3. In all the cases mentioned before it is supposed that the open set Ω has irregular boundary points, so that the DIRICHLET problem has not always a solution. If Ω has only regular boundary points, F and F_W are both identical with the solution of the DIRICHLET problem and there is no question at all. But our proofs do not assure that then in all cases $u^P(e) = \mu^P(e)$ (we have not proved generally that the representation (1) is inique; this is a new problem).

4. If it be assumed that $F = F_W$, we have, as is easily seen,

$$U^*(P; R) = G(P; R),$$

where G means the generalized (in the sense of WIENER) GREEN's function for Ω. From the symmetry of G it then follows that

$$\int \frac{du^P(e_Q)}{RQ} = \int \frac{du^R(e_Q)}{PQ}.$$

$$(P \subset \Omega; R \subset \Omega).$$

Dordrecht, April 28, 1939.

Physiology. — *Studies on phosphorus metabolism in normal and rachitic chickens with a radioactive phosphorus isotope.* III. *The distribution of the injected phosphorus in the organs, especially in the different parts of the leg bones.* By M. J. L. DOLS and B. C. P. JANSEN (Laboratory of Physiological Chemistry, University, Amsterdam) and G. J. SIZOO and G. J. VAN DER MAAS (Natuurkundig Laboratorium, Vrije Universiteit, Amsterdam). (Communicated by Prof. G. VAN RIJNBERK.)

(Communicated at the meeting of May 20, 1939.)

In previous papers [1]) experiments have been published on the phosphorus metabolism in normal and rachitic rats, by which the absorption, excretion, deposition in bone as well as the formation of lipin phosphorus were investigated with a radioactive phosphorus isotope as an indicator.

In this paper, we wish to discuss two series of experiments on the distribution of phosphorus in the leg bones and some organs of normal and rachitic chickens after intraperitoneal injection of labelled sodium phosphate. In both series, a fixed quantity of the active phosphate was injected as an aqueous solution of pH 7.2. After it had been established in rat experiments, that 20 hours after the injection the largest difference in the distribution exists between normal and rachitic rats, in these chicken experiments the animals were decapitated 22 hours after the injection and quickly sectioned. The leg bones then were dissected and cleaned. One of the leg bones of each bird was divided into three parts on the basis of the radiographs, namely the proximal epiphysis, the distal epiphysis and the diaphysis. In the first series with 8 chickens the two epiphysial parts of the bone were carbonized together in an oven at 200 degrees centigrade; the same was done with the diaphysis. Experimentally it was found, that no phosphorus was lost in this procedure. Two preparations now were made for the determination of the radioactivity in the manner described previously, namely a preparation of the two epiphysial parts together and a preparation of the diaphysial part of the leg bone. In the second series with 26 chickens, the leg bones were dried at 105 degrees centigrade for 24 hours, extracted with absolute alcohol for 48 hours and then dried again. Now, one of the leg bones of each bird was divided into three parts, namely the proximal epiphysis, the distal epiphysis and the diaphysis, which were carbonized separately. In this series four preparations of a leg bone of each chicken were made for the determination of the radioactivity,

[1]) Proc. Kon. Akad. v. Wetensch., Amsterdam, **40**, 547 (1937); Proc. Kon. Ned. Akad. v. Wetensch., Amsterdam, **41**, 997 (1938); Nature, **139**, 1068 (1937); **141**, 77 (1938).

namely a preparation of the proximal epiphysis, of the distal epiphysis, of the diaphysis and of the alcohol extract of the whole leg bone.

In our first experiment we had found, that there was a large difference in the phosphorylation of the injected phosphorus in the bones of normal and rachitic animals. Now, one could ask, is it only characteristic for the bones or also for the other organs, containing phosphorylating enzymes. Therefore, in this series it was also investigated, whether there is any difference between the phosphorus content and the distribution of injected phosphorus in the liver and the spleen of normal and rachitic chickens. So, preparations for the estimation of the radioactivity in these organs were made. In addition, a part of the residue of carbonization of the various parts of the leg bones and of the livers was used for the determination of the total phosphorus content according to FISKE and SUBBAROW.

