STUDIES ON BONE DEVELOPMENT IN RATS

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HISTORICAL REVIEW

"A perfect experiment in any field of science may be said to be one that has been planned and conducted in such a way that the results obtained are susceptible of only one interpretation." (1) Such is the aim which H. H. Mitchell has set for experimental work expecially in the biological field. This general idea has been used in judging the applicability of the conclusions reached by previous workers and in setting up the experiment which is to be reported in this paper.

Rickets has long been known to be a disturbance in the calcium and phosphorus deposition in the bone of animals suffering from this malady. As generally spoken of now, it refers specifically to the low phosphorus type i.e. the blood is usually low in inorganic P. The disease is produced in rats by feeding the Steenbock and Black ration which is now being widely used in the experimental study of rickets. It was found necessary to have a high Ca:P ratio in the dietary to produce the typical change in bone structure. In the Steenbock diet this ratio is approximately 4 to 1.

The blood picture was one of the first factors to be studied. Since the blood carries all nutritive materials to every portion of the body, a drop in calcium or phosphorus content would, of course, influence bone development. Dutcher, Creighton, and Rothrock reported in 1925 that in normal young rats the phosphorus in the blood was about 10 mg./100cc. of serum, and that this level continued, falling slowly

over an eight week period, to 8 mg./100 cc. of serum. The ash of the femur correspondingly rose from 40% to 62%. On the other hand, with rats on the Steenbock ration, the phosphorus dropped to 1.6 mg./100 cc. of serum within three weeks, rising slightly thereafter presumably due to a lessened food intake. The ash of the femur became 26.5% in three weeks and values even as low as 24% have been obtained. (2) Koch and Cohan found results comparable with these. They, however, did some work on the method of obtaining the blood and on the influence which any variation would have on the phosphorus content. They concluded that deep anesthesia increased the inorganic blood phosphate very rapidly, light anesthesia increasing it only 2-6%. They also found that the struggle and injury from stunning the animal increased the blood phosphorus even more than anesthesia. (3) Shohl and his co-workers have found this same condition in rickets - low phosphorus content of the blood serum with normal calcium. (4) Later, they tried adding NaH_2PO_4 to the basal Steenbock ration changing the Ca:P ratio from 4:1 to 1:1. The blood calcium remained the same but the blood phosphate became suddenly very high and then came down to a normal value. This was accompanied by rapid healing of the bone with a tendency toward tentany. (5) Working further along this line, these experimenters came to the conclusion that the ratio of Ca:P and their salt level were interdependent. At any given level of calcium or phosphorus, increasing the Ca:P ratio intensified the degree of rickets and at any given ratio of Ca:P,

increasing the level of the salts diminished the degree of rickets. (6)

After producing the disease, the next important thing is to effect a cure. This can be accomplished by irradiation of the animal or feeding phosphate or vitamin D. Schultzer found the following change in the phosphorus of the blood: In rachitic rats, the phosphorus was 5.6 mg./100 cc. of serum rising to 8.6 mg./100 cc. on treatment with ultraviolet and as high as 10.6 mg. by feeding cod liver oil. The calcium content of the blood rose slightly from 11.9 mg./100 cc. of serum in the basal diet to 15.2 mg. with irradiation and 13.8 mg. with cod liver oil. Normal rats did not show this increase of calcium with ultraviolet treatment or cod liver oil administration. Schultzer also concluded that the healing of rickets by these agencies differed from the healing by added phosphate in that the serum calcium and phosphorus values reached but did not exceed the normal values. (7) This does not exactly agree with Shohl's results as given above. Later, Shohl and coworkers reported that marked cure of rickets in rats was secured in two weeks using cod liver oil or .01 mg. daily doses of irradiated ergosterol. (8) In a parallel experiment, they found increased blood phosphorus, increased growth, and increased calcium and phosphorus retention with diets varying from the basal rachitic ration to the basal plus NaH_2PO_4, basal plus butter fat (replaced lard), basal plus cod liver oil, to the basal of which the cornstarch was irradiated. (9) In another article, they report finding that .01 mg. of irradiated ergosterol improved the normal rat in weight and well being while in the rachitic rat, it effected a cure but not a general improvement. 0.5-2.0 mg. was toxic to the normal rat while the

rachitic rat was much more resistant to toxic amounts which is to be expected. The vitamin D did not, however, alter the ratio of Ca:P retained and, therefore, these workers concluded that the vitamin produced calcification by dissolution and deposition of the bone salts and that the amount of calcium and phosphorus in the diet controlled the retention while the vitamin controlled the intermediary metabolism. (10)

In spite of all this evidence, Hess, Weinstock, et al, recently published an article attempting to prove the lack of relationship between the development and cure of rickets and the inorganic phosphorus of the blood. The question might be asked as to whether they had only one variable in their experiment. Their data indicated very poor growth in their animals. (11)

Other workers have studied the problem of bone development from slightly different angles. LeCocq and Villins report that hypophosphites, furnishing 0.7% phosphorus in the feed, were less effective than 0.13% phosphorus in the form of Na_2HPO_4. (12) Haury made some determinations on the calcium content of striated muscle of rachitic rats. The muscle of the rachitic animals contained 41.6 mg. of Ca/100 g. of dry muscle in comparison with 74.0 mg. of Ca/100 g. of dry muscle in the normal rat. These results may not be comparable to the rachitic conditions as usually produced, for the diet was quite different from the one ordinarily used. (13) Hörste reported an interesting experiment on calcification "in vitro". The epiphyseal cartilege of rachitic rats took up calcium and phosphorus from the surrounding medium in the mol ratio of 1:1. The calcified epiphyseal cartilege from normal rats took up less and the

investigator found that if the phosphorus content of the calcifying solution was lovered, phosphorus actually escaped from the tissue into the solution. (14)

