LVI. THE PARENT SUBSTANCE OF VITAMIN D.

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Two years ago we found that cholesterol, having the characteristics then known of the pure substance, was rendered antirachitic by exposure to ultra-violet light [Rosenheim and Webster, 1925]. This fact was also discovered independently, and at about the same time, by the American observers Steenbock and Black [1925] and Hess, Weinstock and Helman [1925].

Although this discovery has since been confirmed and accepted by numerous investigators in many countries, and has found practical application for the cure of rickets in children, we were able to show last year [Rosenheim and Webster, 1926, 1] that cholesterol when purified still further by a chemical method had lost the property of becoming antirachitic by irradiation. In agreement with this observation, it was found that chemically purified cholesterol no longer possesses the absorption spectrum in the ultra-violet region which is characteristic for cholesterol purified by physical means only.

These facts threw new light on the photo-chemical formation of vitamin D. It became evident that the precursor of vitamin D (= provitamin) is not cholesterol itself, but an unknown substance associated with cholesterol as obtained from all natural sources (gallstones, brain, eggs, etc.)¹.

The fact that the presence of an impurity in such a well crystallised substance as cholesterol has for more than a hundred years escaped recognition by chemists is explained by the minute amount (1:2000) concerned, and emphasises the delicacy of the biological test. It may be pointed out that the same method which enabled Reinitzer [1888] to arrive at the correct empirical formula of cholesterol, *i.e.* bromination, has now led also to the discovery of the biologically pure cholesterol.

The newly established facts proved valuable for the rapid progress of the work on the nature of the provitamin, in so far as they enabled us to rely, to a large extent, on the spectroscopic test in place of the tedious animal experiment. Although such a test had already been tentatively suggested by Schlutz and Morse [1925], a firm basis for its application was only established when we showed by animal experiments that the absence of absorption bands

¹ From the historical point of view, it is interesting to note that the suggestion of an impurity in cholesterol as the causative factor concerned in its activation by ultra-violet light had already been considered by Steenbock and Black [1925] and by Schlutz and Morse [1925], the latter observers basing themselves on spectrographic observations only.

in the ultra-violet coincided with the loss by cholesterol of its property of becoming antirachitic on irradiation. It is evident that the destruction or absence of provitamin can be rapidly proved by a negative spectroscopic test, whilst the animal experiment remains indispensable in confirmation of positive spectroscopic evidence.

In the search for the nature of the provitamin we had the privilege of collaborating with Prof. Windaus (Göttingen). Separate preliminary communications on this part of the work, the outcome of mutual suggestions, were made simultaneously by Prof. Windaus [Windaus and Hess, 1927], and by ourselves [Rosenheim and Webster, 1927, 1], according to a friendly arrangement.

In the first instance we established the fact that not only cholesterol but the vegetable sterols also could be freed from the provitamin by bromination. It was found that stigmasterol ($C_{30}H_{48}O$), containing two double bonds, and sitosterol ($C_{27}H_{46}O$), with one double linkage, when purified by way of the bromides, also yield no vitamin on irradiation; whereas all vegetable oils, and the sterols obtained therefrom and not so purified, readily acquire antirachitic properties under the action of ultra-violet rays.

Ordinary cholesterol may be freed from provitamin, as we have since discovered, not only by bromine, but also by various other chemical as well as physical methods. Thus pure cholesterol may be prepared from cholesteryl chloride, which cannot be activated itself, by conversion into cholesterol acetate [Mauthner and Suida, 1894] and subsequent saponification. Treatment of cholesterol in acetone solution by potassium permanganate, whilst leaving cholesterol unattacked, removes the provitamin by oxidation. Of the physical methods the simplest consists in over-irradiation and subsequent recrystallisation. Further, repeated treatment with an efficient charcoal frees ordinary cholesterol from provitamin, but whether this is due to adsorption or oxidation has not yet been investigated.

Whilst these results afforded no means for the isolation or even concentration of provitamin, more direct evidence as to its nature was furnished by the observation that, unlike the vitamin obtained from it, provitamin forms an addition compound with digitonin. Although the product recovered from the digitonide of ordinary cholesterol by fractional extraction with xylene contains provitamin in increased amount, the complete separation of the latter from cholesterol or other sterols by this method does not appear to be practicable.

