CLXXXIX. INVESTIGATIONS ON THE DETERMINATION OF VITAMIN D. COMPARISON BETWEEN THE PREVENTIVE AND THE CURATIVE METHODS.

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INTRODUCTION.

FOR the biological standardisation of vitamin D, several fundamentally different methods have been devised, but only three of these are in use. These methods are based on the presence or absence of rickets in young rats kept on a rachitogenic diet *plus* a varying addition of vitamin D. The preventive method consists in giving the vitamin D addition to the experimental animals at the same time as they are placed on the rachitogenic diet. The curative method implies the supposition that after rickets is established in the animals they are cured of this lesion by a suitable addition of vitamin D to the rachitogenic diet. Not all investigators insist upon complete recovery, however, but merely a certain degree of recovery. In these two methods the decisive point is, whether at the end of the experiment the animals have rickets or not; yet, as mentioned, a slight degree of rickets may be allowed in tests by the curative method. To decide whether rickets is present or to what extent the condition of rickets has improved, the various authors have used different methods. Macroscopic pathological-anatomical examination is the simplest method, but most authors are agreed that this is relatively rough. It may be supplemented by histological examination of the bones, but this complicates the test considerably.

X-ray examination is just as simple as the macroscopic pathological-anatomical examination. It was first employed by American investigators and it has the great advantage that it is practicable on the living animal. Systematic X-ray examination has been elaborated by Poulsson and Løvenskiold [1928], and others. Methods based on chemical analysis are somewhat more circumstantial, whether concerning the dry substance, ash (including Ca and P) of the bones or the calcium and phosphorus content of the serum.

Of these methods of examination it may be said that the investigators working with them have obtained results they consider satisfactory.

Weighing the two methods, it seems obvious to me that the preventive

method offers several advantages over the curative. It takes less time for the experiment. Moreover a "preventive dose" is more precise than a "curative dose," as several authors differ in their requirements as to the time in which recovery, or relative recovery, is to take place.

The third method to be mentioned in this paper is Shipley's line test [McCollum, Simmonds, Shipley and Park, 1922]. In the last two years considerable attention has been paid to this method. In this method it is not the recovery of rachitic young rats that decides the estimate; the essential feature of the line test is a sign of commencing recovery, namely, the precipitation of calcium phosphate, demonstrable with silver nitrate under influence of the light as a blackening around the epiphyseal lines (tibia). The method has been the subject of thorough investigations [Steenbock and Black, 1925; Coward, 1928; Adams and McCollum, 1928; Bills *et al.* 1931], and judging from these, it appears to be serviceable. Still, several objections may be raised to this method—objections which have indeed been foreseen by several of the authors mentioned, without, I think, being sufficiently refuted.

One weighty objection is that the line test may give a positive result in conditions other than commencing recovery. As has been demonstrated first by Cavins [1924], in young rats there appears on inanition a very distinct precipitation of calcium salts at the epiphyseal line. This precipitation is so marked that it can be demonstrated with X-rays. Of course, the vitamin tests in question do not involve any true inanition, but from the papers quoted it is evident that the weight curve of rachitic young rats will often show a marked change when the diet is supplemented with an addition, for instance, of cod-liver oil. Thus, Adams and McCollum give several instances of such an influence upon the weight curve, the animals showing a natural rise of weight during the fore-period and a loss of weight during the test period. Quite naturally, one will think that these instances involve at least a partial inanition. In going through the tables of Adams and McCollum, it is found in keeping with this, that the most positive results of the line test are obtained in the rats that are losing weight. Of course, rats that are losing weight may be left out of consideration, as well as the rats with a strikingly small weight during the test period; and, in fact, Bills *et al.* [1931] recommend such a procedure. But a method that allows of exclusion of test animals according to arbitrary criteria involves a certain degree of unreliability, and should, therefore, be avoided as far as possible.

Nor is there any doubt that a quantitative estimation of the line test is difficult. I am here leaving out the macroscopic estimation and refer to the microscopic examinations reported by Adams and McCollum and by Bills *et al.* They show that in the line test the histological picture is rather polymorphous. The calcium precipitation may take place in several ways. The last scheme worked out by Bills must be said to show a distinct gradation of the precipitation phenomena. But the fact that there is a rather wide scattering in the results of the line test in animals getting the same addition

makes it evident that the significance of this gradation may still be rather problematic; and it is also questionable whether Bills is justified in working with the average of his arbitrary gradation. Besides, as mentioned before, some animals have already been left out on account of their weight.