Another phase of the rickets problem which was of particular interest in setting up the present experiment was the influence of the acidity or basicity of the ration. Zucker, Johnson, and Barnett reported work along this line in 1922. They thought that they had conclusively proved that rickets did not develop on a rickets - producing ration if calcium chloride or ammonium chloride were used to make the ration acid. They also stated that they could produce rickets by making a suitable normal ration alkaline with sodium carbonate. Here again, the question arose as to whether the acidity was the only variable factor. (15) In 1924, Martha Jones reported the use of hydrocloric acid in the therapy of rickets. In a few cases which came to the hospital in San Francisco, the addition of hydrochloric acid to the diet of the rachitic infants improved their condition. She states that she used this treatment as the result of an idea taken from the fact that puppies on a seemingly good ration but slightly alkaline had developed rickets. With rats, this same ration had not, however, proved rickets producing. Miss Jones also indicated that Schabad had previously shown that in active rickets, the distribution of calcium and phosphorus in the feces and urine was different from the normal, indicating difficulty in the absorption. (16) Samuel and Kugelmass recently found that acid - forming diets lacking in vitamin D produced rickets and generally inhibited growth, development, metabolism, and activity. Again the question arose, and this time quite prominently, as to the

justification of comparing results from two diets so greatly varying as those used. (17) Shohl and coworkers, taking up this work, tried varying the rickets producing diet by adding the same amount of phosphate so that the diet became alkaline, neutral, or acid. The blood picture showed the characteristics of tetany with the alkaline diets and of rickets with the acid diets. The clinical picture corresponded. Ash analyses of the bones showed greatest salt deposition with neutral diet, less with the alkaline diets, and least with acid diets. (18) In a second experiment, they altered the basal diet stepwise by adding phosphate until it became normal. Then parallel diets were made acid, neutral, or basic. Under these conditions, in the intermediate groups, diets were found which produced the mild type of rickets when acid and no rickets when neutral or alkaline. (19)

From the question of the effect of the acidity of the ration, the problem advanced to a study of the gastro-intestional system and the mechanism and paths of absorption and excretion of calcium and phosphorus. In 1924, Zucker and Matzner advanced the theory that the disturbance in rickets was a gastro-intestional one. They came to this conclusion after observing that the pH of the feces of rachitic rats averaged 7.6 and that on the addition of cod liver oil or its active principle to the diet, the pH of the feces changed to 6.2 or even 5.7. The cod liver oil or the active principle injected subcutaneously did not produce healing or change in the pH of the feces. (20) Schultzer made a study of the calcium and phosphorus retention in rickets and gave his results as "retention quotient" which was the number of mg. of either element retained per g. of

body increase. His figures indicated an increased retention on treatment with ultraviolet or feeding of cod liver oil, but his "quotient" has not been used. (21) Shohl et al. have done work along this line by means of metabolism studies. They have shown in their early experiments the paths of excretion of calcium to be 23% in the urine, 77% in the feces; for phosphorus, 5% in the urine and 95% in the feces. The positive balance of calcium during rickets was 50% of the normal and of phosphorus 20% of the normal. The ratio of Ca:P retention in the normal was 1.58 and in the rachitic animal 3.9.(4) Addition of phosphate to the basal diet of rachitic animals increased the phosphorus retention from 20% to 70% and changed the Ca:P retention ratio to 1.7 and 0.8. (5) The cure of rickets by cod liver oil or irradiated ergosterol was accomplished without great increase in the retention of calcium or phosphorus. (8 & 10)

Bergeim studied the absorption of calcium by adding ferric oxide to the diet. Knowing the ratio of Ca:Fe in the ration and determining this ratio in the feces, the amount of absorption could then be calculated. By this method, he found that lactose seemed to increase calcium and phosphorus absorption while starch, glucose, fructose, and maltose had no effect. By killing the animals at the height of digestion, he was able to find that calcium absorption took place in the upper small intestine and an excretion took place in the lower intestine bringing about a negative balance. Phosphorus was actually excreted in the upper intestine. He suggested that this secretion might be important in promoting calcium absorption since it was most rapid where the P:Ca ratio was highest. He also showed that animals receiving cod liver oil showed a

positive calcium balance throughout the intestine while the phosphorus was secreted into the upper tract and was absorbed in the lower intestine, resulting in a positive balance, also. In comparison, the rachitic animal absorbs calcium in the upper intestine and excretes it into the lower while it excretes phosphorus into the upper intestine and fails to reabsorb it. (22 & 23) Peola and Guassardo record similar results studying the calcium and phosphorus absorption by means of an intestional fistula. (24) Hesse reports a storage in the body of 10% of the calcium given in the ration. He also has investigated the effect of emulsifying the calcium salt in gum arabic which seemed to increase the absorption markedly, while in a phosphatide emulsion the absorption remained about the same, 50%. (25)

