obtained. In certain cases, when the extract was not dialyzed and no glutathione was added, the activity proved to be much less than that of dialyzed extract plus glutathione. While the weak activity of the undialyzed extracts might conceivably be due to the presence of some inhibitory material, we found that weak activity goes hand in hand with a weak nitroprusside reaction. Moreover, when to the undialyzed extract 2 mg. of glutathione were added, the activity rose and approximated to that of the dialyzed extract plus glutathione. Thus it seems clear that in the untreated extracts the amount of glutathione present was not sufficient to develop the full activity of the enzyme.

With present knowledge it is, of course, generally assumed that the sole activity of this enzyme is to convert substituted glyoxals into their corresponding hydroxy acids. The absence of satisfactory evidence for the occurrence of such a substrate in the cells and tissues studied has involved disappointment to those interested in the enzyme; this will not be lessened by knowing that the enzyme may exist in certain cells and yet be unable to exert its full activity owing to a deficiency of glutathione.

This failure to gain reliable evidence for any definite function for glyoxalase has now lasted for the thirty years which have elapsed since the discovery of the enzyme by Dakin & Dudley (1913). The realization of this may at the present time be

discouraging further work on the subject. We find that some few still hold that in the case of an agent separated from a living system and studied *in vitro* the assumption that it has a function in life partakes of the teleological. It seems more logical to believe that during the processes of development and evolution a functionless agent would have been eliminated, whereas the glyoxalase system remains with its very wide distribution. We feel that our experiments extending the evidence for this should encourage further work in the field. It is possible, though perhaps unlikely, that the enzyme may prove to have a function of a kind not yet recognized, or that its activity has interrelationships with that of other enzymes. We know that this is true in the case of glutathione, especially in the higher animals and plants.

SUMMARY

1. The results of the experiments described have added considerably to the evidence for the wide distribution of the enzyme glyoxalase in living organisms.

2. While glutathione is also widely distributed, in some cases, especially among the invertebrates, its concentration may be very low and limits the activity of the enzyme actually present.

3. We have isolated glutathione in crystalline form from the earthworm, locust, broad bean and vegetable marrow.

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The Biological Assay of Vitamin D₃. The Effect of the Calcium and Phosphorus Content of the Diet

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That the content and proportion of calcium and phosphorus have a marked effect on the efficacy of rachitogenic diets used in the biological assay of vitamin D on rats and on the responses of the animals to doses of the vitamin, is well known. Investigations of the effect of the same factors in the assay of vitamin D₃ on chicks have been relatively few and in this paper are presented the results

of an extended series of investigations of their effect on the growth, tarso-metatarsal distance, and bone-ash of chicks receiving graded doses of vitamin D₃ and, in some cases, on serum Ca, P and phosphatase.

In rats it has been shown (Bethke, Kick & Wilder, 1932; Brown, Shohl, Chapman, Rose & Saurwein, 1932; Shohl, 1935, 1936) that growth and production of rickets depend on the level of Ca and P as well

as their ratio in the diet. Rachitogenic diets for vitamin D assay usually have a Ca/P about 0.5/0.12*; diets for optimum growth have Ca : P between 2 and 1.

Such studies on the Ca and P content of the diet in relation to the nutrition of chicks have been concerned with the optimum quantities for growth and egg production for commercial purposes. They show some divergency in results, which is not surprising since other factors in the diet, such as acid-base balance (Titus, McNally & Hillberg, 1933) and nature of the diet, especially the cereal portion (Branion, Stackhouse & Hull, 1938; Loy, DeWitt & Knudsen, 1941; Jones & Elliot, 1943) affect the growth. Usually a ratio of Ca : P between 2 and 1 is considered best for growth and bone formation. Vitamin D requirements diminish as the ratio increases. Jones & Elliot (1943) have suggested that growth of chicks on a suitable diet is a satisfactory criterion for the biological assay of vitamin D₃.

The object of the investigations described herein was to determine the optimum conditions for producing maximum differences in response in chicks to graded doses of vitamin D₃ and maximum reliability of assays as affected by the calcium and phosphorus content of the diet. The diet was of a cereal type, commonly used in vitamin D studies and previously found (Jones & Elliot, 1943) satisfactory for such investigations.