EXPERIMENTAL.

In the following I have tabulated a number of experiments by the preventive and the curative methods; and I have also tried to include the line test in these comparative examinations. In the preventive method that dose of cod-liver oil is designated as preventive which is the smallest of the doses employed that is sufficient to keep the test animals free from rickets. In the curative method, the curative dose of cod-liver oil is the dose which is just enough to give the greatest effect, while greater doses have no additional effect under the given experimental conditions.

Technique.

The experiments were made on young white rats, 30 days old, taken direct from the mother. The stock of breeding animals has been kept through 10–11 generations on Gudjónsson's diet No. 4 [1930], so there have been but comparatively slight variations in the size of the litters and the average weight of the young.

Gudjónsson's diet No. 4 consists of:

Skimmed-milk powder	...	...	...	30 %
Rice flour	...	...	...	40 %
Autolysed yeast	...	...	...	15 %
Coconut oil (with 0.3 % shark-liver oil)	...	...	...	15 %

The young rats are transferred from the stock quarters to the experimental room, where daylight is always shut out. The temperature of this room has been recorded by means of a thermograph, and throughout all the experiments it has been 20–25°. Each animal has been alone in its cage. In all cases the diet in the experimental period has been McCollum's diet No. 3143, which consists of:

Whole wheat	...	...	...	33 parts
Whole corn (yellow)	...	...	...	33 „
Wheat gluten, powdered	...	...	...	15 „
Gelatin, powdered	...	...	...	15 „
Calcium carbonate	...	...	...	3 „
Sodium chloride	...	...	...	1 part

The wheat was a mixture of American and Danish wheat (Havnemøllen Ltd., Copenhagen). The yellow corn was La Plata corn (Ballerup Mill Ltd., Copenhagen). The wheat gluten was prepared from a mixture of American and Danish wheat (Leerbeck and Holm, Copenhagen). The gelatin was the

brand V.I. (T. M. Duché and Sons, London). The inorganic salts were chemically pure (*pro analysi*).

All additions of cod-liver oil were given separately in a glass dish as solutions in peanut oil, so that in each case the addition has been 0.20 g.

In this laboratory we prefer to give the addition as mentioned rather than mix it with the food or put it directly into the mouth of the animal. We find it more convenient and just as reliable as the last-mentioned procedure, as the animals devour it completely at once.

X-ray examination. When the animals are entered in the tests, X-ray examination of the left tibia is made at the same time, without anaesthesia. By means of a rubber band the animal is fastened in a wooden box in such a manner that the projection is the same in all the exposures. The X-ray examination is carried out with a Wappler X-ray dental apparatus. The time of exposure is $\frac{1}{4}$ second at 15 cm. with a current of 15 milliamperes and a tension at the tube of *ca.* 50 kilovolts. In judging the X-ray findings importance has been attached to the width of the epiphyseal line and to the density of the metaphysis. The changes are indicated by designations from $\div$ to 3+; or, in case of extreme changes 4+. At the end of the test, X-ray examination is again made of the left tibia under the same conditions.

Autopsy. The animals are killed with illuminating gas and autopsy is performed. This examination has aimed at the following points. (1) Whether the costochondral junctions are swollen, whether there are any so-called costal fractures, and whether there is a "Harrison's groove." (2) Whether the epiphyseal line of the proximal end of the left tibia is wider than normal. The normal width is taken as $\frac{1}{4}$ mm. or less [Schulz, 1929]. The greatest width of this epiphyseal line in rachitic rats has been 1-1 $\frac{1}{4}$ mm. (3) Whether the bone is soft to the knife. No great importance has been attached to this point, however, on account of the subjective character of its estimation. The degree of the changes revealed by autopsy has been designated by values ranging from $\div$ to 3+, in particularly severe degree of rickets 4+.

