as in those experiments in which phosphate was actually added to the serum.

Variations in hydrogen ion concentration.

It is known that in normal serum wide variations in p_H cause a great change in the diffusibility of calcium [Loeb, 1928] (see also Table III, Exps. I and II). With regard to changes in the physiological range Greenberg and Gunther [1929-30] have reported that no change in diffusibility occurs between p_H 7 and 8. Similarly Meysenbug *et al.* [1921] found no change between p_H 7.0 and 7.6. More recently Dillman and Visscher [1933] have reviewed the literature on this subject and have reported results contrary to the above findings. The serum-calcium diffusibility at p_H 7.0 (59 %) differed quite markedly from that at p_H 7.6 (45 %). Table III records the results of a series of ultrafiltration experi-

Table III. *Effect of hydrogen ion concentration on diffusibility of normal serum-calcium.*

Exp. No.	Total Ca mg.	Diffusible Ca %	p_H	Exp. No.	Total Ca mg.	Diffusible Ca %	p_H
I	9.1	17.5	10.1	V	9.5	51	8.0
		54	7.6			55	7.9
		106	4.1			55	7.75
II	8.4	16	10.2			56.5	7.55
		26	9.2			55	7.35
		54	7.7			57	7.15
		68	7.0	VI	10.05	61.5	8.15
		72	5.25			63.5	7.85
		85	5.1			62	7.75
III	10.0	59	8.0			62	7.55
		62	7.8			64	7.25
		68.5	7.5			66	7.0
		68	7.4	VII	9.15	54	7.95
		65	7.2			51	7.77
IV	9.95	64	7.95			59	7.60
		64	7.8			64	7.35
		65	7.6			61	7.20
		71	7.3			66	7.05
		69	7.15				
		69	7.05				

Exps. III-VII. Average maximum change in p_H = 0.92.

Average change in calcium diffusibility = 6.7 %.

ments on normal sera, showing some change in diffusibility between p_H 7 and 8. With slight variations in p_H the differences are well within the limits of experimental error, but the general direction of the change is the same in all experiments. On averaging the results of Exps. III-VII it is found that on decreasing the p_H by 1.0 in this range, approximately 7 % more of the total calcium becomes diffusible. Thus the finding of Dillman and Visscher is confirmed although smaller changes in diffusibility are observed in the present experiments.

It was conceivable that the part played by calcium phosphate in the formation of non-diffusible calcium in hypercalcaemia might also be a factor in calcium diffusibility changes with variations in hydrogen ion concentration. In titration curves of H_3PO_4 with $Ca(OH)_2$, presented by Holt *et al.* [1925], $Ca_3(PO_4)_2$ was precipitated at p_H 5-6. The point of precipitation was definitely changed by the presence of other ions, occurring at a slightly higher p_H when the titration was carried out with $Ca(OH)_2$ in 0.1 *M* NaCl, and at p_H 6.5-7.5 with $Ca(OH)_2$ in 0.1 *M* $MgSO_4$. In blood-serum, $Ca_3(PO_4)_2$ precipitation might

be expected to occur in this range, *i.e.* at a p_H definitely higher than that of the isoelectric point of serum-protein, below which protein-bound calcium is freed [Loeb, 1928]. Thus, variations in p_H in the former range would be expected to have a much greater effect on the diffusibility of calcium in hypercalcaemia if a considerable part of the non-diffusible calcium were due to phosphate, than in normal serum where the non-diffusible calcium is bound only to protein. This supposition was tested experimentally, the results being recorded in Table IV. Here, as predicted, calcium diffusibility is affected to a much greater

Table IV. *Effect of hydrogen ion concentration on calcium diffusibility in high calcium sera.*