Still another angle of this problem is a study of the hydrogen ion concentration of the gastro-intestional tract. Abrahamson and Miller reported a marked increase in the pH of the intestional contents in the rachitic animal and the return to normal acidity on administration of cod liver oil. (26) Yoder studied the relation between pH and calcium and phosphorus utilization. He concluded that irradiation or feeding of cod liver oil decreased the large calcium and phosphorus elimination characteristic of the rachitic animal and lowered the pH throughout the intestional tract. The rickets which he studied could not have been typical since he started his animals at 150 gm. Moreover, his pH values did not seem to vary sufficiently to justify such sweeping conclusions. (27) Redmon, Willimott, and Wokes further corroborate the general observations of these previous workers. (28)

Menville, Ane, and Blackberg have added an interesting note by discovering a general hypomotility of the intestional tract of rats on a D deficient diet. (29)

This pH work has some practical use. It has been suggested that the pH of the feces be used as a test for vitamin D. Jephcott and Bacharach first proposed this method. By using the Zucker ration, they obtained quite significant differences. Their method consisted in feeding this diet until the pH of the feces became constant at 7.2. The supplement was then given and after the third day, it was again tested until the pH became constant at some value below 6.8. They found the rise in fecal pH invariable on the basal ration and the addition of supplementary doses of non-anti-rachitic substances to cause no alteration in the alkaline pH. Restoration of acid pH occured by the addition of the antirachitic vitamin and by irradiation. The latter could prevent the rise of pH in the first place. (30) Shohl and Bing critisized this method severely and showed evidence that the pH of the feces varied too greatly to be used in the quantitative estimation of vitamin D. (31) Bacharach and Jephcott replied defending their method. They did not get sufficient fecal change with the Steenbock ration in the same length of time as with the Zucker ration, but the difference was suggested to be ascribed to the different Ca:P ratio in the two diets. (32 & 33) Oser was inclined to agree with Shohl and Bing. He found that the pH of the feces of the rachitic rat showed insufficient uniformity to distinguish it from the normal, that there were large daily fluctuations on the same ration, and that the time response of different rats varied. (34) Recently, Heller and Caskey have taken up a defense of the method.

They recommend it not as a test for vitamin D but as a complete record of the progress of an animal on an experiment, thus lessening the possibility of error from basing conclusions on a single examination. These workers admit the daily variation in values but point out the fact that the relative slope of the curve is comparable to the skiagrams of bone changes. Examination of the pH curves indicated that the drop in pH precedes recalcification by three or four days. Thus it forms a good indication of the time to take x-ray pictures of the conditions of bone development in the animal. (35)

To complete the picture in the study of rickets, the production of tetany in rachitic rats throws some light on the blood picture and absorption question. McCollum and Cavin had both shown that fasting of rachitic animals caused healing of the bone and was accompanied by a rapid rise in blood phosphorus. Shohl ran into this question in studying the addition of phosphate to rachitic rations. Limiting the food intake to one-third of that normally consumed caused only slight changes in the rachitic animal - a slight increase in blood phosphorus, no healing in the bone, and reduced balances of calcium and phosphorus. (36) Later, he and his coworkers came to the conclusion that rachitic rats develop tetany with high phosphorus and low calcium content of the blood when moderate amounts of phosphate were added to the diet. Rats fed with the same diet plus the antirachitic vitamin did not show tetany or alteration in blood calcium and phosphorus and their bones yielded a normal amount of ash. (37) Again Shohl and Bing found that the administration of cod liver oil or irradiation lessened the irritability of the muscle.

The addition of phosphate, on the contrary, increased the reaction to a condition which could clearly be called tetany. (38) In comparing the conditions of tetany produced by these two methods, Shohl and Brown concluded that the tetany produced from fasting was probably due to phosphate. The tetany resulting from feeding phosphate was demonstrated in animals which gained weight on the rachitic ration. The symptons developed soon after the ingestion of the phosphate supplemented diet. The most marked condition was produced when the Ca:P ratio was changed to 1:1. A change to 2:1 showed slight tetany. (39) Still later Shohl and his coworkers showed that the acid base equilibrium of the blood in rickets bordered on alkalosis and the condition of tetany brought about by either of these methods changed the equilibrium to one of acidosis. (40)

Shipley and Holt have tried to explain this change caused by fasting of rachitic animals by means of experiments "in vitro". They happened to be studying the effect of salts on calficiation. With the control rachitic animals, a certain addition of NaCl to the calcifying solution inhibited calcification of the bones of these animals even to the point of no calcification. Using the bones of rachitic animals which had been starved twenty four hours, rapid calcification took place even in the same solutions which had inhibited the calficiation of the control rachitic bones. These investigators suggested that the rapid rise in blood phosphorus was not the only cause of rapid healing in the case of starvation. (41) Wilder has attempted to determine the source of the increased phosphorus in the blood during fasting. By blood analyses, he showed thatthe phosphorus did not come from the constituents of the blood such as the red cells. A study of the N in the urine

indicated a destruction of body protoplasm sufficient to account for the increase in phosphorus in the blood. A comparison of the N:P ratio in the urine with that of muscle tissue indicated an extensive retention of phosphate liberated by the tissue destruction. As a check on the condition produced, the blood sugar value was determined and it was found that the convulsions produced in the animal could not be due to hypoglycemia. Furthermore, normal rats were checked and did not develop tetany on fasting, did not show an elevation in blood phosphorus, but did have a much longer survival period than the rachitic animals. (42)

Recently, Hess, Weinstock, et al. have produced tetany in rachitic rats by suddenly shifting from the rachitic ration to a normal ration such as dried milk and wheat. These workers felt that the reaction was brought about by the sudden shift in Ca:P ratio and not by any absolute or relative increase in phosphorus in the dietary. Changing the ratio from 4:1 to 1.5:1 or 1:1 caused tetany. If the ratio was decreased to only 2:1 the condition did not follow. In their discussion, the authors make this interesting comment - that "attention should be directed to the effect of marked alterations in the makeup of dietaries since such shifts might help to explain nutritional disturbances which are inexplicable from the standpoint of adequacy." (43)