METHODS

Sex-linked day-old cockerels (Light Sussex and Rhode Island Reds) were fed on the various diets following the procedure described by Jones & Elliot (1943). After 4 weeks on the vitamin-containing diet and after weighing and radiography, the chicks were killed, and the left tibiae dissected, extracted, dried, and the bone ash determined by the method of the Association of Official Agricultural Chemists (1940). The tarso-metatarsal distances (t.m.t.) were measured on prints from the X-ray negatives. In Exp. 4, the chicks were anaesthetized, and blood removed with a syringe through a direct puncture of the heart. The pooled sample of blood from each group was allowed to clot in a refrigerator overnight, and the serum, poured off next day, was analyzed for calcium, inorganic phosphorus and phosphatase. Calcium was determined by microtitration of precipitated calcium oxalate with 0.01 N-KMnO₄ (Hawk & Bergeim, 1942, p. 468), inorganic phosphorus by Kuttner & Cohen's method (Hawk & Bergeim, 1942, p. 461) and phosphatase by Bodansky's method (Hawk & Bergeim, 1942, p. 463; Jones, 1945).

Basal diet. The basal diet used had the composition given below and adjustments of calcium and phosphorus content were made by adding the calculated amounts of CaCO₃, Na₂HPO₄ and bone meal.

* To avoid confusion, all Ca and P contents of the diets are expressed as fractions (e.g. Ca/P, 0.5/0.12) of which the numerator and denominator represent respectively the percentages of Ca and P in the diet.

Composition of basal diet

Material	Percentage
Ground yellow corn	36.34
Middlings	30.02
Dried skim milk	9.94
Dried grass meal	5.82
Soya bean meal	16.28
Iodized salt (0.02% KI)	1.06
Charcoal	0.53

Percentage of calcium and phosphorus in basal diet

Exp.	Ca	P
1	0.32	0.78
2	0.29	0.78
3	0.29	0.77
4	0.30	0.73

Sufficient basal diet, bone meal, and CaCO₃ or Na₂HPO₄ were taken to give a total of 99 parts, and 1 of olive oil containing the required amount of vitamin D₃ was then added, the whole being mixed in a hobart mixer.

Vitamin D₃ standards. In Exps. 1, 2 and 4 the standard preparation of vitamin D₃ of the British Standards Institution (B.S.I. Specification no. 911, 1940) was used; in Exp. 3, a substandard consisting of a commercial preparation of irradiated 7-dehydrocholesterol which had been found by assay on both rats and chicks to contain 1 million B.S.I. units/g.

EXPERIMENTAL

The experiments fall into two groups, in one of which (Exps. 1, 3 and 4) the ratio of Ca to P was varied and in the other (Exp. 2) the levels of Ca and P were maintained at what appeared to be the optimum ratio. Exps. 1 and 4 cover values of Ca/P from 1/2 to 2/1. Exp. 3 covers smaller variations from the optimum ratio. Exp. 2 covers the ratio Ca : P = 1 at levels from 0.75 to 3% of both Ca and P.

Previous experience had shown that the growth-promoting and bone-forming effect of vitamin D₃ commenced at 10 B.S.I. units/100 g. of the basal diet (Ca/P = 1/1) and was without further effect above 25-30 B.S.I. units/100 g.

In Exp. 1 the levels of vitamin D₃ dosage were 0, 10 and 30 units/100 g. of diet; in subsequent experiments the levels were 0, 10 and 22.5 units with additional levels of 15 units in some cases. In Exp. 4 (Ca/P = 1.5/1.5) a range of five levels from 6.7 to 33.75 units was included to check the linear portion of the response curve.

The diets of Exps. 2 and 4 were also fed to rats of 40-50 g. weight newly weaned at 3 weeks of age. Feeding was continued for 4 weeks and gain in weight and bone ash of the femora determined.

The experimental results are summarized in Tables 1 and 2. Table 3 gives relevant statistical data.