Line test. The line test of Shipley has been carried out in the curative experiments. After the upper end of the left tibia has been divided by sagittal section, one of the cut surfaces is immersed in a 1 % silver nitrate solution, and in this solution it is irradiated by sunlight or by a mercury quartz lamp (Alpine Sun, Hanau); with the last source of radiation usually for 40 seconds. Then the cut surface is examined under a lens with 6 $\times$ magnification. Under the given experimental conditions the results obtained with this method have been so inconstant as to make their quantitative estimation questionable. Still the relative values have been entered in the Tables, and their significance will be discussed later. In the estimation of these findings, as far as possible, I have followed the scheme of Bills, but only from $\div$ to 3+; in several instances where such an estimation has been impracticable, this is shown in the table by a question mark. In computing the average values these doubtful animals are not taken into account.

Table I. *Preventive method.*

In every case the experimental period has been 28 days. X-ray examination was made of every animal at the beginning of the experiment, when all the rats were found free of rickets. The X-ray findings recorded are the results of the X-ray examination at the conclusion of each test.

Litter	Rat No.	Addition	Initial weight g.	Gain in weight g.	X-ray examination	Autopsy	Bone analysis	
							Dry substance %	Ash %
491 Age of mother: 10½ months. 3rd litter	1832 ♀	0	69	37	4 +	3 +	48.8	19.8
	1833 ♂	0	76	68	3 +	3 +	45.8	19.3
	1834 ♂	1 mg. cod-liver oil	72	60	3 +	2½ +	62.5	24.5
	1835 ♀	1	64	43	3 +	2½ +	52.8	25.1
	1836 ♀	3	71	44	÷	÷	58.4	30.9
574 Age of mother: 10 months. 4th litter	2098 ♀	0.2 g. peanut oil	46	29	3 +	3 +	45.0	12.7
	2099 ♂	1 mg. cod-liver oil	49	40	2½ +	2½ +	43.0	14.2
	2100 ♂	2	43	39	½ +	2 +	46.9	17.0
	2101 ♂	2	47	31	÷	1 +	50.8	19.4
	2102 ♂	3	47	17	÷	÷	59.4	24.5
	2103 ♀	3	46	25	÷	÷	53.4	22.6
	2104 ♀	5	43	25	÷	÷	56.9	23.1
	2105 ♀	5	46	22	÷	÷	54.0	19.7
	2106 ♂	10	50	31	÷	÷	55.2	23.7
586 Age of mother: 15 months. 7th litter	2167 ♀	0.2 g. peanut oil	59	37	3 +	3 +	45.8	19.2
	2159 ♂	1 mg. cod-liver oil	56	32	2 +	2 +	48.4	21.5
	2161 ♂	1	61	33	1 +	1½ +	52.8	23.9
	2160 ♂	2	59	38	÷	÷	53.8	25.5
	2162 ♀	2	50	29	÷	÷	53.7	24.9
	2164 ♀	3	63	40	÷	÷	53.8	25.5
	2165 ♂	3	53	39	÷	÷	53.6	24.4
	2163 ♀	5	50	29	÷	÷	56.2	27.8
	2166 ♀	10	53	42	÷	÷	54.9	28.0
640 Age of mother: 4 months. 1st litter	2373 ♂	0.2 g. peanut oil	39	32	3 +	3 +	43.8	15.2
	2374 ♀	1 mg. cod-liver oil	42	25	1½ +	1½ +	45.0	19.8
	2375 ♀	2	42	35	½ +	½ +	51.1	22.3
	2376 ♀	2	38	34	÷	½ +	52.2	23.1
	2377 ♀	3	38	34	÷	÷	53.0	24.5
	2378 ♀	3	38	32	÷	÷	54.0	25.0
	2379 ♂	5	46	36	÷	÷	55.1	26.6
	2380 ♂	10	38	36	÷	÷	53.4	21.8
641 Age of mother: 8 months. 2nd litter	2381 ♀	0.2 g. peanut oil	46	34	3 +	3 +	45.3	17.5
	2382 ♂	1 mg. cod-liver oil	51	30	2 +	2 +	48.5	19.8
	2383 ♀	1	48	33	2 +	2 +	47.8	20.4
	2384 ♀	2	47	27	÷	1 +	51.4	22.9
	2385 ♂	3	46	33	÷	÷	52.4	22.0
	2386 ♂	5	48	38	÷	÷	53.4	25.7
	2387 ♀	10	50	34	÷	÷	54.0	23.7
661 Age of mother: 9 months. 3rd litter	2448 ♂	0.2 g. peanut oil	42	30	3 +	3 +	44.7	14.8
	2447 ♀	1 mg. cod-liver oil	36	26	2½ +	2½ +	49.0	19.9
	2449 ♀	5	35	27	÷	÷	55.0	24.8
	2451 ♀	5	39	33	÷	÷	54.7	24.6
	2450 ♀	10	36	20	÷	÷	54.4	24.7
	2452 ♂	10	39	30	÷	÷	54.4	23.5
682 Age of mother: 4 months. 1st litter	2546 ♂	0.2 g. peanut oil	30	28	4 +	4 +	40.0	12.9
	2540 ♀	2 mg. cod-liver oil	32	33	÷	÷	55.2	23.4
	2541 ♀	5	29	32	÷	÷	54.7	24.8
	2542 ♀	10	32	42	÷	÷	52.1	24.5
	2543 ♀	10	30	34	÷	÷	54.1	23.0
	2544 ♀	20	30	43	÷	÷	54.1	25.6
	2545 ♀	20	29	39	÷	÷	52.4	23.9
689 Age of mother: 7½ months. 3rd litter	2563 ♂	0.2 g. peanut oil	38	44	4 +	4 +	38.9	13.2
	2564 ♂	2 mg. cod-liver oil	37	37	3 +	3 +	44.6	17.8
	2565 ♂	5	42	49	÷	÷	51.6	24.2
	2566 ♀	10	41	49	÷	÷	50.0	21.7
	2567 ♀	20	36	39	÷	÷	50.5	24.2
	2568 ♂	20	42	38	÷	÷	52.3	23.9
	2569 ♂	40	39	46	÷	÷	51.7	25.0
	2570 ♂	40	40	55	÷	÷	51.6	23.8