The determination of the radioactivity in the different preparations was also done as described previously [2]) with an ionization chamber, whereby the ionization current produced by the β-particles, emitted by the radioactive phosphorus in the bone preparations, was compared with the ionization current of a constant source, in this case the γ-activity of a piece of pitch blend.

The provisional figures of the first series with 5 normal and 3 rachitic chickens, which are tabulated in table I, show, that the phosphorus content of the carbonized residue in the epiphysial parts and in the diaphysial part is larger in the normal than in the rachitic chickens. Furthermore it was established, that both in the normal and in the rachitic chickens, the phosphorus content from the diaphysial part of the bone seems to be larger than that from the epiphysial parts of the same bone. With regard to the distribution of the active phosphorus administered it was observed, that

TABLE 1.

	Number of chickens	Percentage phosphorus carbonized residue	Percentage of the active phosphorus administered present in 1 mgm of the bone phosphorus
Normal chickens	5		
Epiphysis		7.84	0.0255 ± 0.009
Diaphysis		11.10	0.0085 ± 0.003
Rachitic chickens	3		
Epiphysis		5.55	0.0619 ± 0.014
Diaphysis		8.61	0.0251 ± 0.008

both in the normal and in the rachitic chickens decapitated 22 hours after the injection of the active phosphorus, the quantity of the active phosphorus present in 1 mgm bone phosphorus was larger in the epiphysis than in the diaphysis. Furthermore, it was found, that both the epiphysis and the

[2]) G. J. SIZOO and C. P. KOENE, Physica **3**, 1053 (1936).

diaphysis of the rachitic birds contained a much larger quantity of the active phosphorus in 1 mgm bone phosphorus than the normal chickens do.

These experiments were continued and repeated with 26 birds in the second series mentioned before; the results are fully in accordance with those of the first series and produced in table 2.

As is shown in this table, the phosphorus content of the residue of carbonization, both in the epiphysial parts and in the diaphysial part from the leg bones is larger in the normal than in the rachitic chickens, whereas in both groups the diaphysial part of the leg bones seems to contain a larger phosphorus content than the epiphysial parts.

TABLE 2.

Percentage of phosphorus in the residue of carbonization from the leg bones.

Normal Chickens			Rachitic Chickens		
Proximal Epiphysis	Diaphysis	Distal Epiphysis	Proximal Epiphysis	Diaphysis	Distal Epiphysis
8.62	12.81	9.14	4.78	10.10	5.71
6.41	11.64	7.17	4.82	9.78	4.96
8.30	13.13	8.26	5.34	10.45	5.84
7.00	12.51	8.44	5.83	10.27	6.41
9.33	12.94	7.58	6.23	10.16	6.18
9.75	13.25	10.13	5.93	9.53	6.19
10.39	14.55	10.34	4.17	9.05	4.66
8.39	12.50	9.34	5.14	9.46	4.28
9.40	13.12	9.53	5.23	9.78	5.51
9.70	13.74	9.27	5.54	9.96	6.03
8.79	13.99	9.44	5.19	9.73	5.13
9.11	13.37	9.92			
7.98	13.42	8.98			
8.73	13.71	7.02			
Total 121.90	184.68	124.56	58.20	108.27	60.90
Mean 8.71	13.19	8.90	5.29	9.84	5.54
Standard Deviation 0.28	0.20	0.26	0.18	0.18	0.20

No difference exists in both groups between the phosphorus content of the proximal and the distal epiphysis.

A statistical treatment of the obtained dates established that the mentioned differences are clearly significant.

The difference between the phosphorus content of the diaphysis and the epiphysis in the group of the normal chickens is:
$D = 13.19 \pm 0.20 - 8.90 \pm 0.26 = 4.29 \pm 0.33$. The t-value according to FISHER [3]) is 13, whereas the corresponding value for $P = 0.00$.

The same fact was established in the rachitic group; the difference between the diaphysis and epiphysis here was:

[3]) R. A. FISHER, Statistical Methods for Research Workers, 4th Ed. 1932.