The diets of Exps. 2 and 4, when fed to rats, produced no significant differences in weight gains or bone ash. There was normal calcification of the bones on all diets with bone ash averaging 54.7%.

Table 1. *Experimental data*

(The figures in brackets in the column 'No. of birds' is the number at the commencement of the test. Birds that died were discarded. The high mortality rate on low Ca: P ratios is noteworthy.)

Exp. no.	Group no.	Percentage of		Vitamin D (B.S.I. units/100 g. of food)	No. of birds	Mean gain in weight (g.)	Mean t.m.t.* (mm.)	Mean percentage of bone ash	Standard deviations			Average food consumption (g.)	Average gain in weight (g./100 g. of food)	Average final weight (g.)
		Ca	P						Gain in weight	T.m.t.*	Bone ash			
1	1a	0.32	0.78	0	0 (7)	—	—	—	—	—	—	—	—	—
	b			10	0 (17)	—	—	—	—	—	—	—	—	—
	c			30	12 (17)	109.0	2.875	—	30.9	0.853	—	611	17.84	180
	2a	1.0	2.0	0	0 (12)	—	—	—	—	—	—	—	—	—
	b			10	9 (16)	106.3	2.017	—	34.2	0.473	—	566	19.16	165
	c			30	15 (17)	185.0	1.200	—	38.2	0.244	—	688	26.88	245
	3a	1.0	1.0	0	5	141.2	4.170	—	28.2	0.701	—	833	16.95	208
	b			10	16 (17)	206.2	3.566	—	49.8	0.787	—	747	27.63	277
	c			20	16	353.1	1.316	—	53.2	0.813	—	1017	34.72	424
	d	1.5	1.5	30	15	385.8	0.867	—	24.6	0.208	—	1069	36.09	456
	4a			0	5	167.2	3.880	—	18.7	1.460	—	868	19.26	242
	b			10	14 (15)	251.8	2.511	—	51.6	0.768	—	859	29.33	326
	c	2.0	1.0	30	16	385.3	0.769	—	49.1	0.191	—	1093	35.25	460
	5a			0	6	272.2	3.417	—	76.6	0.605	—	1179	23.08	344
	b			10	17	337.5	1.277	—	51.2	0.573	—	1013	33.33	411
	c			30	13 (15)	342.9	0.754	—	64.5	0.161	—	1069	32.07	419
4	1a	1.0	2.0	0	0 (10)	—	—	—	—	—	—	—	—	—
	b			10	9 (18)	133.3	2.589	39.14	32.6	1.047	2.10	683	21.21	193
	c			22.5	16 (17)	204.3	1.200	45.75	39.9	0.330	1.95	825	24.76	264
	2a	1.0	1.0	0	7	149.0	3.400	36.86	31.1	0.914	1.44	733	20.33	212
	b			10	15 (16)	226.9	2.823	37.94	67.7	0.779	1.40	893	25.42	297
	c			22.5	16	344.1	0.981	43.77	54.8	0.467	2.47	1108	31.05	411
	3a	1.5	1.5	0	8	150.6	3.744	34.43	32.3	1.030	2.06	756	19.92	222
	b			6.7	14 (17)	205.3	2.950	36.76	49.4	1.050	2.09	898	22.86	277
	c			10	16 (17)	200.8	3.087	36.10	68.5	1.111	2.27	788	25.46	270
	d	2.0	1.0	15	17	321.5	1.759	41.67	67.0	0.924	2.65	1028	31.28	389
	e			22.5	17	364.6	0.712	44.76	38.7	0.303	1.84	1092	33.38	437
	f			33.75	17	360.5	0.626	45.41	57.5	0.669	1.38	1059	34.04	432
