CCI. ORIGIN OF VITAMIN D IN COD-LIVER OIL: VITAMIN D CONTENT OF ZOOPLANKTON.

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The abundance of the fat-soluble vitamins A and D in the liver of the cod has led many investigators to examine the food supplies of the cod for the source of these vitamins. In the case of vitamin A these researches have been successful. Drummond and Zilva [1922] searching for the ultimate source of vitamin A in nature found that the marine diatom, *Nitzschia closterium*, was capable of synthesising a fat-soluble growth factor. Jameson *et al.* [1922] also demonstrated that *N. closterium* could synthesise this factor under controlled conditions in the laboratory, but Leigh-Clare [1927], while confirming the synthesis of vitamin A by this organism, was unable to prove the presence of vitamin D in such cultures. Thus while the diatom might be the ultimate source of vitamin A in the food of the cod, it was extremely improbable that it could be the source of vitamin D.

As the cod is a deep-water fish, it is unlikely that any synthesis of vitamin D in the cod itself can take place by the action of ultraviolet irradiation, since such irradiation has been shown to be reduced to 1 % of its sub-surface value at a depth of 1·1–2·2 m. and to 0·001 % at 2·9–5·5 m. [Atkins and Poole, 1933]. Bills [1927] has suggested that the cod might be able to synthesise vitamin D in its liver by some process as yet undetermined, since no rich source of the vitamin has been found in its food, but Hess *et al.* [1932] were unable to show any increase of vitamin D in the livers of fish which had received ergosterol intramuscularly or by mouth. Drummond and Hilditch [1930], in examining the relative vitamin values of cod-liver oils from various sources, also investigated the food supplies of the fishing grounds and reported that there was no significant content of vitamin D in the zooplankton (copepods, *etc.*) and only very little in the small fish on which the cod was known to feed. In the same year and later, Drummond and Gunther [1930; 1934] tested extracts of zoo- and phyto-plankton, prepared as described by Collin *et al.* [1934] and showed that vitamin D was probably absent from the phytoplankton but present in the zooplankton extract, to the extent of less than 100 "Coward" (International) units per ml. (equivalence of extract in terms of original matter not stated), by giving small doses to rats in curative tests. They suggested that the zooplankton might derive the vitamin D from irradiation while in surface waters, rather than from ingestion of phytoplankton. Belloc *et al.* [1930] tested the antirachitic potency of the sterols of plankton. They found that the sterols obtained by the saponification of the oils extracted from copepods were antirachitically active without previous irradiation, but that the sterols from ctenophores required to be treated with ultraviolet light before they had any activity. The crystalline sterol from copepods was active in doses of 0·01 mg., but no figures are given by means of which this potency can be correlated with the amount of original material from which the sterol was derived.

These observations, suggesting that copepods, or the sterols extracted from them, possessed antirachitic activity, are in accord with the observation of Russell [1930] that copepods came up to the surface of the water during July, August and September, while earlier in the season they were found at depths of 10–15 m. Russell also observed that the females of the copepod *Calanus finmarchicus* were always at a slightly higher level in the water than the males during the summer.

Thus there seemed to be some evidence for the existence of vitamin D in the copepod but no quantitative data as to the amount of vitamin contained in the natural material.

Collection and preservation of material.

Copepods are minute crustaceans which abound in the cold northern seas and form a large source of food for the young cod during the fishing season. They are not caught in the trawl, but can be collected in special fine-mesh nets. A full description of their nature and the method of collecting them is given by Gunther [1934]. Dr Atkins of the Marine Biological Station at Plymouth undertook to obtain material during the summer of 1931 and again in 1932. Numerous small catches were sent to us packed in ice.