PLAN OF EXPERIMENT

In setting up the present series of experiments, the original plan was to study the effect of varying the acid-base value of the rachitic diet. The study of such a variation did not seem to be thoroughly brought out in the literature. In fact, there was considerable controversy on the

subject as has already been indicated and the comparison of experiments by different individuals was difficult. The plan was to use the same basal rachitic ration and to vary the alkalinity by the addition of equivalent amounts of mono-, di-, and tri-sodium phosphates. After running the preliminary experiments, the scope of the work was increased in an attempt to correlate the amount of blood phosphorus, the alkalinity of the blood, the percentage of bone ash, and the pH of the intestional tract.

EXPERIMENT

The basal ration used throughout this series of experiments was the M.S.C. variation of the Steenbock and Black rachitic ration. Its composition is as follows:

Corn meal	38%
Oats, ground	38%
Gluten	20%
$CaCO_3$	3%
NaCl	1%

By titrating the total alkalinity of this ration as represented by the ash, a value of 400 cc. of N/10 alkali per 100 g. of ration was obtained. Shohl gives 510 cc of N/10 alkali for the Steenbock - Black ration.

The animals used for these experiments were from the laboratory here. Both albinos and piebald rats were used. The animals were started when they were 28 days old and weighed approximately 50 - 60 g.

The rations fed to the various groups are listed below.

Group S 1 Basal ration

Group S 2 " " plus 1% $NaH_2PO \cdot H_2O$

Group S 3 " " plus 1.02% Na_2HPO_4

Group S 4 Basal ration plus 2.75 % $Na_3PO_4 \cdot 12H_2O$

" S 5 " " plus 1% sodium acid pyrophosphate

" S 6 " " plus 1% sodium metaphosphate

" S 7 " " plus 1% yeast

" S 8 " " with 1% of oats replaced by 1% non-irradiated yeast. Referred to as Y_1

" S 9 " " with 1% oats replaced by 1% irradiated yeast. Referred to as Y_2

" S10 Y_1 plus 1% $NaH_2PO_4 \cdot H_2O$

" S11 Y_2 plus " " " "

" S12 Y_1 plus 1.02 % Na_2HPO_4

" S13 Y_2 plus " "

" S14 Y_1 plus 2.75 % $Na_3PO_4 \cdot 12H_2O$

" S15 Y_2 plus " " " "

" S16 Y_1 plus N/20 HCl for drinking water

These rations were given ad libitum to the rats from the time they were first started on the experiment. They were kept on the experiment for four weeks and then were killed for the various analyses.

A final series was run in comparison with these to determine the effect of adding the phosphate after the animal had been on a rachitic diet for three weeks and to compare any rise in blood phosphate to that caused by fasting after a similar period on the basal diet. The plan of this experiment was as follows showing the change of ration after three weeks on the basal:

Sg1 Basal ration for 4 days

Sg17 Fasted for 24 hours

Sg18 Fasted for 48 hours

Sg19 Basal plus 1% $NaH_2PO \cdot H_2O$ for 24 hours

Sg20 " " " " " " " 48 hours

Sg21 " " " " " " " 4 days

Sg22 " " " " " " plus 1% irradiated yeast for 48 hours

Bone analyses were run on all of the animals. The percentage ash of the femur was determined on the fat-free bone. In the first groups, the amount of calcification was compared by use of the silver nitrate test on longitudinal sections of the wrist bones.

For blood analysis, it was found most practical to anesthetize the animals slightly and obtain blood by cutting directly into the heart. Anesthesia seemed to cause very little change in the blood values and to cause less struggle and excitement than stunning. The blood of all animals of any one group was pooled to obtain sufficient for analysis. The blood was citrated and immediately centrifuged to obtain the plasma. The carbon dioxide combining power and the phosphorous content were determined on this plasma. The carbon dioxide combining power was determined immediately after obtaining the blood by using the VanSlyke apparatus. The phosphorus content of the plasma was determined colorimetrically. The protein in this sample was precipitated with trichloracetic acid immediately after obtaining the plasma.

In making pH determinations on the intestional tract, the animals were killed between eight and ten o'clock in the morning. Another worker in the laboratory had found this the time for most uniform contents in the stomach and intestional tract. Both openings to the stomach were

tied off immediately and also both openings of the caecum. The tract was then cut just above the stomach and just below the caecum and the stomach and intestine placed in physiological salt solution to prevent drying of the tissue. Two sections of the stomach, four equal portions of the small intestine and the caecum were the divisions made. The contents were emptied into beakers and enough distilled water was added to give uniform suspensions. The determination of the pH was made electrometrically by use of the quinhydrone electrode.

On two series, the pH of the urine was determined. The separation of urine and feces was made by means of special metabolism cages in use in the laboratory. The pH was determined electrometrically as above.