Table II. *Curative method.*

The fore-period of every litter has been 28 days, except litter 533 (26 days) and litter 600 (31 days). The after-period of every litter has been 7 days. R I means the X-ray examination at the beginning of the after-period, R II at its conclusion.

			Weight increase								Dry substance %	Ash %
Litter	Rat No.	Addition	Initial weight g.	Fore-period g.	After-period g	R I	R II	Autopsy	Line test			
524	1945 ♂	0	49	35	6	3+	3+	3+	÷	46.8	16.4	
Age of mother:	1946 ♂	0	48	37	11	3+	3+	3+	÷	48.8	17.4	
6½ months.	1947 ♂	5 mg. cod-liver oil	46	38	12	3+	2½+	3+	2+	46.5	17.7	
3rd litter	1948 ♂	5	49	43	6	3+	2+	2+	3+	46.8	17.2	
	1949 ♂	10	50	41	7	3+	2+	2+	3+	47.4	18.1	
	1950 ♂	10	53	41	13	3+	2+	2+	3+	45.5	14.4	
	1951 ♀	20	51	31	11	3+	½+	1+	3+	51.7	22.0	
	1952 ♂	20	51	36	6	2+	½+	½+	3+	50.5	20.5	
533	1962 ♀	0	62	39	÷2	2+	3+	3+	?	55.3	23.8	
Age of mother:	1963 ♂	0	69	42	3	2+	3+	3+	÷	50.5	18.0	
14 months.	1966 ♀	20 mg. cod-liver oil	59	38	2	3+	2+	1+	3+	53.2	21.8	
6th litter	1965 ♂	20	63	46	6	2½+	½+	2+	?	52.6	22.9	
	1964 ♂	40	62	43	÷4	3+	÷	½+	?	52.8	19.9	
	1967 ♀	40	54	38	÷2	2+	÷	÷	3+	56.2	24.0	
542	1991 ♂	0	58	45	6	3+	3+	3+	÷	45.1	13.3	
Age of mother:	1992 ♀	0	51	42	5	2½+	2½+	2½+	?	49.8	20.4	
7½ months.	1993 ♀	0	52	34	2	2+	2+	3+	÷	51.3	20.4	
3rd litter	1994 ♂	10 mg. cod-liver oil	56	48	8	2+	1+	2½+	1+	52.0	19.4	
	1995 ♂	20	54	45	4	3+	1½+	2+	2+	49.7	19.7	
	1996 ♂	40	55	42	0	3+	1½+	2+	1+	48.8	17.7	
	1997 ♂	40	52	42	7	3+	÷	1½+	3+	50.8	17.1	
555	2052 ♀	0	50	25	5	3+	3+	3+	÷	49.8	17.7	
Age of mother:	2053 ♂	10 mg. cod-liver oil	52	40	3	3+	1+	2+	2+	48.6	18.0	
16 months.	2054 ♂	10	50	37	3	3+	½+	1+	½+	53.5	18.7	
7th litter	2055 ♂	20	52	38	1	3+	½+	2+	2+	50.7	16.2	
	2056 ♀	20	48	36	1	3+	1½+	2+	1+	50.9	21.3	
	2057 ♀	40	51	36	2	3+	2+	1+	1+	49.8	19.1	
566	2094 ♂	0.2 g. peanut oil	50	24	9	3+	3+	3+	÷	46.3	15.0	
Age of mother:	2092 ♂	10 mg. cod-liver oil	50	32	5	3+	2+	2+	3+	49.1	20.0	
8 months.	2093 ♀	10	45	33	3	3+	2+	3+	2+	45.9	18.7	
4th litter	2091 ♂	20	47	37	6	3+	2+	2+	3+	44.3	17.8	
	2095 ♀	20	47	30	3	3+	1+	1½+	3+	49.3	19.5	
	2096 ♂	40	49	42	6	3+	2+	2½+	2+	42.8	16.2	
	2097 ♂	40	49	39	3	3+	1½+	2½+	?	45.8	16.0	
600	2230 ♂	0.2 g. peanut oil	50	45	8	3+	3+	3+	÷	43.3	15.0	
Age of mother:	2236 ♀	10 mg. cod-liver oil	46	44	8	3+	2½+	3+	?	43.6	17.8	
6½ months.	2235 ♂	10	48	46	8	3+	1+	1½+	?	46.8	19.6	
2nd litter	2234 ♀	20	42	41	8	3+	1½+	½+	3+	50.2	23.6	
	2233 ♀	20	52	41	10	3+	1½+	1+	3+	47.7	20.7	
