CALCIUM AND PHOSPHORUS STUDIES IN THE CHICK*

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The majority of the published data dealing with blood calcium and phosphorus changes in rickets is based upon blood determinations made after the appearance of rickets. Values obtained after the symptoms of rickets are evident do not show when the reduction in the calcium and phosphorus first takes place. A true understanding of the relation of vitamin D to these blood changes can be obtained only if the analyses are made at frequent intervals.

It is the purpose of this paper to show the changes in the calcium and phosphorus content of the blood and the ash content of the bone of chicks during the first 6 weeks of life when reared on rachitic rations and on the same rations plus vitamin D. The chick was used as the experimental animal for several reasons. Owing to the high vitamin D requirement of this species, the development of rickets takes place very rapidly on a diet of natural food materials without resorting to a distorted calcium-phosphorus ratio. A sufficient quantity of blood for analysis could be obtained from one animal which eliminated the necessity of pooling the blood from several individuals. A sufficient number of animals could be placed on each ration to allow several determinations during the 6 week period.

Steenbock, Hart, Jones, and Black (1), Ackerson, Blish, and Mussehl (2), and Hughes and Titus (3) were the first workers to report low inorganic phosphorus and calcium in the blood serum

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of rachitic chicks. Since then a large number of workers have reported similar results but the actual values vary to such an extent that it is impossible to compare the results of different investigators. For example, the normal inorganic phosphorus values vary from 3.00 mg. per 100 cc. of plasma as reported by Heller, Hunter, and Thompson (4) to 8 mg. per 100 cc. of serum as given by McFarlane, Graham, and Richardson (5). We have compared, therefore, the value for chicks on a rachitic diet with the results obtained with chicks on the same diet plus a source of vitamin D. In one case the vitamin D was supplied by cod liver oil and in the other by irradiation. Two different rachitogenic diets were used, one low in phosphorus and one containing added phosphate.

EXPERIMENTAL

Day-old white Leghorn chicks were used for all the studies. They were housed in the usual way in warmed cages with 2 mesh to the inch screen bottoms. In the first part of our study the blood and bone analyses were made at weekly intervals. For this work three different experiments were conducted, started on the following dates: March 3, 1932, May 4, 1932, and October 27, 1932. Each series consisted of six groups with fifteen to twenty chicks in each group. Three of the groups received the following basal ration.

59 parts ground yellow corn
25 " wheat middlings (standard)
12 " crude casein
1 part common salt
2 parts precipitated calcium carbonate
1 part dried yeast (Northwestern)

The composition is very similar to the ration recommended by Hart, Kline, and Keenan (6) except that the calcium phosphate is replaced by calcium carbonate. This was done in order to supply all the phosphorus from the grains and casein. This ration contains 0.91 per cent Ca, 0.51 per cent P, with a Ca:P ratio of 1.8:1. The other groups received the same ration plus 0.8 per cent NaH$_2$PO$_4$·H$_2$O, which raised the phosphorus content to 0.70 per cent and changed the ratio to 1.3:1.
The chicks were divided into the following groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Basal + 0.8 per cent NaH$_2$PO$_4$·H$_2$O + 1 per cent cod liver oil</td>
</tr>
<tr>
<td>II.</td>
<td>Basal + 0.8 per cent NaH$_2$PO$_4$·H$_2$O</td>
</tr>
<tr>
<td>III.</td>
<td>&quot; + 0.8 &quot; + irradiation</td>
</tr>
<tr>
<td>IV.</td>
<td>&quot; + 1 &quot; + irradiation + cod liver oil</td>
</tr>
<tr>
<td>V.</td>
<td>&quot; + irradiation</td>
</tr>
<tr>
<td>VI.</td>
<td>&quot; + irradiation</td>
</tr>
</tbody>
</table>

Two or three chicks from each group were killed for blood and bone analyses weekly for a period of 6 weeks. The blood was drawn from the external jugular vein directly into trichloroacetic acid as described below. The left tibia was removed from each chick, dried, extracted with hot alcohol for 72 hours, and ashed for 1 hour in an electric furnace. The percentage of ash was calculated on the moisture- and fat-free basis.

