trations of H^+ and of substrate, maximum phosphatase activity was obtained at a concentration of 0.005M-magnesium chloride with the enzymes of bone, kidney and liver, and a concentration of 0.01M with the intestinal phosphatase.

2. The phosphatase activities of the plasma and of bone extracts were found to increase markedly and in an approximately parallel manner with diminishing consumption of vitamin D, but those of

the extracts of liver, kidney and intestinal mucosa were not related to the vitamin D intake. These findings support the view of other workers that the increase in plasma phosphatase in rachitic conditions is chiefly the result of 'leakage' of the enzyme from the bones into the blood stream.

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REFERENCES

- Association of Official Agricultural Chemists (1940). *Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists*, 5th ed., p. 371. Washington: The Association of Official Agricultural Chemists.
- Bakwin, H. & Bodansky, O. (1933). *J. biol. Chem.* **101**, 641.
- Delory, G. E. & King, E. J. (1944). *Biochem. J.* **38**, 50.
- Folley, S. J. & Kay, H. D. (1936). *Ergebn. Enzymforsch.* **5**, 159.
- Gould, B. S. (1944). *Arch. Biochem.* **4**, 175.
- Jaffe, H. L. & Bodansky, A. (1943). *Bull. N.Y. Acad. Med.* **19**, 831.
- Kay, H. D. (1933). *Physiol. Rev.* **12**, 384.
- Macpherson, E. (1942). Ph.D. Thesis, University of Toronto.
- Moog, F. (1946). *Biol. Rev.* **21**, 41.
- Morris, N. (1944). *Glasg. med. J.* **24**, 31.
- Motzok, I. (1950). *Biochem. J.* **47**, 196.
- Motzok, I. & Wynne, A. M. (1950). *Biochem. J.* **47**, 187.
- Robison, R. (1932). *The Significance of Phosphoric Esters in Metabolism*. New York: The New York University Press.
- Thannhauser, S. J., Reichel, M., Grattan, J. F. & Maddock, S. J. (1938). *J. biol. Chem.* **124**, 631.
- U.S.A. National Research Council (1944). *Recommended Nutrient Allowances for Domestic Animals*. I. *Recommended Nutrient Allowances for Poultry*, p. 4. Washington, D.C.: National Research Council.
- Williams, H. L. & Watson, E. M. (1940). *J. biol. Chem.* **135**, 337.
- Williams, H. L. & Watson, E. M. (1941). *J. Lab. clin. Med.* **26**, 1333.

Studies on the Plasma Phosphatase of Normal and Rachitic Chicks

3. THE ASSAY OF ANTIRACHITIC PREPARATIONS BY A METHOD BASED ON THE DETERMINATION OF PLASMA PHOSPHATASE ACTIVITY

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For the determination of vitamin D potency of fish liver oils and other vitamin D-containing substances used in poultry feeding, the Association of Official Agricultural Chemists (1940), and the British Standards Institute (1940) recommend a procedure based on the determination of bone ash. In this method the potency of a vitamin D preparation is determined by comparing the bone-ash values, obtained with the material of unknown potency, with the values obtained with the standard vitamin D preparation. While this is perhaps the most accurate and commonly used chick method, the freeing of the bones from adhering tissue and the extraction of the lipid materials are tedious and time-consuming processes. In comparison with the preparation and

treatment of the bones, the determination of plasma phosphatase activity is a relatively rapid procedure.

As was pointed out previously (Motzok & Wynne, 1950), the increase in the alkaline phosphatase activity of blood has been correlated with the degree of rickets by many investigators. In the case of both animals and man, however, no intensive study of the quantitative relationship between the severity of the rachitic condition and the amount of alkaline phosphatase in the plasma has been reported in the literature. The investigations described in this paper are concerned with the comparison of two methods for the estimation of antirachitic potency of vitamin D preparations: (a) the bone-ash method as outlined

by the Association of Official Agricultural Chemists (1940), (b) a method based on the determination of phosphatase activity of the plasma of the chicks used in method (a).