	2232 ♀	40	48	37	10	3+	½+	1+	3+	51.5	23.4	
	2231 ♀	40	46	38	11	3+	÷	÷	3+	50.3	24.7	
617	2282 ♂	0.2 g. peanut oil	43	27	3	2½+	2½+	3+	÷	43.9	15.2	
Age of mother:	2283 ♀	10 mg. cod-liver oil	42	31	2	2½+	½+	1+	1+	46.1	18.5	
11 months.	2284 ♀	10	42	28	4	3+	1½+	2+	1+	45.4	18.1	
4th litter	2285 ♀	20	47	30	0	3+	2+	3+	2+	42.0	15.2	
	2286 ♀	20	48	37	1	3+	1+	2+	3+	44.4	17.0	
	2287 ♂	40	46	30	4	3+	÷	1+	2+	45.7	18.1	
	2288 ♂	40	48	25	6	3+	1+	3+	2+	43.9	15.8	

Analysis of the bones. At conclusion of the autopsy, the right tibia is dissected out rapidly. The greatest care has been employed in every instance to prepare the bone in the same way. The periosteum, menisci and ligaments are removed. The fibula is always cut off in the same place. The weighing of the bone is done as quickly as possible and the bone is then placed in an electrical drying-oven, where it is left for 72 hours at 100°. Such an intensive drying process is required, in order to ascertain the dry substance content of the bone. After 72 hours, the bones are constant in weight.

The incineration is done in a muffle-oven heated by 4 Méker burners. The temperature corresponds with that of light red heat. In some experiments with the purest CaCO_3 from Kahlbaum, it was ascertained that this temperature was sufficient to calcine this substance into CaO in less than $\frac{1}{4}$ hour. The bone is placed in the oven in a small porcelain crucible and calcined for $\frac{1}{4}$ hour. It is then taken out of the oven, cooled and moistened with 2 drops of concentrated nitric acid, after which it is again heated in the oven to a red heat for 10 minutes. In every instance this procedure has yielded a firm snow-white ash which does not lose in weight on further heating. After cooling in the desiccator, the final weight is recorded. The results of these examinations are given in detail in Tables I and II and are summarised in Tables III and IV, arranged according to the nature of the addition to the diet. Thus, each group comprises all the rats on the respective addition, giving the limits of variation of the dry substance percentage of the bones, ash percentage, X-ray findings, autopsy findings, and line test. The tables give further the average values obtained in these examinations, but under the last three headings the averages are entered in parentheses, as, I think, no great significance is attributable to these averages.