RESULTS

The results of the first two preliminary experiments are shown in Table I. The percentage ash of the fat-free bone from the animals on the basal rachitic ration varies over a large range but is usually below 30%. The addition of phosphate in the quantities used produced uniform bone ash within groups. The mono-, di-, and tri-sodium phosphates were utilized equally well. The pyrophosphate in the amount used gave as good calcification as the orthophosphate indicating the utilization of phosphorus in this compound. The metaphosphate seemed to be utilized slightly less readily than the other compounds. The addition of yeast to the basal ration had no positive effect on calcification. From the silver nitrate test and also from the bone ash values, it seemed evident that yeast causes a more severe degree

Group	Addition to Basal	Animal	Initial Weight	Final Weight	% ash Femur	AgNo$_3$ test on wrist bones
Sa1	None	1 m.	60	90	22.66	Typical wide rachitic metaphysis
	"	2 m.	57	90	20.61	" " " "
Sb1	"	15 m.	65	110	27.27	" " " "
	"	16 f.	79	129	32.13	
					29.59	" " " "
Sa2	1% $NaH_2PO_4 \cdot H_2O$	3 f.	50	99	47.54	Metaphysis wide but half width basal group
	" " " "	4 m.	55	110	49.71	Slightly narrower than #3
Sb2	" " "	17 m.	60	108	43.68	Metaphysis half width of basal type
	" " "	18 f.	73	126	45.78	" " " " " "
Sa3	1.02% Na_2HPO_4	5 f.	52	91	41.23	Metaphysis comparable to gr. 2 type
					38.46	
	" "	6 m.	57	117	49.50	" " " " " "
Sb3	" "	19 m.	59	115	47.19	" " " " " "
	" "	20 f.	71	122	43.79	" " " " " "
Sa4	2.75% $Na_3PO_4 \cdot 12H_2O$	7 m.	57	103	47.64	Metaphysis narrow - practically normal
	" " "	8 m.	55	112	51.64	" " " "
Sb4	" " "	21 m.	59	101	46.31	" " " "
	" " "	22 m.	75	132	49.38	" " " "
Sa5	1% sodium acid phro-phosphate	9 f.	58	96	43.90	Slightly wider metaphysis than gr. 2 type
	" " " "	10 m.	56	110	45.24	" " " " " " "
Sb5	" " " "	23 m.	66	112	45.40	" narrower " " " " "
	" " " "	24 f.	67	109	44.20	" " " " " " "

Continued

Group	Addition to Basal	Animal	Initial Weight	Final Weight	% ash Femur	AgNo$_3$ test on wrist bones
Sa6	1% sodium metaphosphate				38.42	Comparable to group Sa5
		11 m.	53	97	39.37	
	" " "	12 f.	51	110	44.81	" " " "
sb6	" " "	25 m.	63	105	46.09	Comparable to group Sb5
	" " "	26 m.	73	143	44.60	" " " "
Sa7	1% yeast	13 f.	57	84	26.32	Metaphysis slightly wider than basal
	" "	14 m.	53	94	25.80	" " " " "
					20.73	" " " " "
Sb7	" "	27 m.	57	106	21.26	" " " " "
	" "	28 m.	75	122	23.61	" " " " "

TABLE I. Bone Analysis of Animals Fed Phosphorus Containing Salts in Addition to the Basal Rachitic Ration.

of rickets. This may perhaps be explained by the slightly increased rate of growth that is effected by supplementing the rachitic ration with yeast.

Table II shows the growth and ash analyses of the bones of the animals fed the orthophosphate salts with and without the addition of vitamin D, the latter supplied by irradiation of the yeast. The ash of the bones of animals receiving the mono-, di-, and tri-sodium phosphate without the vitamin show the same composition as in the previous series. The addition to the basal ration of the vitamin by means of irradiated yeast increased the percentage of bone ash but not as much as produced by the combined use of phosphate and vitamin D. The unusually close agreement of ash values on the mono-, di-, and tri-sodium phosphate diets plus the irradiated yeast, indicate normal calcification. The combination of the phosphate and vitamin D increased the percentage ash above that obtained by the addition of either irradiated yeast or phosphate alone.

In parallel, the CO_2 combining power of the blood and the phosphorus content of the blood plasma of these same animals was determined. The results are shown in Table III. For some reason, the inorganic phosphorus content of the plasma did now show any uniformity. This may have been due to errors in technique. With one series, difficulty was encountered in the actual colorimetric comparison. The reason for this was not discovered, but seemed to involve a fading of color in the standard solution. Checks on the latter did not clear up the difficulty. However, the inorganic phosphorus content of the plasma of the rats on the basal ration showed a surprising uniformity and the addition of irradiated

TABLE II - Growth and Percentage Bone Ash of Animals Given Phosphate in Addition to the Basal Rachitic Ration With and Without Vitamin D

Group	Addition to basal	Animal	Initial Weight	Final Weight	% ash Femur
Sc1	None	29 m.	54	99	18.61
	"	30 f.	64	101	27.43
	"	31 f.	59	90	-
	"	32 m.	60	93	19.67
	"	33 f.	65	89	27.55
Sd8	1% non-irr. yeast	49 m.	66	117	28.42
	" " "	50 f.	75	108	32.72
	" " "	51 f.	61	130	-
	" " "	52 m.	77	137	24.33
	" " "	53 m.	65	127	-
Se8	1% non-irr. yeast	68 f.	61	92	26.55
	" " "	69 m.	68	126	24.47
	" " "	70 f.	69	113	27.53
Se16	1% non-irr. yeast	92 f.	52	88	26.73
	plus HCl for water	93 f.	62	102	29.10
	"	94 f.	63	61	34.25
Sc2	1% $NaH_2PO_4 \cdot H_2O$	34 m.	62	109	48.72
	" " "	35 f.	58	104	44.09
	" " "	36 f.	56	91	46.04
	" " "	37 m.	64	123	44.87
	" " "	38 m.	68	110	47.83
Sd10	(1% non-irr. yeast	54 m.	63	130	47.89
	(plus 1% $NaH_2PO_4 \cdot$	55 f.	59	124	53.09
	(H_2O	56 f.	64	129	44.43
	"	57 m.	69	130	50.64
Se10	(1% non-irr. yeast	74 f.	52	100	40.89
	(plus 1% $NaH_2PO_4 \cdot$	75 f.	54	109	50.11
	(H_2O	76 m.	63	141	51.82
Sc3	1.02% Na_2HPO_4	39 m.	63	118	39.05
	" "	40 f.	66	90	46.29
	" "	41 m.	67	127	42.34
	" "	42 f.	57	101	40.60
	" "	43 m.	65	109	41.96