These observations afford significant information with regard to the chemical nature of provitamin. We may conclude that it is destroyed by oxidation and by bromine, and that it possesses the properties of a sterol, in so far as it forms an addition product with digitonin. The sensitiveness of provitamin to oxidative processes is a characteristic of only one of the known sterols, *i.e.* the highly unsaturated ergosterol ($C_{27}H_{42}O$). Its discoverer, Tanret [1890, 1908] had already studied the oxidation of ergosterol under the influence

of light and air, as evidenced by its change in colour, fall of melting point and optical rotation [see also Schulze and Winterstein, 1906]. Two other properties of provitamin are characteristic also for ergosterol, namely its destruction by bromine¹ and the formation of an insoluble digitonide [Smedley-MacLean and Thomas, 1920].

This similarity in their known properties suggested to Prof. Windaus that provitamin may be nearly related to, if not identical with, ergosterol and we therefore took up again, at his suggestion, the study of ergosterol which we had previously found [Rosenheim and Webster, 1926, 2], after irradiation "to be highly protective even in doses of one milligram" when comparing it under identical conditions with 5 mg. doses of irradiated cholesterol on the same batch of rats.

In view of the more recent work on the absorption spectrum of cholesterol by Heilbron, Kamm and Morton [1926] and by Pohl [1926], we compared the absorption spectrum of ergosterol with that of a specimen of ordinary cholesterol. One would expect ergosterol, with its three double bonds, to show a greater absorption in the ultra-violet region than cholesterol, which has only one double linkage. It was, indeed, found that ergosterol exhibited a very pronounced absorption in the ultra-violet region, the characteristic bands of absorption being identical with those of cholesterol but of enormously greater intensity. The intensity of the absorption was estimated to be 1500–2000 times as strong as that of brain cholesterol². As the result of the irradiation the absorption bands disappear just as in the case of cholesterol; at the same time the product loses its property of being precipitated by digitonin. If provitamin were ergosterol, or an unsaturated sterol of similar constitution, the amount present in ordinary cholesterol would be of the order of 0.05 %, or one part in 2000, and irradiated ergosterol should in this case possess antirachitic activity in correspondingly small doses.

These assumptions have been fully confirmed by biological tests on rats. A daily dose of 1/10000 mg. of irradiated ergosterol cured and prevented rickets in rats kept on a rachitogenic diet. Tests with still smaller quantities, not yet completed, indicate that the limit of activity will prove to be less than 1/50000 mg. Irradiated ergosterol is therefore the most potent antirachitic substance known, 5 mg. being equivalent to approximately 1 litre of a good cod-liver oil.

It is of interest to note that a dose 10000 times greater than what is at present regarded as an effective dose, produced no obvious ill effects on rats under the experimental conditions used.

These results seem to justify the conclusion that the naturally occurring

¹ See also Rosenthal [1922]. According to previous unpublished observations of Windaus, ergosterol cannot be recovered unchanged from its amorphous bromination products.

² Since the provitamin content of cholesterol, purified by physical methods, is variable (see later) this figure necessarily varies with different specimens and may be as high as 1:10,000 or more.

parent substance of vitamin D is ergosterol or a sterol possessing the same absorption spectrum and physiological activity. The admixture in cholesterol from all animal sources (brain, eggs, gallstones, etc.) of a sterol with more than one double bond seems likely, in view of the well-known occurrence in phytosterols from vegetable oils, seeds, etc., of other representatives of this class, containing two double bonds. In this connection the recent work of Anderson [1927] is of interest; he drew attention to the fact that cholesterol preparations obtained from different sources show differences in physical properties, which are ascribed to the probable admixture of isomeric or different sterols. Their partial separation from cholesterol should be possible not only by fractional distillation in a high vacuum, as found by Windaus, but also by fractionation in superheated steam [see the suggestive results of Gardner, 1921].

It should be added that although ergosterol was originally isolated by Tanret from ergot, and named accordingly, similar or identical sterols ("mycosterols") have been obtained from a wide range of the lower plants, occurring, for example, abundantly in the fat extracted from certain yeasts [Smedley-MacLean and Thomas, 1920; Windaus and Grosskopf, 1922]. The ergosterol employed in our own experiments above quoted was actually prepared from ergot, whilst that used by Prof. Windaus was obtained from yeast.

The action of ultra-violet light on ergosterol leads to an obvious physical change and the production of a yellowish resin. The nature of the intramolecular change which gives rise to the vitamin formation is at present unknown.

Experimental.