EXPERIMENTAL

Fifteen preparations of vitamin D were assayed by both the bone-ash and plasma-phosphatase methods. The potency of three of the antirachitic substances, a cod liver oil, a preparation of vitamin D₃ in corn oil, and a sample of dihydro-tachysterol (samples nos. 1-3), had been previously assayed by the A.O.A.C. chick method (1940), using forty chicks for each of three levels of vitamin D in the ration. In the present study the basal ration was supplemented with known amounts of the vitamin. The other antirachitic substances included four preparations of cod liver oil (samples nos. 4-7), one preparation of vitamin D₃ in a fish meal base (sample no. 8), three preparations of vitamin D₃ in crude wheat flour (samples nos. 9-11) and four preparations of vitamin D₃ in corn oil (samples nos. 12-15). The last twelve preparations had not been assayed previously, and were fed at levels determined by the assumed potencies designated by the manufacturers. All vitamin D₃ preparations employed were synthetic products.

The procedure outlined by the A.O.A.C. (1940) was followed in the treatment of the chicks and in the determination of percentage bone ash. The ash content was determined on pooled tibiae for each test.

In the present study approximately 3600 chicks were employed. The A.O.A.C. (1940) recommends the use of twenty chicks for each level of vitamin D for both reference and assay preparations. However, Motzok, Hill & Branion (1943) and the Animal Vitamin Research Council (1944) have pointed out that one of the greatest sources of error in this method is the difference in the response of individual chicks to a given dose of vitamin D. Accordingly, for the purpose of reducing the error due to variation in the response of individuals, approximately forty chicks were used in the present study for each of three levels of dietary vitamin D (10, 15 and 20 units/100 g. of ration) for both the assay and the reference oils.

The plasma for the phosphatase tests and the tibiae for the bone-ash determinations were obtained from the same chicks. Calculations of the potency from both the bone-ash data and the plasma-phosphatase activities were made by the statistical method employed by Knudsen & Tolle (1940).

The method for the preparation of plasma phosphatase has been described previously (Motzok & Wynne, 1950). Test tubes of approximately 30 ml. capacity were used for the reaction mixtures. To each tube were added the following reagents: 5 ml. of 0.05M-veronal-carbonate buffer, pH 10.1; 4 ml. of 0.4M-Na glycerophosphate and 1 ml. of 0.01M-MgCl₂. The tubes were fitted with rubber stoppers, placed in a water bath at 30°, and allowed to reach this temperature. The enzyme preparations were warmed to 30° separately in suitable flasks. Exactly 10 ml. of the enzyme solution were added to each test tube containing the buffer, substrate and MgCl₂, and after replacing the stoppers, the contents of the tubes were mixed thoroughly by inversion and shaking. The pH of the mixture was 9.88-9.90 (measured at room temperature, approx. 25°). The tubes were again placed in the water bath, and the hydrolysis was allowed to proceed for exactly 15 min. At the end of the digestion

period the contents were mixed by shaking. A 4 ml. sample was drawn from each tube and transferred to 3 ml. of 15% (w/v) trichloroacetic acid in a centrifuge tube. The contents of the tube were thoroughly mixed and the precipitated proteins were thrown down by centrifugation. Control tubes containing 4 ml. of distilled water in place of substrate solution were included in all experiments. The amount of liberated inorganic phosphate was determined by the method outlined previously (Motzok & Wynne, 1950).

RESULTS AND DISCUSSION

Under the conditions specified in the A.O.A.C. (1940) chick method of vitamin D assay, the percentage ash of the tibiae varies with the amount of vitamin D fed; the regression of percentage ash on the dosage of vitamin D is curvilinear and is of an exponential nature. The logarithm of the weight of the dose of vitamin D gives a direct linear relationship with the percentage ash in the bone (Knudsen & Tolle, 1940). In the present study the phosphatase activities were also found to give a linear relationship when plotted against the logarithm of the weight of the dose of vitamin D (Fig. 1), but the proportionality was inverse in nature.

Fig. 1. Relationship between plasma phosphatase activity of chicks and the logarithm of the weight of dose of reference cod liver oil.

The Association of Official Agricultural Chemists, in its recommendation of the chick bone-ash procedure (1940), does not specify the accuracy of the method. When this technique was adopted in 1940 by the accredited laboratories* for vitamin D assay in Canada, the limits of error were arbitrarily set at $\pm 20\%$. These same limits of error are used in comparing the potencies given by the bone-ash and plasma-phosphatase methods in the present study.