On arrival the material was dried on trays in the hot room at 37° under an electric fan. The drying was usually complete in 24 hours, after which the material, which amounted to about 15–16 % of the original wet weight, was stored in the cold room at approximately 0° in stoppered bottles. Each batch was divided into two portions, one to be stored in the dry condition and the other after mixing with its own weight of hardened cotton-seed oil. This latter procedure was used in 1931 and the only positive result then obtained was with material stored in oil, so it was thought probable that vitamin D was more stable in oily solution. The process was therefore repeated with the 1932 material.

The catches from Plymouth consisted of mixed zooplankton from the coarse and medium mesh silk plankton nets, mainly small crustacea—copepods, with *Pseudocalanus elongatus* Boeck in largest amount, but including other common copepods as in the Plymouth Marine Fauna List.

Experimental methods and results.

Two different methods were applied to the testing of the copepod material, but the most trustworthy results were obtained with the prophylactic method as described by Hume *et al.* [1932]. In this method young rats, at 21 days of age, were put on McCollum rachitogenic Diet 3143 [McCollum *et al.*, 1921] and given doses of the test material, or of standard vitamin D preparation or no dose (to serve as negative control) for a period of 28 days. The rats were then killed and the femur, tibia and fibula of both legs removed, dried, extracted and ashed. The degree of rickets is then calculated by the percentage of ash reckoned on the fat-extracted bone.

The line test method as modified by Coward [1928] was also used in some preliminary experiments in 1932. Young rats weaned at 21 days of age and weighing about 40 g. were kept for three weeks on McCollum Diet 3143. At the end of this period moderately severe rickets had usually developed, and doses of the materials to be tested were then given for a period of 10 days and the results assessed by examination of slices of the distal end of radius and ulna.

The copepod material received during the summer of 1931 was tested by M. M. Gaffikin and E. M. Hume, using the prophylactic method of feeding small

daily doses to young rats on a rachitogenic diet for 28 days. There was some difficulty in persuading the rats to take the copepod doses, and an extract was therefore prepared with light petroleum. Neither the extract nor the residual material was found active, but the dried copepod material preserved in cotton-seed oil proved to be a fairly good source of vitamin D in the small number of experimental animals for which it was available; 1 g. dry copepod daily produced a definite raising of the percentage ash of the bones as compared with controls receiving cotton-seed oil. The results of these tests are set out in Table I.

Table I. *Antirachitic activity of copepod material. Prophylactic experiment, 28 days on McCollum rachitogenic Diet 3143, 1931.*

No. of rats	Supplement given daily	% ash on extracted bone (average)
3	1 unit vitamin D (international standard)	51·2
3	1 g. dried copepod in cotton-seed oil	50·6
3	2 g. cotton-seed oil	47·9

From May to September 1932 larger supplies of material were received and stored, the total dry weight collected being nearly 400 g. All the different samples of dry material preserved respectively alone and with cotton-seed oil, were combined into two main batches in November 1932, and some preliminary experiments using the line test curative technique were carried out. A definitely positive result was obtained by this method, 1 g. daily of either dried copepod or of copepod in cotton-seed oil when administered to rachitic rats produced rather better healing in the epiphysis than 0·25 unit of vitamin D international standard. There appeared to be no difference in antirachitic activity of the dried material whether preserved with or without cotton-seed oil.

Table II. *Antirachitic activity of copepod material. Curative experiment using rats maintained on McCollum rachitogenic Diet 3143 for line test, 1932.*

No. of rats	Supplement given daily	Result of line test
3	0·25 unit vitamin D (international standard)	Good healing
3	1 g. dried copepod	Good healing
3	1 g. dried copepod in cotton-seed oil	Good healing
3	1 g. cotton-seed oil	No healing
3	No supplement	No healing

The phosphorus content of the extracted copepod had been estimated as 0·8 % in 1931; the 1932 dried copepod material was found to contain about 1 % of phosphorus. It was therefore possible that the healing effects observed were due to the phosphorus administered rather than to the vitamin D in the material, since the diet used was of the high-calcium low-phosphorus type. A prophylactic experiment was therefore planned to investigate the effect, if any, of such amount of phosphorus as was given with the test material, and if necessary to allow for it.