Total Ca and total PO_4 (P) mg.	Calcium		Inorganic PO_4 (P)		Non-diffusible Ca as		p_H
	Diffusible %	Non- diffusible mg.	Diffusible %	Non- diffusible mg.	$Ca_3(PO_4)_2$ mg.	Protein- bound mg.	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Ca 23.2	57	10.0	62.5	1.2	2.3	7.7	8.05
P 3.2	58	9.8	59.5	1.3	2.5	7.3	7.98
	58.5	9.6	65.5	1.1	2.1	7.5	7.88
	59.5	9.4	69	1.0	1.9	7.5	7.72
	64.5	8.2	101	—	0	8.2	7.52
	65.5	8.0	103	—	0	8.0	7.20
Ca 23.7	47	12.5	42	2.9	5.5	7.0	8.05
P 5.0	45	13.0	46	2.7	5.1	7.9	7.95
	52.5	11.2	60	2.0	3.8	7.4	7.75
	57.5	10.1	64	1.8	3.4	6.7	7.62
	63.5	8.7	90	0.5	0.9	7.8	7.05
Ca 28.3	42	16.4	45	4.0	7.6	8.8	8.05
P 7.3	42	16.3	45	4.0	7.6	8.7	7.92
	51	13.8	63	2.7	5.1	8.7	7.73
	49	14.5	57.5	3.1	5.9	8.6	7.55
	60	11.4	86.5	1.0	1.9	9.5	7.42
	64	10.1	87.5	0.9	1.7	8.4	7.25
Ca 26.7	51	13.1	46.5	3.1	5.9	7.2	8.10
P 5.8	53	12.5	51.5	2.8	5.3	7.2	7.70
	61	10.4	72.5	1.6	3.0	7.4	7.30
	71	7.7	99	0.05	0.1	7.6	6.88
Ca 37.0	48.5	19.0	28	2.6	4.9	14.1	8.20
P 3.6	50	18.4	28	2.6	4.9	13.5	7.85
	54	16.9	36	2.3	4.4	12.5	7.48
	59	15.2	69.5	1.1	2.1	13.1	7.05
Ca 31.4	55.5	14.0	39	2.7	5.1	8.9	8.10
P 4.4	61.5	12.1	54.5	2.0	3.8	8.3	7.75
	64.5	11.2	61	1.7	3.2	8.0	7.35
	71	9.1	75	1.1	2.1	7.0	7.00
	79	6.7	98	0.1	0.2	6.5	6.60

Average maximum change in $p_H = 1.11$.

Average change in calcium diffusibility = 16.8 %.

extent by such changes in p_H . By averaging the changes in the six experiments it is found that approximately 15 % more of the total calcium becomes diffusible with a decrease in p_H of 1.0 in this range. In a general way the magnitude of the change varies directly with the inorganic phosphate concentration and proceeds parallel with the liberation of the non-diffusible phosphate. In column (6) the non-diffusible calcium presumably present as $Ca_3(PO_4)_2$ is the product—non-diffusible phosphate $\times 1.9$. That present in combination with protein, column (7), is the difference between $Ca_3(PO_4)_2$ -calcium and the total non-diffusible calcium.

Such calculations show that the increase in calcium diffusibility with decrease in p_H may be explained entirely by the disappearance of $\text{Ca}_3(\text{PO}_4)_2$. The protein-bound calcium shows little change, unlike its behaviour in normal serum. It is conceivable that any decrease with lowered p_H would be counteracted by the greater concentration of diffusible calcium freed from $\text{Ca}_3(\text{PO}_4)_2$. The variations in column (7), with the possible exception of the last experiment, are not considered significant, as the values involve the errors of two calcium and two phosphate determinations as well as the ultrafiltration process.

If in the presence of excess phosphate the non-diffusible calcium, formed by the addition of calcium to serum, is due only to the formation of $\text{Ca}_3(\text{PO}_4)_2$, as indicated in Table II, similar changes in p_H under such conditions should have a still greater effect. That such is the case is evident from Table V. Approxi-

Table V. *Effect of hydrogen ion concentration on calcium diffusibility in high calcium serums in the presence of excess phosphate.*