The potencies of the fifteen antirachitic substances as estimated by the bone-ash and plasma-phosphatase methods are recorded in Table 1. The

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Table 1. *The potency, in units of vitamin D per g., of fifteen antirachitic substances determined by the official bone-ash method and by the plasma-phosphatase method*

Sample no.	Description of vitamin D preparation	Assumed potency	Assayed potency	
			Bone-ash method	Phosphatase method
1	Cod liver oil	115*	119	116
2	Vitamin D ₃ in corn oil	400†	360	392
3	Dihydrotachysterol in oil	800‡	815	782
4	Unfortified cod liver oil	100	78	66
5	Unfortified cod liver oil	130	163	175
6	Unfortified cod liver oil	100	110	131
7	Fortified cod liver oil	400	300	329
8	Vitamin D ₃ in fish meal	200	300	265
9	Vitamin D ₃ in crude wheat flour	2000	4100	3380
10	Vitamin D ₃ in crude wheat flour	2000	3640	3760
11	Vitamin D ₃ in crude wheat flour	5000	9750	6900
12	Vitamin D ₃ in corn oil	400	430	380
13	Vitamin D ₃ in corn oil	550	347	307
14	Vitamin D ₃ in corn oil	400	294	220
15	Vitamin D ₃ in corn oil	400	400	560

* U.S.P. Standard Reference Cod Liver Oil No. 2, stated to contain 115 A.O.A.C. chick units of vitamin D per g. of oil.

† Previously assayed in this laboratory and found to contain 400 A.O.A.C. chick units of vitamin D per g. of preparation.

‡ Hytakerol, purchased from the Winthrop Chemical Company, Inc., U.S.A. stated to contain the equivalent of 1.25 mg. crystalline dihydrotachysterol per ml. and previously assayed in this laboratory and found to have an antirachitic potency equivalent to 800 A.O.A.C. chick units of vitamin D per g. of the commercial preparation.

potencies of the first three preparations of vitamin D (samples nos. 1-3) estimated by the bone-ash and plasma-phosphatase methods are in very good agreement with each other, as well as with the potencies previously established. Sample no. 1, a cod liver oil, stated by the U.S. Pharmacopoeia to contain 115 A.O.A.C. chick units per g., was assayed to contain 119 and 116 A.O.A.C. chick units per g. by the bone-ash and the phosphatase methods, respectively. The potencies of sample no. 2, a vitamin D₃ preparation in corn oil, and sample no. 3, a preparation of dihydrotachysterol, were previously established in this laboratory to be 400 and 800 A.O.A.C. chick units per g. respectively. In the present study these two preparations were found by the bone-ash method to contain 360 and 815 units and by the phosphatase method 392 and 782 units, respectively.

In the case of three samples of unfortified cod liver oil and one of fortified cod liver oil (samples nos. 4-7) the values obtained by the phosphatase method were within the arbitrarily chosen limits of agreement of $\pm 20\%$ of the potencies obtained by the bone-ash method.

The two methods were also within the limits of agreement of $\pm 20\%$ in the determination of one sample of vitamin D₃ in a fish-meal base and of two preparations of vitamin D₃ in crude wheat flour (samples nos. 8-10). In the assay of a third preparation of vitamin D₃ in crude wheat flour (sample no. 11) the potency given by the phosphatase method was approximately 29% below that given by the bone-ash procedure. But the values obtained by both methods were considerably higher than the potency stated by the manufacturer. In the assay

of two of the preparations of vitamin D₃ in corn oil (samples nos. 12 and 13) the two methods were within the limits of agreement of $\pm 12\%$, whereas in the assay of the other two preparations of vitamin D₃ in corn oil the potencies given by the phosphatase method were approximately 25% lower for sample no. 14 and 40% higher for sample no. 15 than the values obtained by the bone-ash method.

From these data it is observed that the agreement between the values obtained by the bone-ash and phosphatase methods was within $\pm 12\%$ for each of nine of the vitamin D preparations and within the range ± 12 to $\pm 20\%$ for three of the preparations. In the case of the other three preparations the values obtained by the two methods did not fall within the arbitrarily chosen limits of agreement of $\pm 20\%$.