TABLE II - continued

Group	Addition to basal	Animal	Initial Weight	Final Weight	% ash Femur
Sd12	(1% non-irr yeast plus	59 f.	73	140	59.67
	(1.02% Na_2HPO_4	60 m.	63	148	47.95
	(" "	61 m.	65	148	47.62
	(" "	62 f.	73	134	50.21
	(" "	63 f.	66	104	50.82
Se12	(1% non-irr. yeast plus	80 f.	57	106	44.71
	(1.02% Na_2HPO_4	81 m.	59	137	51.01
	(" "	82 m.	75	175	45.65
Sc4	2.75% $Na_3PO_4 \cdot 12H_2O$	44 f.	57	95	49.70
	" " "	45 m.	65	99	48.06
	" " "	46 f.	55	97	50.16
	" " "	47 m.	66	107	47.75
	" " "	48 f.	69	104	50.73
Sd14	(1% non-irr yeast plus	64 f.	66	131	51.67
	(2.75% $Na_3PO_4 \cdot 12 H_2O$	65 m.	85	210	52.24
	(" " " "	66 m.	74	184	51.94
	(" " " "	67 f.	70	103	50;73
Se14	(1% non-irr. yeast plus	86 f.	51	110	46.48
	(2.75% $Na_3PO_4 \cdot 12 H_2O$	87 f.	47	89	50.49
	(" " " "	88 m.	71	198	51.80
Se9	1% irr. yeast	71 f.	54	108	48.17
	" " "	72 m.	73	145	47.78
	" " "	73 f.	68	104	50.55
Se11	(1% irr. yeast plus	77 f.	61	138	56.90
	(1% $NaH_2PO_4 \cdot H_2O$	78 m.	59	166	56.14
	(" " "	79 f.	71	140	56.79
Se13	(1% irr. yeast plus 1	83 m.	57	150	54.23
	(1.02 % Na_2HPO_4	84 m.	56	157	57.62
	(" "	85 f.	65	175	56.55
Se15	(1% irr. yeast plus	89 m.	59	164	56.09
	(2.75% $Na_3PO_4 \cdot 12H_2O$	90 f.	64	130	52.81
	(" " " "	91 f.	64	151	56.79

TABLE III - Carbon Dioxide Combining Power and Inorganic Phosphorus Content of the Blood of Animals Given Phosphate in Addition to the Basal Rachitic Ration

Group	Addition to Basal		Mg. inorganic P per 100 cc. plasma	cc. of Co_2 absorbed by 100 cc. plasma
Sc1	None		3.33	48.1 **
Sd8	1% non-irr. yeast	(2.66)	1.04	42.6 **
Se8	" " " "		2.90	54.2
Sf8	" " " "		2.69	54.8
Sel6	" " " " plus 1/20 HCl to drink		2.91	55.8
Se9	1% irr. yeast		5.52	55.6
Sf9	" " "		5.84	56.7
Sc2	1% $NaH_2PO_4 \cdot H_2O$		6.35	47.1**
Sdl0	1% non-irr. yeast plus $NaH_2PO_4 \cdot H_2O$	(6.16)	7.28	40.8**
Sel0	" " " "		4.00	58.1
Sf10	" " " "		4.64*	61.6
Sell	1% Irr. yeast plus $NaH_2PO_4 \cdot H_2O$		8.08	66.7
Sfll	" " " " " "		4.96*	58.9
Sc3	1.02% Na_2HPO_4		3.70	53.2**
Sdl2	1% non. irr. yeast plus 1.02% Na_2HPO_4	(6.48)	7.04	-
Sel2	" " " " " "		5.68	58.6
Sf12	" " " " " "		4.48	55.7
Sel2	1% irr. yeast plus 1.02% Na_2HPO_4		7.44	65.4
Sf13	" " " " " "		6.56	63.1
Sc4	2.75% $Na_3PO_4 \cdot 12 H_2O$		4.50	48.0**
Sdl4	1% non-irr. yeast plus 2.75% $Na_3PO_4 \cdot 12 H_2O$	(7.76)	7.44	43.6**
Sel4	" " " "		7.12	56.2
Sf14	" " " "		5.6*	60.7
Sel5	1% Irr. yeast plus 2.75% $Na_3PO_4 \cdot 12 H_2O$	(5.36)	7.28	61.7
Sf15	" " " " " "		6.4*	63.4

* Value not accurate due to difficulty in making color comparison.

** Exhaled air supplied by different person.

yeast to the basal ration also gave consistent results. In general, it can be said that the addition of the vitamin increased the inorganic phosphorus content of the blood even for those animals already receiving phosphate.