Table III. *Results obtained by the preventive method (Table I) arranged according to dose of addition.*

V means Extent of variation. A means Average.					
Addition	Number of animals	Dry substance of bone %	Ash of fresh bone %	X-ray examination	Autopsy
Peanut oil or no addition	9	V: 38.9/48.8 A: 44.3	V: 12.7/19.8 A: 16.1	V: 3+/4+ (A: 3.3+)	V: 3+/4+ (A: 3.2+)
1 mg. cod-liver oil	9	V: 43.0/62.5 A: 49.5	V: 14.2/25.1 A: 20.6	V: 1+/3+ (A: 2.3+)	V: 1½+/3+ (A: 2.2+)
2 ,,	9	V: 46.9/55.2 A: 51.9	V: 17.0/25.5 A: 22.3	V: ÷/½+ (A: 0.1+)	V: ÷/2+ (A: 0.6+)
3 ,,	8	V: 52.4/59.4 A: 54.8	V: 22.0/30.9 A: 24.9	V: 0 (A: ÷)	V: 0 (A: ÷)
5 ,,	9	V: 51.6/56.9 A: 54.7	V: 19.7/27.8 A: 24.6	V: 0 (A: ÷)	V: 0 (A: ÷)
10 ,,	9	V: 50.0/57.4 A: 54.0	V: 21.7/28.0 A: 23.8	V: 0 (A: ÷)	V: 0 (A: ÷)
20 ,,	4	V: 50.5/54.1 A: 52.3	V: 23.9/25.6 A: 24.4	V: 0 (A: ÷)	V: 0 (A: ÷)
40 ,,	2	V: 51.6/51.7 A: 51.7	V: 23.8/25.0 A: 24.4	V: 0 (A: ÷)	V: 0 (A: ÷)

Table III shows a gradual rise of the averages with increase of the dose up to 3 mg. cod-liver oil, which is thus the preventive dose. The X-ray and the autopsy findings run very nearly parallel, falling off in + values up to the 3 mg. dose, at which both the X-ray examination and the autopsy give invariably negative results. From percentage of dry substance and of ash, X-ray and autopsy findings, it is consistently evident that cod-liver oil in doses above 3 mg. has had no additional effect. If, with this information in

mind, one turns back to Table I and surveys the material, litter by litter, it becomes strikingly obvious that practically every one of the litters that had a suitable dose of cod-liver oil allows of the same conclusion with regard to the size of the preventive dose. This applies in particular to the X-ray and the autopsy findings. This point is illustrated especially by the litters 574, 640, 661, 682 and 689, whereas litters 586 and 641 leave some doubt whether 3 mg. cod-liver oil has really produced the greatest possible effect on the dry substance and ash content of the bone. In these two litters, however, the X-ray and autopsy findings are in keeping with the findings in the other litters.

Table IV. *Results obtained by the curative method (Table II) arranged according to dose of addition.*

V means Extent of variation. A means Average.						
Addition	Number of animals	Dry substance of bone %	Ash of fresh bone %	X-ray examination	Autopsy	Line test
Peanut oil or no addition	11	V: 43.3/55.3 A: 48.3	V: 13.3/23.8 A: 17.5	V: $2\frac{1}{2} + /3 +$ (A: 2.8 +)	V: $2\frac{1}{2} + /3 +$ (A: 3.0 +)	V: 0 (A: ÷)
5 mg. cod-liver oil	2	V: 46.5/46.8 A: 46.7	V: 17.2/17.7 A: 17.5	V: $2 + /2\frac{1}{2} +$ (A: 2.3 +)	V: $2 + /3 +$ (A: 2.5 +)	V: $2 + /3 +$ (A: 2.5 +)
10 mg. cod-liver oil	11	V: 43.6/53.5 A: 47.6	V: 14.4/20.0 A: 18.3	V: $\frac{1}{2} + /2 +$ (A: 1.5 +)	V: $1 + /3 +$ (A: 2.0 +)	V: $\frac{1}{2} + /3 +$ (A: 1.8 +)
20 mg. cod-liver oil	13	V: 42.0/53.2 A: 49.0	V: 15.2/23.6 A: 21.4	V: $\frac{1}{2} + /2 +$ (A: 1.2 +)	V: $\frac{1}{2} + /3 +$ (A: 1.6 +)	V: $1 + /3 +$ (A: 2.6 +)
40 mg. cod-liver oil	11	V: 42.8/56.2 A: 48.9	V: 15.8/24.7 A: 19.3	V: $\div /2 +$ (A: 0.8 +)	V: $\div /3 +$ (A: 1.4 +)	V: $1 + /3 +$ (A: 2.2 +)