	4a	2.0	1.0	0	9 (10)	289.8	3.089	36.33	52.8	0.848	0.94	1106	26.21	359
	b			10	18	366.0	0.858	43.31	47.3	0.367	2.75	1087	33.67	438
	c			22.5	16 (17)	345.3	0.481	45.58	47.6	0.168	1.32	1066	32.38	415
3	1a	1.0	1.5	0	2 (7)	81.0	2.725	34.31	11.3	1.025	4.24	586	13.82	158
	b			10	12 (18)	106.8	3.350	37.85	38.9	1.690	4.53	621	17.20	189
	c			22.5	17	192.9	2.768	39.19	80.3	0.665	2.84	739	26.09	271
	2a	1.25	1.5	0	7 (8)	102.5	3.188	36.51	21.9	1.518	4.04	735	13.94	184
	b			10	15 (17)	139.1	3.023	38.94	52.3	1.122	3.35	690	20.15	216
	c			22.5	16	310.8	2.053	41.54	74.7	0.609	1.71	955	32.54	388
	3a	1.5	1.5	0	3 (5)	170.7	3.467	35.15	21.4	0.813	2.74	875	19.50	241
	b			10	14	174.0	3.086	38.27	42.5	0.996	2.45	813	21.41	251
	c			15	14	248.9	2.396	38.43	79.9	0.828	1.91	853	29.18	323
	d	1.5	1.25	22.5	14	358.7	1.314	43.50	84.7	0.757	2.06	1077	33.30	434
	4a			0	7	190.3	3.557	35.40	33.4	0.686	2.45	854	22.28	271
	b			10	14	232.3	2.836	35.84	36.4	1.064	2.32	771	30.15	313
	c	1.5	1.0	22.5	16	400.8	0.728	42.98	63.6	0.370	2.07	1129	35.51	480
	5a			0	7	225.1	3.150	33.28	33.3	0.539	1.63	907	24.82	302
	b			10	15	353.3	2.060	36.96	60.7	0.854	2.33	1115	31.68	431
	c			22.5	15	426.7	0.757	43.44	59.2	0.301	1.46	1230	34.69	507
2	1a	0.76	0.76	0	9 (10)	121.0	4.511	28.64	24.3	0.786	2.93	675	17.93	194
	b			10	18 (19)	139.3	3.833	29.92	32.1	0.969	2.25	652	21.36	212
	c			22.5	18	225.4	2.378	36.58	67.8	0.964	2.83	865	26.07	297
	2a	1.0	1.0	0	9 (10)	134.1	3.767	31.15	33.4	0.612	2.34	704	19.05	209
	b			10	16 (19)	162.4	3.219	32.91	35.6	0.785	2.23	753	21.56	236
	c			22.5	17 (18)	315.7	1.385	40.83	71.1	0.920	2.83	975	32.39	376
	3a	1.25	1.25	0	9	160.4	3.767	33.20	39.1	1.011	1.80	Not taken		229
	b			10	18 (20)	163.2	2.908	35.03	54.6	1.047	2.25	685	23.84	242
	c			22.5	18 (19)	344.0	0.942	43.80	60.6	0.556	2.03	979	35.13	404
	4a	1.5	1.5	0	10	139.7	3.420	32.57	21.0	1.103	1.95	750	18.64	216
	b			10	19	178.2	2.884	34.60	49.7	0.661	1.09	724	24.62	256
	c			22.5	20	365.4	0.760	43.46	40.5	0.210	2.01	1002	36.49	431
	5a	2.0	2.0	0	10	133.3	3.130	35.57	23.2	1.307	1.99	697	19.12	197
	b			10	19	148.6	2.584	36.88	33.8	0.845	2.48	704	21.12	224
	c			22.5	20	255.2	1.395	43.63	50.7	0.754	2.35	901	28.32	329
	6a	3.0	3.0	0	2 (8)	89.0	2.400	40.83	1.41	0.707	1.71	565	15.76	152
	b			10	11 (14)	118.2	2.091	40.91	26.3	0.480	1.95	715	16.52	186
	c			22.5	18 (19)	185.8	1.419	46.22	49.6	0.384	3.14	731	25.40	253

* t.m.t.=tarsometatarsal distances.