$D = 9.84 \pm 0.18 - 5.54 \pm 0.20 = 4.30 \pm 0.27$; the t-value is 16 and the corresponding value for $P = 0.00$.

The difference between the epiphysial parts of the leg bones from the normal and the rachitic chickens is also significant; the same thing can be said from the diaphysis of these two groups.

The differences with their t-values and corresponding values for P according to FISHER are:

Proximal epiphysis:

$8.71 \pm 0.28 - 5.29 \pm 0.18 = 3.42 \pm 0.33$; $t = 10.4$; $P = 0.00$

Distal epiphysis:

$8.90 \pm 0.26 - 5.54 \pm 0.20 = 3.36 \pm 0.33$; $t = 10.2$; $P = 0.00$

Diaphysis:

$13.19 \pm 0.20 - 9.84 \pm 0.18 = 3.35 \pm 0.26$; $t = 13.0$; $P = 0.00$

With regard to the distribution of the active phosphorus in the bone, tabulated in table 3, it was found that both in the normal and in the rachitic group the two epiphysial parts of the leg bone contain more of the active phosphorus than the diaphysial part does. Furthermore, it was found, that the proximal epiphysis in both groups contains more of the active phosphorus than the distal epiphysis.

TABLE 3.

Percentage of the active phosphorus administered present in 1 mgm of the bone phosphorus.

Normal chickens			Rachitic chickens		
Proximal Epiphysis	Diaphysis	Distal Epiphysis	Proximal Epiphysis	Diaphysis	Distal Epiphysis
0.0435	0.0169	0.0308	0.0622	0.0267	0.0442
0.0808	0.0207	0.0568	0.0665	0.0196	0.0454
0.0316	0.0081	0.0196	0.0593	0.0187	0.0448
0.0748	0.0233	0.0630	0.0539	0.0183	0.0444
0.0525	0.0154	0.0608	0.0697	0.0240	0.0315
0.0332	0.0071	0.0233	0.0525	0.0203	0.0291
0.0437	0.0153	0.0382	0.0724	0.0226	0.0477
0.0456	0.0132	0.0327	0.0503	0.0182	0.0456
0.0384	0.0068	0.0202	0.0679	0.0210	0.0393
0.0307	0.0107	0.0206	0.0653	0.0218	0.0395
0.0434	0.0090	0.0234			
0.0287	0.0092	0.0233			
0.0631	0.0187	0.0484			
0.0315	0.0092	0.0246			
0.6415	0.1836	0.4857	0.6200	0.2112	0.4115
0.0458	0.0131	0.0347	0.0620	0.0211	0.0411
0.0044	0.0014	0.0043	0.0024	0.0009	0.0021

The comparison of the figures of the normal and the rachitic chickens shows, that the different parts from the leg bones of the rachitic chickens

contain more of the active phosphorus administered than the same parts of the normal group. With the exception of the difference between the distal epiphysis of the normal and the rachitic chickens all the other differences are clearly significant.

The statistical treatment of these figures shows the following results:

Normal chickens.

Difference between the proximal and distal epiphysis:

$D = 0.0458 \pm 0.0044 - 0.0347 \pm 0.0043 =$

$= 0.0111 \pm 0.0062$; $t = 1.79$; $P = 0.08$

Difference between the proximal epiphysis and the diaphysis:

$D = 0.0458 \pm 0.0044 - 0.0131 \pm 0.0014 =$

$= 0.0327 \pm 0.0046$; $t = 7.1$; $P = 0.00$

Difference between the distal epiphysis and the diaphysis:

$D = 0.0347 \pm 0.0043 - 0.0131 \pm 0.014 =$

$= 0.0216 \pm 0.0045$; $t = 4.8$; $P = 0.00$

Rachitic chickens.

Difference between the proximal and distal epiphysis:

$D = 0.0620 \pm 0.0024 - 0.0411 \pm 0.0021 =$

$= 0.0209 \pm 0.0032$; $t = 6.6$; $P = 0.00$

Difference between the proximal epiphysis and diaphysis:

$D = 0.0620 \pm 0.0024 - 0.0211 \pm 0.0009 =$

$= 0.0409 \pm 0.0026$; $t = 15$; $P = 0.00$

Difference between the distal epiphysis and diaphysis:

$D = 0.0411 \pm 0.0021 - 0.0211 \pm 0.0009 =$

$= 0.0200 \pm 0.0023$; $t = 8.7$; $P = 0.00$

Normal and rachitic chickens.