The CO_2 combining power of the blood was the same regardless of the phosphate used. Here, again, the irradiation of the yeast increased the value slightly. An interesting point was encountered that is somewhat of a side issue but is important in drawing conclusions from the data. In supplying the CO_2 for the saturation of the blood sample, two different people ran the experiment. The CO_2 supplied by one of these gave results uniformly 5 to 10 cc. lower than the values obtained by the other person. This result is again shown in Table V. It is important because in order to compare results from several different types of rations, the same person should run the determinations.

Table IV shows the results of the determinations of the pH of the intestional tract of individual animals from the same groups as given above. In general, these results indicate that the addition of the most alkaline salt caused the greatest increase in acidity. This is rather unexpected inasmuch as the addition of a basic salt to an already decidedly basic ration should probably increase the alkalinity. The mono-sodium phosphate gave values corresponding to the basal. Even in the upper part of the small intestine, the contents of the tract were slightly alkaline. Normally this would be acid as a result of the presence of the gastric juices. The lower part of the small intestine was quite alkaline, approaching neutrality again

in the caecum. The di-sodium phosphate seemed to lower the pH throughout the tract. The tri-sodium salt lowered the pH decidedly with about three quarters of the small intestine showing a pH less than 7.0. The ration did not seem to affect the pH of the stomach to any degree. Again the irradiated yeast in any of these diets increased the amount of lowering of the pH.

With two series of animals, the pH of the urine was determined. The values obtained were as follows:

	pH urine
Basal ration	
Group Sc1	8.30
Group Sd8	8.89
Group se8	8.38
Basal plus 1% irr. yst.	
Group Se9	7.37
1% $NaH_2PO_4 \cdot H_2O$ addition	
Group Sd10	8.72
Group Sc2	8.55
1% $NaH_2PO_4 \cdot H_2O$ plus 1% irr. yeast.	
Group Se11	8.38
1.02% Na_2HPO_4 addition	
Group Sc3	8.89
Group Sd12	9.16
1.02% Na_2HPO_4 plus 1% irr. yeast	
Group Se13	8.29
2.75% Na_3PO_4 addition	
Group Sc4	8.63
Group Sd14	9.06
2.75% Na_3PO_4 plus 1% irr. yeast	
Group Se15	9.06

Since the values were all so extremely alkaline and appeared to give no definite information, no further work was done along this line.

Group	Addition to Basal	Animal	pH Intestional Tract: Stomach Fundus	Stomach Pyloric End	Small Intestine 1st.Qt.	2nd. Qt.	3rd. Qt.	4th. Qt.	Caecum
Sd8	1% non-irr. yeast	51	5.75		7.28	8.72	9.06	8.92	7.45
"	" " " "	53	3.64		7.12	8.38	8.12	8.72	7.20
Se8	" " " "	69	3.38		7.37	8.51	8.63	8.55	7.28
Sf8	" " " "	96	4.40	2.88	7.11	7.45	7.95	8.04	6.43
Se9	1% Irr. yeast	72	5.25		6.52	6.86	7.11	7.70	6.94
Sf9	" " "	98	5.17	3.55	-	7.03	7.03	7.61	6.09
Sd10	(1% Non-irr. yeast	56	5.08		7.53	7.53	8.21	8.12	7.03
"	(plus 1% NaH_2PO_4 ·	57	5.92		7.70	8.38	8.45	8.55	7.20
Se10	(H_2O	76	5.41		7.45	8.55	8.81	8.72	6.78
Sf10	(" " "	103	5.92	3.72	7.28	7.28	7.53	8.46	7.11
Se11	(1% Irr. yeast plus	78	4.57	4.06	6.35	6.09	6.09	8.38	8.72
Sf11	(1% $NaH_2PO_4 \cdot H_2O$	106	4.49	3.98	6.69	6.69	7.03	8.29	6.69
Sd12	(1% non-irr. yeast	62	2.71		8.38	8.63	8.89	9.06	9.06
Se12	(plus 1.02% Na_2HPO_4	82	2.80		7.11	7.37	7.37	8.12	7.37
Sf12	(as above	109	4.66	3.98	7.03	7.20	7.49	7.95	7.15
Se13	(1% Irr. yeast plus	85	4.57	3.13	7.03	7.78	7.87	7.70	6.78
Sf13	(1.02% Na_2HPO_4	111	4.23	2.97	6.77	6.86	7.37	8.29	6.93
Sd14	(1% non-irr. yeast	65	3.13		7.45	7.45	8.89	8.89	7.87
Se14	(plus 2.75% Na_3PO_4 ·	88	4.23	2.80	6.69	6.69	6.69	7.78	6.43
Sf14	(12 H_2O	113	5.25	4.40	6.60	6.35	7.70	8.29	6.77
Se15	(1% irr. yeast plus	91	4.66	3.73	7.11	7.03	7.45	8.12	6.43
Sf15	(2.75% $Na_3PO_4 \cdot 12H_2O$	117	4.66	3.81	6.69	6.93	7.03	8.72	6.77

TABLE IV pH of the Gastro-Intestional Tract of Animals from the Same Groups as Given in Tables II and III.