Table 2. *Serum calcium, phosphorus, and phosphatase (Exp. 4)*

Percentage of		Vita- min D ₃ (B.S.I. units/ 100 g. diet)	Serum Ca (mg./ 100 ml.)	Serum inor- ganic P (mg./ 100 ml.)	Serum phos- phatase (Bodansky unit/ 100 ml.)
Ca	P				
1	2	0	—	—	—
		10	11.5	10.7	127.7
		22.5	15.9	8.5	34.6
1	1	0	9.4	5.9	183.5
		10	9.6	6.2	152.1
		22.5	8.1	7.6	53.3
1.5	1.5	0	7.6	9.4	176.4
		6.7	7.3	6.2	159.4
		10	7.0	6.8	185.6
		15	11.1	7.9	71.3
		22.5	11.6	6.1	33.7
		33.8	12.3	6.8	29.1
2	1	0	7.7	5.2	167.2
		10	11.8	7.6	43.8
		22.5	12.4	14.5	26.4

(b) With adequate dosage of vitamin D₃ the differences produced by variations in the Ca and P content of the diet tend to disappear when the Ca/P is greater than 1/1.

(c) An amount of 1.5% Ca and 1.5% P in the diet gives optimum increase of response with graded increase of vitamin D₃ intake.

(d) With a Ca : P ratio of 1, gain in weight increases with increase in dietary level of Ca and P up to 1.5%, after which it decreases. This occurs at all dosage levels of vitamin D₃. In the case of t.m.t. responses, a corresponding phenomenon occurs on the highest vitamin D₃ dose level, the t.m.t. responses reaching a minimum at 1.5% Ca and 1.5% P and then increasing again. No such reversal occurs in the case of the percentage of bone ash (Exp. 2).

(e) Data for efficiency of food utilization (gain in weight/100 g. of food consumed) run parallel with the absolute gain in weight of the chicks. The increased food consumption with increase in vita-

Table 3. *Regression data*

Exp. no.	Group no.	Percentage of		Gain in weight (g.)		Tarso-metatarsal distances (mm.)		Bone ash (%)	
		Ca	P	<i>b</i>	<i>b</i> ² / <i>s</i> ²	<i>b</i>	<i>b</i> ² / <i>s</i> ²	<i>b</i>	<i>b</i> ² / <i>s</i> ²
1 10 and 30 units	2	1.0	2.0	136.4	13.7	-1.453	17.7	—	—
	3	1.0	1.0	283.7	51.2	-4.264	52.3	—	—
	4	1.5	1.5	229.5	20.8	-2.995	30.6	—	—
	5	2.0	1.0	10.8	0.04	-1.044	5.5	—	—
4 10 and 22.5 units	1	1.0	2.0	150.8	16.1	-2.953	19.3	14.05	49.3
	2	1.0	1.0	262.5	18.4	-4.332	47.7	13.06	41.6
	3	1.5	1.5	336.9	32.1	-4.799	32.2	17.55	72.6
	4	2.0	1.0	-60.3	1.6	-1.097	14.2	6.62	9.1
3 10 and 22.5 units	1	1.0	1.5	201.2	9.1	-1.365	1.3	3.13	0.75
	2	1.25	1.5	348.0	28.8	-1.967	4.8	5.27	4.0
	3	1.5	1.5	387.4	33.4	-3.736	17.9	18.25	83.5
	4	1.5	1.25	325.6	36.1	-4.072	27.6	13.79	39.8
	5	1.5	1.0	185.8	9.6	-3.359	27.3	16.40	71.0
2 10 and 22.5 units	1	0.75	0.76	181.4	11.7	-3.067	10.1	14.02	30.0
	2	1.0	1.0	330.3	33.8	-3.902	20.7	17.05	44.4
	3	1.25	1.25	356.4	38.3	-3.877	21.4	17.28	64.9
	4	1.5	1.5	379.4	70.4	-4.304	78.7	17.96	121.7
	5	2.0	2.0	231.9	28.7	-2.589	10.5	14.68	37.0
	6	3.0	3.0	186.8	19.4	-1.629	14.9	14.65	28.2

Note. *b* is the slope of the regression line. *s*²/*b*² is the major factor influencing the value of the semi-fiducial range of an experiment or assay. (See British Standards Institution Specification 911/1940, App. C, p. 16.) The smaller is this factor, the narrower the fiducial limits or the greater the reliability of the results. The author prefers to use the inverse value *b*²/*s*² which is a direct index of reliability, i.e. the greater the value, the greater the reliability.