**Blood Analysis**—About 10 cc. of 10 per cent trichloroacetic acid are placed in a 25 cc. volumetric centrifuge tube. The tube is stoppered and weighed. 2 to 5 cc. of blood are allowed to drop directly from the animal into the acid. The tube is rotated to insure complete mixing of the blood with the acid. Any blood remaining in the upper part of the neck or on the outside of the flask is removed carefully. The tube is stoppered and weighed to determine the amount of blood taken.

The contents of the flask are stirred with a curved glass rod which is also used to mix any particles of blood adhering to the side of the flask with the acid. Additional trichloroacetic acid is then added to supply about 4 cc. of 10 per cent acid per gm. of blood. The volume is finally made to 25 cc. with distilled water. The contents are mixed thoroughly and the tubes are centrifuged at 3000 R.P.M. for 4 minutes. Aliquot portions of the clear supernatant liquid are then taken for analysis. If four 5 cc. aliquots are needed it is better to pour off the supernatant liquid into another container before the aliquots are taken.

**Calcium**—Aliquots equivalent to approximately 1 gm. of blood are placed in 15 cc. centrifuge tubes and made up to 10 cc. with water. 1 cc. of 20 per cent sodium acetate, 1 to 2 drops of 0.1 per cent brom-cresol green, and 1 cc. of 4 per cent ammonium

1 These tubes may be purchased from Eimer and Amend.
oxalate are added. Care is taken to prevent the oxalate from touching the lip of the tube from which removal by subsequent washing is likely to be incomplete. The mixture is stirred with a thin glass rod and 1:1 ammonia is added drop by drop until the resulting color matches that of a similar volume of acetate buffer solution at pH 5.0. The tube is covered and allowed to stand overnight for complete precipitation. The precipitate is centrifuged off and the calcium determined by the method of Clark and Collip (7).

We have found this procedure for the analysis of blood calcium very satisfactory. Separate determinations on the same sample of blood check very well. Several analyses were made in which definite quantities of calcium were added to one of the samples of blood. Recoveries ranging from 92 to 100 per cent with an average of 97 per cent were obtained.

**Phosphorus**—The inorganic phosphate was determined on similar aliquots according to the method of Fiske and Subbarow (8). All results were calculated as mg. per 100 gm. of whole blood.

**Results**

The weekly changes in the calcium and inorganic phosphorus content of the blood taken from the chicks studied in Series 2 and 3 are given in Chart I. The figures from Series 1 are not included because a slightly different method was used for the blood analyses in that experiment. Each point on the curves was obtained from an average of the values from four to six different chicks. Corresponding values for the ash content of the bones taken from these chicks are given in Table I.

It is evident from these results that the calcium and inorganic phosphorus in the blood of chicks are greatly influenced by age. In all the animals receiving vitamin D (cod liver oil or irradiation) both on the high and low phosphorus diets, Groups I, III, IV, VI, the calcium was 13 to 14 mg. per 100 gm. of blood during the 1st and 2nd weeks of life. By the 3rd week it had decreased to 12 mg. or less and thereafter it remained at about 10 to 11 mg. The high blood calcium during the first 2 weeks of life is undoubtedly associated with the rapid calcification of the bones during this period. It is seen from Table I that a very considerable amount of calcium deposition has taken place by the end of the 2nd week.
Results given later in the paper will show that the chicks are not hatched with a blood calcium content as high as 13 to 14 mg., but that this increase takes place only after the addition of vitamin D to the diet.

The results for Groups II and V without vitamin D supplement are quite different. Here the calcium is down to 9 to 11 mg. during the 1st week. There is a slight increase the 2nd week, but thereafter the values drop rapidly to 6 to 8 mg. at the 4th week. There is very little decrease after the 4th week; in some cases there may even be an increase if the blood is obtained from very rachitic animals that have consumed little food. The low calcium values
during the 1st week are exceedingly interesting because they show that there is insufficient vitamin D in the chicks at birth to allow normal calcium assimilation even during the 1st week of life. This deficiency is evident from the bone analysis (Table I). There is practically no change in the ash content of the bones from the chicks receiving no vitamin D, while the bones from the other chicks show a definite increase in ash content during the same period.