Methods. The irradiation of the substances to be tested, and the feeding experiments, were carried out by the technique described in our former communications. Negative controls with the basal diet only, as well as positive controls (basal diet + 0.1 cc. cod-liver oil), were made in every set of experiments, although not specifically reported in the tables. For the diagnosis of rickets we relied on the evidence of the radiograph and the estimation of the content of inorganic phosphates in the blood. The large number of radiographs necessary for this work were again kindly taken for us in the department of our colleague, Mr J. E. Barnard, F.R.S., to whom we wish to express our best thanks. It might be mentioned that the results were in all cases decisive, the rats showing either severe rickets or complete absence of rickets.

I. Removal of the provitamin from various sterols.

(1) *Phytosterols.* The method originally worked out for the separation of stigmasterol from other phytosterols by Windaus and Hauth [1906] depends on the insolubility of the tetrabromide of stigmasterol acetate. The necessity of using bromine in its preparation explains, in the light of our present knowledge, the previously surprising result that this naturally occurring sterol could not be activated. It may be pointed out that even the presence of two unsaturated linkages in a sterol is apparently not sufficient to enable it to

act as a provitamin. Stigmasterol (M.P. 170°) irradiated in air for one hour and administered in daily doses of 2 mg. and 4 mg. was unable to protect rats against rickets (see Table I).

Table I.

Substance	Stigmasterol	Sitosterol	Cholesterol recovered from over-irradiated ordinary cholesterol	Olive oil; fraction of "unsaponifiable" not precipitated by digitonin	Cholesterol from ordinary cholesterol-digitonide	
					Fraction (1)	Fraction (2)
Time of irradiation	1 hr.	1 hr.	30 min.	30 min.	30 min.	30 min.
Dose	2 mg. 4 mg.	2 mg. 4 mg.	2 mg. 4 mg.	40 mm. ³ 40 mm. ³ 160 mm. ³	2 mg. 4 mg.	2 mg. 4 mg.
Radiographic result	Rickets	Rickets	Rickets	Rickets	Normal	Normal

The dibromide of sitosterol acetate is soluble and remains in the filtrate from the stigmasterol derivative. Sitosterol itself may be prepared, without previous isolation of its bromide, from the above filtrate by the method described by Windaus and Hauth [1907].

The specimen of sitosterol (M.P. 136–137°) used in the feeding test was prepared from the phytosterols of Soja beans by the above method and when tested biologically in the same way as stigmasterol was found to be inactive (see Table I). Both sterols showed absence of absorption bands in the spectroscopic test.

(2) *Cholesterol*. The bromination method has already been described in our former communication [1927, 2].

(a) *Thionyl chloride* (or phosphorus pentachloride) may be used instead of bromine for the removal of provitamin. Cholesteryl chloride is obtained in the first instance and is subsequently converted into the acetate by boiling with zinc dust in acetic acid solution [Mauthner and Suida, 1894]. Cholesterol was recovered by saponification of the acetate, and gave a negative spectroscopic test.

It may be mentioned in explanation of this result that ergosterol is apparently destroyed by PCl_5 [Reindel, Walter and Rauch, 1927].

(b) *Over-irradiation* destroys vitamin D, as has been previously shown. By converting all the provitamin present in cholesterol into vitamin D by irradiation, and subsequently destroying it by over-irradiation, it is possible to recover pure cholesterol after removal of the reaction products by recrystallisation. From an experiment devised to ascertain the possibility of reactivating cholesterol which had been deactivated by over-irradiation in the dry state, Hess, Weinstock and Sherman [1925] drew the conclusion that a substance "quite different from the original cholesterol had been formed." We found it necessary to irradiate a solution, and not the crystals, of ordinary cholesterol in order to induce the complete destruction of both the provitamin and the vitamin.

The substance was dissolved in ether and irradiated in a quartz flask for 10 hours. After evaporation of the ether, the cholesterol was recrystallised from alcohol and the whole process repeated. The final product was again recrystallised, irradiated for 30 minutes, and administered to rats in daily doses of 2 mg. and 4 mg. (see Table I). The spectroscopic, as well as the biological test, gave negative results.

(c) *Charcoal* easily removes the provitamin from ordinary cholesterol. Five g. cholesterol were dissolved in 50 cc. 96 % alcohol and boiled with 1 g. norit for half an hour, under reflux. The filtrate was treated again with 1 g. norit. The recovered cholesterol was found to be free from provitamin by the spectroscopic test (see Fig. 1, Plate I). The same result was obtained by using Merck's blood charcoal.