DISCUSSION

Consideration of the data of Tables 1 and 3 shows that:

(a) Gain in weight and t.m.t. were found to vary with the Ca : P ratio (within the range of 0.5–2.0) of the diet, the former rising and the latter falling with increasing ratio. The percentage of ash in the bones did not vary with the Ca : P ratio within the same range. The effect of vitamin D₃ on increase in weight diminished with increase in the Ca : P ratio.

min D₃ content of the diet is most marked and is accompanied by an increased efficiency of utilization.

(f) The dietary Ca does not appear to affect the serum Ca to any great extent under the conditions of Exp. 4, the value being 7.5–9.5 mg./100 ml. of serum in the absence of vitamin D₃ and increasing to 11–12 mg. with intake of the vitamin. Inorganic phosphorus does not appear to be affected, but serum phosphatase follows the same course as in other animals, being considerably reduced with increased

intake of vitamin D and showing an S type of curve analogous to those for other criteria of response (Table 2). The figures confirm those of Correll & Wise (1938).

(g) The regression of gain in weight, t.m.t., and bone ash on log vitamin intake for Ca/P = 1.5/1.5 is statistically linear over the range 10–22.5 B.S.I. units/100 g. of diet. This confirms the findings of Motzok, Hill, Branion & Slinger (1943). The data of the bone ash from Exp. 3, Ca/P = 1.5/1.5, provide the sole anomaly.

(h) Table 3 shows that the slope of the regression line and the reliability of assays carried out under these experimental conditions, judged from values of b^2/s^2 , rises to a maximum at Ca/P = 1.5/1.5 and then falls (Exps. 2 and 4). This is partially confirmed in Exp. 3 in which there is a finer gradation of Ca : P ratios. Covariance analysis reveals a significant variation at the 99.9 % level of probability among the diets, which is associated with Ca and P content. The data of the bone ash from Exp. 3 are an exception.

Differences in the slopes of the regression lines are significant in all cases for $P = 0.95$ in Exps. 1 and 4. In Exp. 3, the following differences are significant: gain in weight among groups 1, 3 and 5; t.m.t. among groups 1, 3 and 5; bone ash among groups 1, 2 and 3. In Exp. 2, the bone ash slopes do not differ significantly, but the slopes for groups 1, 5 and 6 (gain in weight) are significantly less than those for groups 2, 3 and 4, which do not differ significantly among themselves. Also, the slopes for groups 5 and 6 (t.m.t.) are significantly less than those for groups 1–4.

No significant difference is found between the regressions on the same diet in different experiments.

The standard deviations of percentages of bone

ash compare favourably with published values, viz. ± 2.49 and ± 3.63 (Oser (1939) and Motzok *et al.* (1943) respectively).

The present study confirms the suggestion previously made (Jones & Elliot, 1943) that the chick-growth method is a simple, economic and accurate method of biological assay for vitamin D₃.

SUMMARY

1. The effect of variation of the Ca and P content of a standard diet on the responses of chicks to graded doses of vitamin D has been studied. The response criteria utilized were gain in weight, tarso-metatarsal distance and percentage of bone ash of tibiae.

2. The maximum spread of average responses and consequently the steepest slope of the response line and the maximum reliability of assays was obtained with 1.5 % Ca and 1.5 % P in the diet. Variations of ± 20 % in these amounts showed no significant change in the slope and reliability.

3. The regression of response on log dose on this diet is linear between 10 and 22.5 B.S.I. units of vitamin D₃/100 g. of food.

4. The bone ash criterion of response gives a greater reliability of assays carried out under these experimental conditions than the gain in weight or t.m.t. methods. Both the latter methods are simpler and more economical. The gain in weight method requires no special apparatus and is suggested as very suitable for routine assays.

5. Data for serum Ca, P and phosphatase are also presented. A marked fall in serum phosphatase with increase in vitamin D₃ intake is shown.

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