Animal	Initial Weight	Weight 3 wks.	Final Weight	Change in basal Ration	Time on change	Mg.P /100cc. blood plasma	cc.CO_2 per 100cc. plasma
119 m.	76	108	101	Fasted	24 hrs.	4.29	45.0
120 f.	52	73	66	"	"		
121 f.	73	103	86	"	48 hrs.	9.28	-
122 m.	60	97	78	"	"		
123 f.	69	105	105	1% $NaH_2PO_4 \cdot H_2O$ added	24 hrs.	3.33	63.3
124 m.	56	95	97	" " " " "	"		
125 f.	66	96	88	" " " " "	48 hrs.	3.90	53.1 *
126 m.	60	103	106	" " " " "	"		
127 m.	55	77	78	" " " " "	4 days	4.40	60.7
128 m.	55	103	111	" " " " "	" "		
129 m.	68	93	93	" " " " 1% irr.yst.	48 hrs.	3.84	64.3
130 m.	62	99	104	" " " " " " " "	"		
131 f.	62	90	86	No addition	4 days	2.00	58.0
132 m.	56	90	91	" "	"		
133 f.	59	92	90	" "	"		

* Exhaled air supplied by different person

TABLE V. Study of Blood Changes in Rachitic Animals Starved and Rachitic Animals Given Phosphate

The last series was suggested by the studies of tetany in rachitic animals already referred to in the literature. It was planned to study the increase in inorganic phosphorus content of the blood caused by fasting and by the addition of the same amount of phosphate that had been used in the other rations. Since no appreciable difference had been found in the three orthophosphates, the mono-sodium phosphate was used in this experiment. The results are given in Table V. Tetany in the fasting animals was not observed while the animals were alive. The first group, however, which were killed after 24 hours, showed intense muscular contraction after anesthesia and bleeding but of a more violent type from that ordinarily shown by the drying of tissues. The second group showed this same phenomenon but to a much greater degree. One animal completely doubled up as soon as it was anesthesitized. The muscular contraction in this latter group consisted of general twitching, movement of toes, ears, and jaw. The blood picture of these animals showed rapid rise of the inorganic phosphorus to an unduly high degree. The value doubled within the first 24 hours. The CO_2 combining power of the blood of the first group showed a very low value. Unfortunately, there was not sufficient blood obtained from the later group to make this determination.

Addition of phosphate to the ration of the rachitic rat resulted in a slow but decided increase in inorganic phosphorus. Likewise, the CO_2 combining power of the blood increased immediately. No ill effects seemed evident from this change. The new ratio of Ca:P in the ratio was approximately 1.5:1. A change of the ratio to 1:1 is

supposed to produce tetany and Shohl has obtained slight tetany with a change to 2:1.

DISCUSSION

The limiting factor in bone development on a rickets producing diet seems to be the amount of available phosphorus. The Steenbock - Black ration is known to be low in phosphorus content. The availability of the element is further hampered by the presence of a large excess of calcium. As a result, the animals placed on such a ration are unable to absorb enough phosphorus to supply the needs of calcification during growth, and the condition known as rickets is produced. Addition of vitamin D to the basal ration increases the amount of inorganic phosphorus in the blood and thereby increases the amount of the calcium phosphate salt deposited in the bones. Since the amount of phosphorus in the ration has not been altered in this case, the vitamin has, therefore, increased the availability and the absorption of the element. In the present experiment it has been shown that the alkalinity of the intestional tract decreased from the values obtained on the basal diet when the vitamin was fed. The indication is that the vitamin aided in the absorption of the phosphorus by decreasing the alkalinity of the digestive tract and thereby increasing the solubility of the phosphorus. Supplying vitamin D does not give the best possible calcification of the bone, however. Evidently, the small amount of phosphorus in the basal ration is insufficient for the needs of the growing animal even under the best conditions. So, the addition of any form of phosphate to

the basal ration already supplemented with vitamin D increased the mineral deposition in the bone.

On the other hand, the absorption of phosphorus is dependent on the acidity of the intestional tract only when that element is present in the ration in limited quantities. If the phosphorus is supplied in a soluble form, it is readily absorbed and utilized. The theory might be advanced that phosphate in an alkaline medium, such as is found in the intestional tract of the animal on the basal rachitic ration, in the presence of a large amount of calcium would form the insoluble calcium phosphate which would then be in an unavailable form to the animal. However, even though the reaction of the intestional tract showed no increase in acidity, if sufficient soluble phosphate is added, enough is absorbed to increase the calcification above that found in the bone of the rachitic animal. Nevertheless the amount of phosphorus and, therefore, the amount of bone ash absorbed is limited after a certain point by the alkalinity. Addition of vitamin D to the phosphate supplemented diet increased the inorganic phosphorus in the blood and also the bone ash values above that obtained on the phosphate supplemented diets. Here again, the change was accompanied by decrease in the alkalinity of the intestional tract. Beyond a certain point, the absorption of the phosphorus seems again to be dependent on the acidity of the intestional tract.

CONCLUSIONS

The results of these series of experiments may be summarized as follows:

1. Development of rickets on the Steenbock - Black ration seems to be due to a deficiency in available phosphorus which in turn is influenced by the reaction of the intestional tract.

2. Addition of vitamin D to the diet increased the acidity and probably, therefore, increased the amount of available phosphorus.

3. Bone development was improved by simply supplementing the rachitic ration with soluble phosphates. In this case, the acidity of the intestional tract seemed to be of relatively little importance.

4. The mono-, di-, and tri-sodium orthophosphates as well as sodium pyrophosphate were apparently equally effective in improving bone development. Sodium metaphosphate was slightly less effective.

5. In all cases, the addition of a phosphate increased the inorganic phosphorus content of the blood.

6. Similarly an increase in the alkalinity as determined by the CO_2 combining power was observed with each phosphorus supplement.

7. Healing of rickets during fasting is probably due to an increase in inorganic blood phosphorus.

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