The difference between the values of the normal and rachitic chickens in favour of the rachitic animals are:

Difference between the proximal epiphysis of rachitic and normal animals:

$D = 0.0620 \pm 0.0024 - 0.0458 \pm 0.0044 =$

$= 0.0162 \pm 0.005$; $t = 3.23$; $P = 0.00$

Difference between the diaphysis of rachitic and normal animals:

$D = 0.0211 \pm 0.0009 - 0.0131 \pm 0.0014 =$

$= 0.008 \pm 0.0017$; $t = 4.7$; $P = 0.00$

Difference between the distal epiphysis of rachitic and normal animals:

$D = 0.0411 \pm 0.0021 - 0.0347 \pm 0.0043 =$

$= 0.0064 \pm 0.0048$; $t = 1.33$; $P = 0.20$

In the last case the difference cannot be regarded as significant.

The values for the quantity of phosphorus administered present in the alcohol extract of the leg bones are calculated on 100 mgm dried matter and not on 1 mgm bone-extract phosphorus, because the quantity of the

bone extract available for the estimation of phosphorus according to FISKE and SUBBAROW was too small. Though the activity in this bone extract was low, it seems that the extract of the rachitic bones contains more of the active phosphorus than the extract of the normal chickens does.

However, the divergency in the individual results was very large, as was shown in the standard deviations of the means.

The mean percentage of the active phosphorus administered present in 100 mgm of the dried matter of alcohol extract of the whole legbone was for the normal chickens: 0.00159 ± 0.00053 %, whereas for the rachitic chickens this value was 0.00619 ± 0.00246 %.

The difference and corresponding values for t and P were:

$$D = 0.00460 \pm 0.00252;\ t = 1.83;\ P = 0.08$$

The statistical treatment of the figures shows that the significance of this difference is not quite convincing; nevertheless the probability is high that the difference in favour of the rachitic birds has not been caused by chance.

Besides the leg bones, in the second series the radioactivity was also estimated in preparations of the spleen and the livers. The spleen preparations contain hardly any activity; therefore, no determinations of the phosphorus content in the preparations have been done. However, the liver preparations were very active. The mean and the standard deviation of the phosphorus content of the carbonized residue as well as the mean and the standard deviation of the percentage of the phosphorus administered present in 1 mgm of the liver phosphorus are reproduced in table 4.

TABLE 4.

Percentage of the phosphorus in the carbonized residue of the liver		Percentage of the administered phosphorus present in 1 mgm of the liver phosphorus	
Normal chickens	Rachitic chickens	Normal chickens	Rachitic chickens
1.93 ± 0.046	1.79 ± 0.089	0.0551 ± 0.0057	0.0580 ± 0.0065

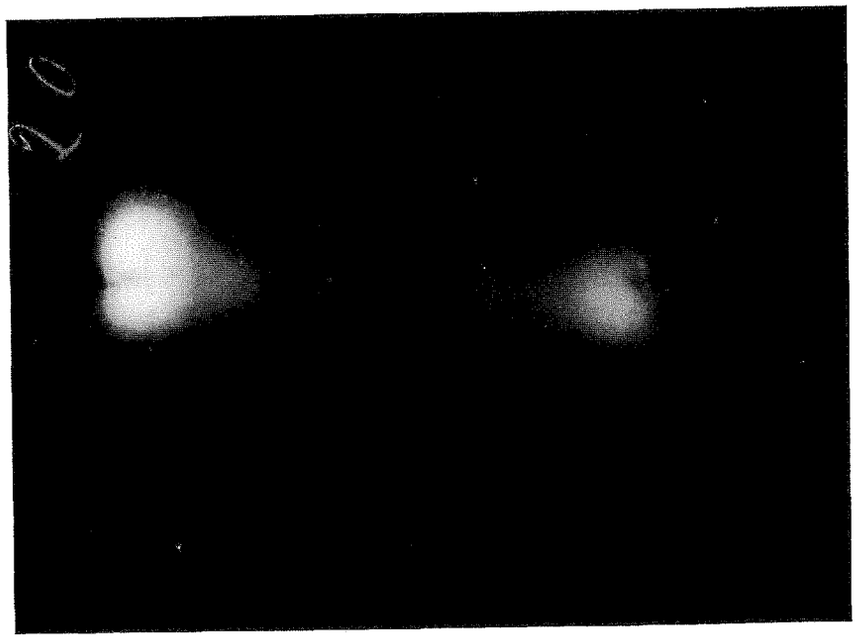
As is clearly shown by the figures, no significant difference exists between the phosphorus content of the carbonized residue from the normal and the rachitic chickens. The difference $D = 0.14 \pm 0.10$; the t value is 1.4 and the corresponding value $P = 0.18$.