Three litters of rats, 27 animals, at 21 days of age and weighing about 40 g. were fed on McCollum Diet 3143 without any supplementary dose for one week. At the end of this time the animals were divided, as evenly as possible, with regard to weight and sex, into four groups as follows:

(1) Rats receiving 0·125 unit of vitamin D international standard daily (6).
(2) Rats receiving 0·5 g. dried copepod daily (7).
(3) Rats receiving 5 mg. of phosphorus daily as NaH_2PO_4 (7).
(4) Negative control rats with no supplement (7).

The phosphorus dose was the amount calculated to be present in the dose of dried copepod and was given as a 20 % solution of sodium dihydrogen phosphate administered by means of a capillary pipette. The results are shown in Table III, which gives the percentage ash of the extracted bone and clearly indicates that the copepod dose produces good calcification.

Table III. *Antirachitic activity of copepod material. Prophylactic experiment, 1933.*

Percentage ash of extracted bones (femur, tibia and fibula) of rats on McCollum rachitogenic Diet 3143, with and without various supplements.

Litter and sex		Supplement given daily: 0·125 unit vitamin D	0·5 g. dried copepod	5 mg. phosphorus as NaH_2PO_4	No dose
2836	♂	40·90	49·27	44·40	—
	♀	46·22	51·53	44·36	30·37
	♀	—	52·16	—	32·52
2840	♂	42·63	48·57	44·92	41·30
	♀	42·93	53·35	46·94	30·24
	♀	—	—	—	35·76
2841	♂	43·57	50·31	37·60	36·99
	♂	44·18	—	38·02	41·80
	♀	—	52·01	*26·00	—
	Average	43·41	51·03	*40·32	35·56

* 42·70, omitting the one low figure.

Discussion.

In spite of the recommendations of Bills *et al.* [1931] and of Dyer [1931] the line test method did not give sufficiently clear-cut results for quantitative conclusions to be drawn. In the animals used here the calcification during healing tended to be on the head of the epiphysis and not as a line in the metaphysis, so that the results were very difficult to interpret and could only be used as a very rough guide to the antirachitic potency of the doses tested. It was, however, obvious that the dried copepod material produced a marked healing effect on the rachitic animals.

In the prophylactic experiments (Tables I and III) the percentage ash of the bones of the rats which had received the copepod doses was probably a maximum figure for the ash content of bones of animals fed on a high-calcium low-phosphorus diet, and the value of the dose in the second experiment was higher than that of the standard vitamin D dose with which it was compared. As in the main experiment the rats received the supplementary doses for only three weeks out of the four-week test period the results are not strictly comparable with those obtained in the researches of Hume *et al.* [1932] and cannot be referred to the curves given in their paper. No absolute value for the antirachitic potency of the copepod material can therefore be stated. There is good agreement between figures obtained for percentage ash of the bones for any one group of rats derived from the three litters, except for one very low result on a phosphorus dose in litter 2841. This animal was difficult to dose and seemed to fare badly, and it would be reasonable to discard this result, in which case the average for the phosphorus group would be 42·70 instead of 40·45, but even so this figure is no higher than that obtained with the dose of vitamin D (0·125 unit international standard). The copepod material in the daily dose (0·5 g. dry weight) given had

therefore a much higher antirachitic potency than can be explained by its phosphorus content alone and must be regarded as a very significant source of vitamin D.

SUMMARY.

1. When tested by the line test method dried copepod material showed some indication of possessing antirachitic activity but the line test was not found satisfactory for the purpose.

2. Prophylactic experiments with a low phosphorus rachitogenic diet showed a degree of antirachitic activity in the copepod material, which was considerably greater than could be accounted for by its phosphorus content.

3. It is therefore concluded that dried copepod, while not one of the richest sources of vitamin D, yet contains sufficient of the vitamin to make this constituent of the zooplankton a good source of vitamin D in the food of the cod.

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