Although the slight increase in the blood calcium of the chicks on the rachitic diet during the 2nd week has been observed in chicks on both the low and high phosphorus diets, many more results are necessary before the significance of this increase can be definitely established. The history of the eggs from which the chicks were hatched may have some influence on these slight changes. However, it is entirely possible that the increase is due to an over-activity of the parathyroid gland at this point in an attempt to compensate for the low blood calcium.

The inorganic phosphorus values are also high at birth and fall with age. On the high phosphorus ration plus vitamin D, the values start at 9.5 mg. and decrease to 6.5 mg. at 6 weeks of age. On the low phosphorus ration plus vitamin D the values start at 7.5 mg. and decrease to 5 mg. It is interesting to note that in spite of the fact that both groups were receiving vitamin D, and the blood calcium values for the two groups are quite comparable, the phosphorus content of the blood from chicks on the basal

<table>
<thead>
<tr>
<th>Age</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
<th>Group VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>wks.</td>
<td>per cent</td>
<td>per cent</td>
<td>per cent</td>
<td>per cent</td>
<td>per cent</td>
<td>per cent</td>
</tr>
<tr>
<td>1</td>
<td>36.40</td>
<td>32.57</td>
<td></td>
<td>32.20</td>
<td>30.07</td>
<td>40.12</td>
</tr>
<tr>
<td>2</td>
<td>38.31</td>
<td>31.57</td>
<td>37.75</td>
<td>34.94</td>
<td>31.00</td>
<td>40.12</td>
</tr>
<tr>
<td>3</td>
<td>42.11</td>
<td>31.92</td>
<td>39.75</td>
<td>36.87</td>
<td>28.70</td>
<td>39.17</td>
</tr>
<tr>
<td>4</td>
<td>42.35</td>
<td>27.71</td>
<td>40.54</td>
<td>38.88</td>
<td>29.30</td>
<td>39.57</td>
</tr>
<tr>
<td>5</td>
<td>43.31</td>
<td>27.46</td>
<td>41.82</td>
<td>39.03</td>
<td>27.47</td>
<td>40.11</td>
</tr>
<tr>
<td>6</td>
<td>43.02</td>
<td>29.20</td>
<td>40.56</td>
<td>40.66</td>
<td>27.70</td>
<td>40.42</td>
</tr>
<tr>
<td>7</td>
<td>44.65</td>
<td>41.72</td>
<td>42.87</td>
<td></td>
<td></td>
<td>39.30</td>
</tr>
</tbody>
</table>
ration is definitely lower than the values for the chicks on the same ration plus sodium phosphate. Shohl and coworkers (9) have recently observed a similar decrease in the blood phosphorus of rats on a low phosphorus diet containing cod liver oil. The observed difference may be due merely to a difference in the total phosphorus of the ration or the presence of the inorganic phosphorus may exert a specific effect. This may explain the high values obtained by Steenbock, Hart, Jones, and Black (1) for normal chicks on a ration of corn and milk, and the low values reported by Ackerson, Blish, and Mussehl (2) on a ration of cereals and meat.

In spite of the low blood phosphorus the animals were able to produce normal bones (Table I). The ash content of the bones from the chicks on the low phosphorus ration plus cod liver oil was slightly lower, especially during the 2nd and 3rd weeks, than from the group receiving added phosphate and cod liver oil. However, the values for the low phosphorus group plus irradiation compare very well with the high phosphorus group plus irradiation. These results are in agreement with the values given by Shohl (9) for rats on a low phosphorus diet.

It is also important to point out that the phosphorus values for chicks on this ration are only slightly lower in the absence of vitamin D. This fact emphasizes again the importance of controlling the phosphorus content of the ration if blood phosphorus is to be used for the diagnosis of rickets. The difference is much greater in the case of the high phosphorus diet. During the 1st week the results for the chicks with and without vitamin D are about the same but the figure for the rachitic chicks drops more rapidly thereafter, reaching the low point of 5.0 mg. at 4 weeks. All these results indicate that during rickets the blood calcium decreases first and the phosphorus changes are dependent upon the decrease in calcium.