In view of the variable content in provitamin of commercial samples of cholesterol, possibly purified by means of charcoal, caution is indicated against the indiscriminate use of irradiated cholesterol as a source of vitamin D, when testing for the presence of vitamin A. We experienced a failure, unexplained at the time, of a series of feeding experiments dealing with vitamin A estimations owing to the employment of a cholesterol prepared from cod-liver oil by ourselves. The specimen in question had been carefully purified by charcoal in order to adsorb the persistent yellow pigments, which could not be removed by recrystallisation alone. We have since come across two commercial samples of cholesterol equally deficient in provitamin. We propose to use a solution of irradiated ergosterol in future work on vitamin A.

(d) *Potassium permanganate* in acetone solution may also be used. The procedure need not be described in detail, and it is sufficient to state that cholesterol obtained on concentration of the solution (after the filtration from MnO_2) is spectroscopically inert.

II. *Precipitation of provitamin by digitonin.*

We obtained decisive evidence for the formation of a digitonide of provitamin in the first instance by an indirect route. Olive oil (200 cc.) was saponified in the usual way. The unsaponifiable fraction could easily be activated by means of irradiation, as shown by a separate experiment. The alcoholic solution of the unsaponifiable fraction (0.64 g.) was precipitated with excess of digitonin. The filtrate from the digitonide, after evaporation *in vacuo*, was taken up in water and extracted with ether. The ether-residue was irradiated for 30 minutes, dissolved in 3 cc. liquid paraffin and administered in daily doses of 40 mm.³ and 160 mm.³ These amounts, corresponding to 2.5 cc. and 10 cc. of irradiated olive oil, were unable to prevent severe rickets, thus proving that provitamin had been completely removed by digitonin (see Table I).

Direct evidence of the presence of provitamin digitonide in ordinary cholesterol digitonide was furnished by precipitating ordinary cholesterol (from gallstones) with digitonin. The digitonide was recrystallised from

pyridine-alcohol, washed with ether and dried. The product was fractionally extracted with boiling xylene, fraction (1) representing cholesterol obtained after the first eight hours' extraction. The extraction was continued for another eight hours and the product labelled fraction (2). Spectroscopic examination of fraction (1) showed a considerable increase of absorption in the ultra-violet, indicating about double that of the original cholesterol. The animal experiment proved that both fractions prevented rickets after irradiation, when administered in daily doses of 2 mg. and 4 mg. (see Table I). No attempt was made to assess the difference in antirachitic activity of the two fractions by the animal experiment.

III. *Ergosterol as provitamin D.*

The specimen of ergosterol used in this work was part of the same preparation, kindly put at our disposal by Dr H. A. D. Jowett, which we had previously found to be easily activated [1926, 2]. It had been prepared from ergot and since it possessed the constants given by Tanret [1908] for pure ergosterol, M.P. 162° , $[\alpha]_{5461}^{19^{\circ}} = -127^{\circ}$, must be considered as free from fungi-sterol.

(1) *Spectroscopic evidence.* The absorption spectra were photographically recorded by means of a Hilger's quartz spectrograph, using as a source of light a quartz mercury vapour lamp. An all-quartz cell of 10 mm. width was used, pure ether (free from peroxides) serving as the solvent.

The absorption of ultra-violet light by ergosterol was found to be enormous, even a solution of 1 : 2000 absorbing the whole of the region from $310\mu\mu$ onwards (see 5, Fig. 1, Plate I). Many photographs were taken in which the absorption spectra of a series of dilutions were recorded, the exposure being kept constant. In Fig. 2 (Plate I) the strength of the solutions varied from 1 : 5000 (1) to 1 : 50000 (10), even this extreme dilution still showing distinct absorption between $280-310\mu\mu$. It is interesting to note that the absorption extends well into the ultra-violet region of sunlight, which is able to activate ordinary cholesterol, as we have previously shown.

In Fig. 1 (2) is shown for comparison the absorption spectrum of a solution 1 : 10 of ordinary cholesterol. The removal of the provitamin by charcoal treatment is illustrated by Fig. 1 (3), whilst (4) brings out the striking influence of the addition of ergosterol 1 : 20000, to a solution of pure cholesterol 1 : 10. By comparing (2) and (4) it will be seen that the absorption in the critical region of from $280-310\mu\mu$ is almost identical, and the conclusion may be drawn that the absorption in the ultra-violet of ergosterol is about 2000 times that of ordinary cholesterol. This merely qualitative judgment was confirmed by quantitative measurements made in Göttingen (see footnote, p. 391).