Table IV tells a rather different story. From the averages it will be noticed that the curative dose is about 20 mg. cod-liver oil. The averages of the percentage dry substance and ash show that the outcome of the test falls considerably short of complete recovery, these averages corresponding approximately with the results obtained with the 1 mg. dose in the preventive method. Besides, the difference of the averages is not so distinct by far as that in Table I, and the significance of the average values loses in weight on account of the wide range of the variations; moreover, the limits of variation for the different doses overlap to such an extent that they do not by themselves give any distinction between large and small doses. This is decidedly in contrast with the findings under the preventive method. With the preventive method the lower level of variation shows a gradual rise till the preventive dose is reached, after which this limit does not change to any significant degree.

In other words an attempt to estimate the curative dose by considering a single litter would be impossible; it would be necessary to work with the averages for several litters.

The X-ray and autopsy findings leave a similar impression. The average values of these examinations convey in all essentials the same impression as do the bone analyses; yet it should be pointed out that in these examinations

the lower limit of variation (corresponding to the highest degree of recovery) gives more exact information than does the corresponding limit in the bone analyses. Judging from this lower limit, 40 mg. cod-liver oil would give a greater effect than 20 mg., but I do not think that one may draw any conclusions as to this point from the relatively few animals, especially when the upper limit of variation comes as high as it does in these tests.

Of the line test, really, there is only this to be said, that it turned out negative in the negative control animals and positive in all the cases where cod-liver oil was given, leaving out those cases where the estimation was difficult on account of irregularities. The extent of variation and the average values do not allow of any conclusions.

DISCUSSION.

It seems to me that these experiments suggest that in estimation of vitamin D the preventive method is preferable to the curative method. The experimental period is shorter, and there is less variation in the results from animals on the same addition. The latter point means that the estimation may be carried out with a smaller number of animals. In their employment, upon the whole, the preventive method is the more simple of the two. Here the point is to ascertain whether the test animal has rickets or not—which is comparatively easy with the criteria employed in this investigation. In the curative method the degree of rickets is the important point; and, as is evident from these experiments, this is not easy to estimate. One difficulty is involved in the relatively wide variation of the results in test animals on the same addition; in the bone analyses one may be somewhat justified in judging by an average value. The other difficulty arises if one wants an average estimate on the basis of the X-ray and autopsy findings together with the line test. In relying on an average of one's arbitrary gradations, it is prerequisite that these gradations express the degree of rickets in a certain regular progression—a presupposition that is naturally uncertain. The less arithmetic employed in these biological tests, the safer is the method.

If the question is raised whether the preventive method requires a comparison of the bone analyses, the X-ray examinations and the autopsy findings, in order to settle the presence or absence of rickets, a review of Table I will show that the X-ray examination and the autopsy have separately given the same result as the bone analysis.

SUMMARY.

1. A precise description is given of the technique employed in the preventive and the curative methods for estimation of vitamin D. The results are judged by bone analysis, X-ray examination and autopsy.
2. Parallel series of experiments are made by the preventive and curative methods; the same cod-liver oil is used in both series.

3. A comparison of the results shows that the preventive method is superior to the curative, especially because the animals behave more alike in tests with the first method, so that each estimation can be carried out with a smaller number of animals.

4. A review of the records shows that the bone analyses do not give other or more uniform results than are obtained by X-ray examination and autopsy.

5. On the basis of these findings together with some general considerations, the preventive method is recommended as the standard method for estimation of vitamin D. X-ray examination or autopsy gives sufficient information for judging the results of the test.

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