Since such definite changes in blood calcium were observed during the 1st week of life, a further study was made on the daily changes in the blood calcium and bone ash of chicks on the basal ration plus NaH₂PO₄ and the same ration plus 1 per cent cod liver oil. These results are given in Table II. Since no definite number of chicks was taken each day the number of values used for calculating the average is given after each figure. The calcium value
at 2 days of age is approximately 12 mg. per 100 gm. of blood. In the presence of vitamin D this value increases to 13 to 14 mg. during the period of rapid calcification then drops to 10 to 11 mg. There is a very striking increase in bone ash during the first few days of life provided sufficient vitamin D is supplied. The bones contain 32 per cent ash at 2 days and increase to 38 per cent at 7 days and to about 40 per cent at 12 days.

Without vitamin D the blood calcium shows a decrease from 12 mg. at 2 days of age to values of 7 to 8 mg. per 100 gm. of blood at 12 days of age. The bone ash starts at 32 per cent, shows no increase with age, and at the end of 12 days is slightly less than 32 per cent.

### TABLE II

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Basal + NaH₂PO₄ + cod liver oil Group I</th>
<th>Basal + NaH₂PO₄ Group II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca per 100 gm. blood (mg.)</td>
<td>Bone ash (per cent)</td>
</tr>
<tr>
<td>2</td>
<td>11.94 (3)</td>
<td>30.05 (2)</td>
</tr>
<tr>
<td>3</td>
<td>13.65 (5)</td>
<td>33.95 (5)</td>
</tr>
<tr>
<td>4</td>
<td>13.92 (5)</td>
<td>36.40 (4)</td>
</tr>
<tr>
<td>5</td>
<td>13.09 (6)</td>
<td>36.47 (4)</td>
</tr>
<tr>
<td>6</td>
<td>9.57 (3)</td>
<td>37.47 (2)</td>
</tr>
<tr>
<td>7</td>
<td>10.36 (5)</td>
<td>38.33 (5)</td>
</tr>
<tr>
<td>8</td>
<td>11.27 (2)</td>
<td>35.85 (2)</td>
</tr>
<tr>
<td>9</td>
<td>10.20 (3)</td>
<td>38.11 (3)</td>
</tr>
<tr>
<td>10</td>
<td>11.98 (3)</td>
<td>38.51 (3)</td>
</tr>
<tr>
<td>11</td>
<td>10.41 (4)</td>
<td>41.04 (4)</td>
</tr>
<tr>
<td>12</td>
<td>10.97 (3)</td>
<td>39.63 (3)</td>
</tr>
</tbody>
</table>

The numbers in parentheses indicate the number of chicks.

The DISCUSSION

Like many other studies of this nature much more work is necessary before a complete standardization of all variables is possible. We have used chicks hatched from eggs produced by hens fed rations containing ordinary amounts of vitamin D. Although preliminary trials have shown that small variations in
the vitamin D intake of the hen do not affect the blood and bone analysis of the chick, more extensive studies are necessary. Only two different rations were used in this study. Rations containing still different amounts of calcium and phosphorus may give quite different results. Different levels of vitamin D in the ration may also reveal other interesting facts.

All the blood analyses were made on whole blood rather than on serum or plasma. This method was necessary in order to allow calcium and phosphorus determinations on the blood from one chick. It also has certain other advantages, especially in the case of phosphorus. However, the relative merits of the two methods will not be discussed in this paper. Suffice it to say, that the values reported here cannot be compared directly with those obtained for blood serum or plasma.

In spite of these limitations, the results presented have a number of significant applications. They definitely illustrate the necessity of controlling a number of factors if the results for blood calcium and phosphorus are to be of any value in experimental animals such as the chick. Most workers delay the blood analysis until the animals on the rachitic diet show profound symptoms of rickets. Hall and King (10) and McFarlane, Graham, and Richardson (5) are the only workers we know of who have made weekly blood analyses. The latter workers, however, did not place the chicks on experiment until they were 16 weeks old.