Although the absorption of pure cholesterol in the ultra-violet is powerfully affected by the addition of this minute amount of ergosterol, it might be mentioned that no change in physical properties, such as melting point and

specific rotatory power which usually serve to the chemist as a criterion of purity, can be demonstrated in an artificial mixture of pure cholesterol containing 0.05 % ergosterol. The admixture of ergosterol cannot be detected by the usual colour reactions, since no specific colour reaction for it in the presence of other sterols is so far known.

The gradual disappearance of the characteristic absorption bands, which takes place on irradiation, may be taken to coincide with the formation of vitamin D, which is also brought into evidence by the coincident decrease of digitonin-precipitability. By taking a series of absorption spectra after exposing a 1 : 1000 ergosterol solution to the prolonged action of ultra-violet light, it was found that an exposure of approximately five hours was necessary to cause the complete "bleaching out" of the absorption. After the same period the product did not possess the property of forming an insoluble digitonide.

(2) *Biological evidence.* Two main series of experiments were carried out. In the first, ergosterol was irradiated as a solid in air for 30 minutes, and in the second series a solution in pure ether was irradiated. A quartz cell ($10 \times 25 \times 25$ mm.) with a narrow tubular neck was employed in order to restrict the exposure of the solution to air as much as possible. The ether solution was irradiated in the closed cell for 30 minutes and 5 hours respectively. After evaporation of the solvent, the irradiated material was dissolved in olive oil, which had previously been tested for the absence of vitamin D by the animal experiment. The daily doses varied from 0.025 mg. to 0.0001 mg. ($= 1/40$ to $1/10000$ mg.). The results were identical in all cases, *i.e.* complete protection and cure were effected even by the smallest daily dose of $1/10000$ mg. (see Table II). Experiments are in progress with still smaller doses. We have reason to believe that the limiting dose will be considerably less than $1/50000$ mg. when the irradiation is carried out under the most favourable conditions in the absence of oxygen.

Table II.

Substance	Ergosterol (in ethereal solution)		Ergosterol (as a solid)
	30 minutes	5 hours	
Time of irradiation	1/40, 1/80,	1/40, 1/80,	30 minutes
Dose in mg.	1/500, 1/1000	1/500, 1/1000	1/40, 1/80, 1/125,
	1/10000	1/10000	1/250, 1/500, 1/1000
			1/10000
Radiographic result	Normal	Normal	Normal

An interesting proof of the stability of irradiated ergosterol is afforded by the fact that a solution in an inactive oil kept for $1\frac{1}{2}$ years was found to be still active when tested in daily doses of $1/1000$ mg. This solution had served for our original work on the activation of ergosterol and had been kept in a closed flask in a cupboard.

Fig. 1.

Fig 2.

SUMMARY.

(1) Cholesterol and phytosterols, purified by the methods described, can no longer be rendered antirachitic by ultra-violet light.

(2) The nature of the impurity present in ordinary sterols which is responsible for their activation by ultra-violet light has been investigated. The evidence obtained leads to the view that the impurity is a sterol of an unsaturated and labile type, of which ergosterol is the only known representative.

(3) Ergosterol was found to possess the same characteristic absorption spectrum in the ultra-violet as non-purified cholesterol, the intensity of the absorption, however, being enormously increased. Assuming it to be ergosterol, the amount of impurity present in ordinary cholesterol was judged by comparative spectroscopic examination to be of the order of 1 : 2000.

(4) Irradiated ergosterol in daily doses of 1/10000 mg. cures and prevents rickets in rats on a rachitogenic diet.

(5) It is concluded that the natural parent substance of vitamin D is ergosterol, or a highly unsaturated sterol of similar constitution, which is converted into vitamin D by irradiation.

EXPLANATION OF PLATE I

Fig. 1. Comparative absorption spectra of cholesterol and ergosterol in solution in ether.

(1) Ether. (2) Ord. cholesterol in ether, 1 : 10. (3) Same after charcoal treatment. (4) Pure cholesterol, 1 : 10, plus ergosterol 1 : 20000. (5) Ergosterol, 1 : 2000.

Fig. 2. Absorption spectra of ergosterol.

(1-10) Ergosterol in ether, 1 : 5000 to 1 : 50000. (11) Ether.

Source of light: quartz mercury lamp.

Width of cell: 10 mm.

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