The same can be said of the percentage of the active phosphorus administered present in 1 mgm of the liver phosphorus; the difference is not significant.

The figures for the difference, standard deviation and corresponding t and P values are:

$$D = 0.003 \pm 0.0086;\ t = 0.35;\ P = 0.76.$$

So it has been established, that the bone seems to play a unique rôle in the phosphorus metabolism as has also been seen in the rat experiments; only in the bones a difference was found in the phosphorus metabolism; in the liver and the spleen no difference could be observed. In further



"Auto-radiograph" of the tibia of a normal (20) and of a rachitic chicken (2).

Proc. Kon. Ned. Akad. v. Wetensch., Amsterdam, Vol. XLII, 1939.

experiments we wish to investigate also the muscle, the kidneys and the brain.

Summarizing the mentioned results, it may be concluded, that the figures convincingly show, that both in the normal and in the rachitic chickens the phosphorus metabolism is more intensive in the epiphysis than in the diaphysis of the leg bones.

With regard to the difference between the normal and the rachitic chickens the figures show, that generally the phosphorus metabolism is more intensive in the bones of the rachitic birds than in the bones of the normal chickens. These conclusions are fully in accordance with those obtained in our experiments on the formation of lipin phosphorus in normal and rachitic rats and also with the fact that the blood plasma and the bones of rachitic animals contain a larger amount of phosphatase than those of the normal animals [4]). In further experiments we now have started to investigate in vitro the influence of vitamin D on bone phosphatase.

The second leg bone of each chicken was not carbonized, but after being cleaned it was placed in both series on a double coated x-ray film. It remained on this film for some days according to the quantity of active phosphorus injected. The film then was developed; a clear picture "Auto-radiograph" of the whole bone was visible as is shown by the accompanying photographs. It is convincingly shown in these, that the epiphysis contains a larger quantity of the active phosphorus than the diaphysis does. Hence the results of the measurements of the radioactivity of the different parts of the bones are confirmed by the obtained photographs.

Summary.

Experiments have been discussed in which particularly the distribution of phosphorus in the leg bones of chickens was investigated, with a radioactive phosphorus isotope as an indicator.

The figures obtained convincingly showed, that both in the normal and in the rachitic chickens the phosphorus metabolism is more intensive in the epiphysis than in the diaphysis of the leg bones, whereas the phosphorus metabolism in the bones of the rachitic birds is more intensive than in the bones of the normal chickens.

Moreover a method has been published by which it is possible to photograph the distribution of the phosphorus in the leg bones of animals.

We are much indebted to Prof. LAWRENCE from the University of California for a generous gift of radioactive phosphorus; to Mr. J. C. DE BACK, Mr. W. G. LINDENBERG and Mr. W. SOMBROEK for their assistance. Finally, we wish to express our heartfelt thanks to the "Vereeniging tot het Bevorderen van de Beoefening der Wetenschap onder de Katholieken in Nederland" and to the "Stichting voor Biophysica" for a grant for our researches.

4) H. D. KAY, Physiol. Reviews **12**, 384 (1932); J. Biol. Chem. **89**, 249 (1930).