The results presented in Chart I show that many of the most important changes take place before the 5th and 6th weeks of life, and that the greatest difference between the blood of normal and of rachitic chicks in both the calcium and the phosphorus may be expected about the 4th week. Similar relations hold in the case of bone analysis. The percentage of ash in the bones from the rachitic chicks is as low at 4 weeks as any other time and the ash in the bones from the normal chicks has practically reached the maximum level at this age. Miller, Dutcher, and Knandel (11) and Russell and Massengale (12) seem to be the only workers who have followed the changes in bone ash during the first few weeks of life.

The phosphorus results clearly indicate the importance of controlling the phosphorus content of the ration if the analysis of inorganic phosphorus in the blood is to be used in the diagnosis of
rickets. When no additional phosphate was added to the grain ration, the blood inorganic phosphorus was only slightly higher in the presence of vitamin D than in its absence. However, when sodium phosphate was added to the ration, more distinct differences were noticeable.

The early changes in the bone ash may be of considerable practical importance in the assay of vitamin D-containing materials. Instead of feeding the chicks for 5 to 6 weeks, the experiments certainly can be terminated at 4 weeks and very probably at 2 weeks. Lachat, Halvorson, and Palmer (13) have recently shown that with the method used by them the results for the bone ash analyses were as accurate after a 4 week feeding period as a feeding period of 5 weeks. This modification will mean a big saving in time and cost in all assay work involving chicks.

Our results also indicate that the chick may be a valuable animal for fundamental studies concerning the mechanism of vitamin D activity. The rapid and profound changes brought about in the blood calcium by the absence or presence of vitamin D demonstrate a very close relation of this vitamin to the absorption of calcium from the gut. The fact that the young chick is immediately dependent upon external supplies of calcium and vitamin D should simplify the problem to a large extent. If vitamin D affects phosphate absorption in the chick, its action on this ion must be much delayed in comparison to its action on calcium. The bones taken from chicks during the first 12 days of life, during which time the ash increases from 32 to 40 per cent, should yield valuable material for studying ossification.

**SUMMARY**

1. The average calcium content of the blood of chicks 1 to 2 days old is 12 mg. per 100 gm. of whole blood. If the chicks receive a diet containing ample vitamin D, the calcium increases to 13 to 14 mg. per 100 gm. during the 1st week of life, then drops gradually, reaching values of 10 to 11 mg. at 3 to 6 weeks of age. The calcium content of the blood is not affected by a decrease in the phosphorus content of the diet. When no vitamin D is added the calcium drops to 9 to 10 mg. per 100 gm. of blood during the 1st week. A definite increase is noted during the 2nd week, which is followed by a drop to 7 to 8 mg. at 4 weeks of age. The lack of
vitamin D shows its effect on the calcium content of the blood during the 1st week of life.

2. The inorganic phosphorus content of the blood of chicks on a standard rachitogenic diet plus cod liver oil or irradiation decreases from an average of 9 mg. per 100 gm. of whole blood during the 1st week of life to 6 to 7 mg. at 6 weeks of age. In the absence of added vitamin D the values drop from 9 mg. at 1 week to 5 mg. at 4 weeks of age and continue at this level until the animals die of rickets. If the added inorganic phosphorus is removed from this ration the average value drops to 7 mg. at 1 week of age and decreases to 5 mg. at 6 weeks of age in the presence of vitamin D. If the vitamin D is also omitted only slightly lower values are obtained.

3. The ash content of the bones of the chicks reared on this rachitogenic diet decreases very slowly from 30 to 32 per cent at 1 week of age to 27 to 29 per cent at 5 weeks. In the presence of ample vitamin D the ash increases to 36 per cent during the 1st week and continues to increase to 40 to 42 per cent at 6 weeks. The ash content of the bones of the chicks on the low phosphorus diet plus vitamin D may be slightly lower than that of the bones from chicks on a high phosphorus diet, but there is no indication of any serious disturbance in calcification.

4. A discussion of the application of these results to the interpretation of blood analysis in the study of rickets, to the use of shorter feeding periods for vitamin D assay with chicks, and to the study of the mechanism of vitamin D activity is included.

BIBLIOGRAPHY

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