Total Ca and total PO_4 (P) mg.	Calcium		Inorganic PO_4 (P)		Non-diffusible Ca as		p_H
	Diffusible %	Non- diffusible mg.	Diffusible %	Non- diffusible mg.	$\text{Ca}_3(\text{PO}_4)_2$ mg.	Protein- bound mg.	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Ca 20.8	26	15.4	70	6.6	12.5	2.9	8.35
P 22.0	26	15.4	71	6.4	12.1	3.3	8.23
	36.5	13.2	76.5	5.2	9.9	3.3	8.05
	51	10.2	88.5	2.5	4.7	5.5	7.72
Ca 28.0	30	19.5	50	9.4	17.8	1.7	7.62
P 23.3	32	19.0	63.5	8.5	16.1	2.9	7.28
	41.5	16.4	68.5	7.3	13.8	2.6	7.02
	69	8.7	90	2.3	4.4	4.3	6.68
Ca 31.3	26	23.2	49	10.1	19.2	4.0	8.10
P 19.7	30.5	21.7	49	10.1	19.2	2.5	7.80
	31.5	21.5	52	9.4	17.8	3.7	7.45
	49	16.1	70.5	5.8	11.0	5.1	6.90
Ca 37.2	19	30.0	36.5	14.2	27.0	3.0	8.10
P 22.4	27	27.3	44	12.5	23.8	3.5	7.85
	33	25.0	50	11.2	21.3	3.7	7.45
	46.5	19.9	63.5	8.2	15.6	4.3	7.03
	71	10.7	86	3.1	5.9	4.8	6.60

Average maximum change in $p_H = 1.07$.

Average change in calcium diffusibility = 34.7 %.

mately 32 % more of the total calcium becomes diffusible by a decrease in p_H of 1.0 in the 7-8 range, in contrast with 7 % with normal sera and 15 % in hypercalcaemia without added phosphate. Here also (Table V) the change in calcium diffusibility is entirely accounted for by the liberation of $\text{Ca}_3(\text{PO}_4)_2$. In fact the protein-bound calcium even increases slightly in spite of the decrease in p_H , which is readily accounted for by the increased concentration of diffusible calcium as it is freed from $\text{Ca}_3(\text{PO}_4)_2$.

DISCUSSION.

Brull [1930] by *in vivo* ultrafiltration showed that when serum-calcium is increased the non-diffusible Ca increases to a much greater extent than the non-diffusible phosphate, in contrast with the results when phosphate alone,

or when both calcium and phosphate, are increased, in which case the non-diffusible calcium and phosphate increase in parallel. This was presented as evidence that the greater part of the serum-inorganic phosphate existed in a non-ionised state. In the light of the present work this postulation is unnecessary. When the serum-calcium is increased non-diffusible calcium increases to a much greater extent than the non-diffusible phosphate, as only a part of it combines with phosphate, the remainder being bound directly by protein. However, when both calcium and phosphate are added the calcium reacts only with the phosphate, provided an excess is present; thus the non-diffusible fraction of each increases in close parallel. If sufficient calcium is added, approximately all the normal serum-phosphate becomes non-diffusible, and there is nothing to indicate that it behaves differently from added phosphate.

Greenberg and Gunther [1929-30] have expressed the protein-bound calcium as a function of the protein and ionic calcium concentrations. It must be recognised that this applies only to protein-bound calcium and not necessarily to the total non-diffusible calcium. As these authors state, according to the equation presented, even when the ionic calcium approaches infinity the protein-bound calcium becomes only of the same order as the total calcium of blood-serum. In a number of experiments of the present study the non-diffusible calcium rises much beyond this level, explicable, of course, by the fact that a considerable part of it is due to calcium phosphate formation.

SUMMARY.

1. When non-diffusible serum-calcium is increased by the addition of calcium to serum, the two processes accountable for this action (a binding of added calcium by protein and the formation of calcium phosphate which may in turn form a colloidal complex with protein) occur simultaneously.

2. Further evidence is presented for the formation of $\text{Ca}_3(\text{PO}_4)_2$, in that, in the presence of excess phosphate the increase in non-diffusible calcium is wholly explicable by the formation of this compound.

3. A change in p_{H} from 8 to 7 causes a slight increase in the diffusibility of normal serum-calcium. With increased calcium concentrations this increase is much greater, as a considerable part of the non-diffusible calcium fraction exists as $\text{Ca}_3(\text{PO}_4)_2$ which breaks down over this range of p_{H} .

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