The results obtained in the present investigation indicate that the phosphatase method for the assay of antirachitic substances may prove to be a useful alternative procedure. Further work is necessary, however, to establish fully the reliability of the method.

SUMMARY

1. The vitamin D potencies of fifteen antirachitic preparations were determined by two methods: (a) the bone-ash method, as outlined by the A.O.A.C., (b) a method based on determinations of the phosphatase activity of the plasma of the chicks employed in method (a).

2. For each of twelve of the preparations the two methods yielded potency values which agreed within the limits of $\pm 20\%$. For nine of the twelve preparations the values obtained by the two methods

agreed within $\pm 12\%$. For three preparations of vitamin D₃, one in crude wheat flour and two in corn oil, the potencies obtained by the phosphatase method differed from the value given by the bone-ash method by amounts varying from 25 to 40 %.

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REFERENCES

- Animal Vitamin Research Council (1944). *Bulletin*, p. 13.
- Association of Official Agricultural Chemists (1940). *Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists*, 5th ed., p. 371. Washington: The Association of Official Agricultural Chemists.
- British Standards Institute (1940). *British Standard Method for the Biological Assay of Vitamin D₃ by the Chick Method*. British Standard 911.
- Knudsen, L. F. & Tolle, D. C. (1940). *J. Ass. off. agric. Chem., Wash.*, **23**, 665.
- Motzok, I., Hill, D. C. & Branion, H. D. (1943). *J. Ass. off. agric. Chem., Wash.*, **26**, 236.
- Motzok, I. & Wynne, A. M. (1950). *Biochem. J.* **47**, 187.

Metabolism of Spermatozoa. The Formation and Elimination of Hydrogen Peroxide by Spermatozoa and Effects on Motility and Survival.

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In previous papers, Tonic & Walton (1945, 1946*a*) described the effect of egg-yolk medium and some of its fractions on the respiration of bull spermatozoa, and found that the addition of egg yolk to whole semen (spermatozoa and seminal plasma) at first greatly increased the oxygen uptake, but as the reaction proceeded, respiration gradually decreased. This decrease was completely abolished by the addition of catalase or peroxidase. By dialysing the egg yolk it was further demonstrated that the non-dialysable portion contained substances which enhanced the respiration and preserved the fertility of the spermatozoa. On the other hand, the dialysable portion contained a substance (or substances), the addition of which gave rise to inhibition of respiration.

In the present paper further experiments are described in which washed suspension of spermatozoa, instead of whole semen, and fractions separated from egg yolk, instead of egg-yolk medium, were used. The use of washed cells and separated egg-yolk fractions made it possible to analyse still further the mechanism of inhibition. Preliminary reports of this work have been published (Tonic & Walton, 1946*b*, Tonic, 1947*a*).

MATERIALS AND METHODS

Semen. The semen was obtained from Dexter bulls, kept for experimental work. Three or more days' sexual rest was given to the bulls before each experiment. Samples were taken with the short type of artificial vagina (Walton, 1945), and precautions were taken to protect the spermatozoa from sudden falls in temperature (Chang & Walton, 1940). Two or three successive collections were taken from one bull for each experiment.

Preparation of sperm suspensions. Provided the samples of semen were satisfactory as regards density and motility the separate ejaculations were mixed, and centrifuged at 2000 rev./min. for about 25 min. The top layer of seminal plasma was removed by suction and replaced by the same volume of 0.066M-phosphate buffer (pH 7.4) at 37°. The spermatozoa were gently resuspended by means of a thin glass stirrer. The suspension was again centrifuged for about 10 min. at 2000 rev./min., the opalescent liquid above the spermatozoa sucked off, and the sperm resuspended as before in sufficient volume of buffer to give a density of $0.6-1.0 \times 10^9$ spermatozoa/ml. suspension. This is comparable with the density of a good sample of bull semen. Damage to the spermatozoa is caused mechanically during resuspension, and therefore particular care should be taken during this operation. The washed sperm suspensions were used without delay for the manometric experiments. The density of spermatozoa in the semen and the suspensions