

CXI. THE VITAMIN D PROBLEM.

II. OPTICAL ROTATION OF VITAMIN D.

BY ENGBERT HARMEN REERINK AND AART VAN WIJK.

*Natuurkundig Laboratorium der N. V. Philips' Gloeilampenfabrieken
Eindhoven-Holland.*

(Received June 2nd, 1931.)

THE most simple reaction, that can take place when ergosterol is irradiated with ultraviolet rays, is, of course, Ergosterol $\rightarrow$ L, i.e. the ergosterol is transformed into one single substance, L.

In a previous paper [Reerink and van Wijk, 1929] we have been able to show that under certain conditions the reaction proceeds in such a way that the resulting phenomena are in full agreement with this reaction scheme. This was concluded from a study of the changes in the absorption spectrum, the necessary conditions being:

- (1) the light used for the irradiation must not contain rays of wave-length less than $284\text{ m}\mu^1$;
- (2) the irradiation has to be stopped before more than about 50 % of the ergosterol has been transformed;
- (3) oxygen must be completely absent;
- (4) the solution must be vigorously stirred.

This result was of some importance, since the preparations obtained by irradiation of ergosterol under these conditions show intense antirachitic activity, the degree of activity being proportional to the degree of transformation of the ergosterol [Everse and van Niekerk, 1931, 1, 2]. Therefore, if indeed a single substance is formed under these conditions, this substance must be vitamin D. This conclusion being very important, we have tried to prove its correctness in an independent way, viz. by the measurement of the changes in the optical rotation on irradiation.

EXPERIMENTAL.

Since the complete absence of oxygen is a very important condition for the reproducibility of experiments on the irradiation of ergosterol, we have taken care to perform all operations *in vacuo*. In order to be able to do so, we have made extensive use of an implement well known in vacuum technique, the working of which will be easily understood from Fig. 1 *a*. In this way it is quite easy to connect an evacuated vessel with another one,

¹ Formerly we put the limit at $275\text{ m}\mu$, but since then we have found it better to cut off at a somewhat greater wave-length [see Reerink and van Wijk, 1931].

which in its turn can be evacuated and sealed off from the pump; by breaking the partition-wall by means of an iron ball, operated magnetically, the communication between the two vessels is brought about. Thus it is possible *e.g.* to distil a liquid from one vessel into another, or to recrystallise a substance from a solvent and filter off the crystals with a sealed-in filtering disc of sintered glass *etc.*, all operations being performed under air-free conditions.

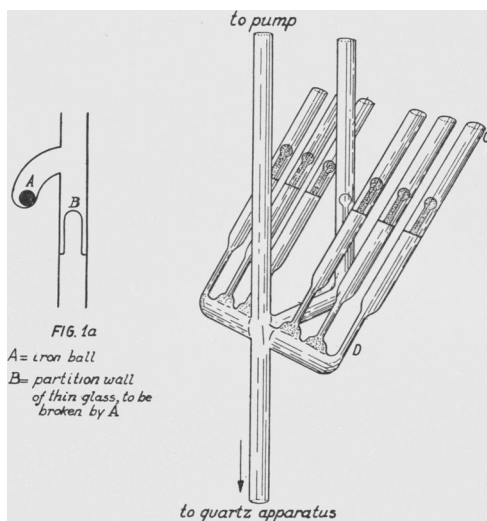


Fig. 1 b.

The solutions were irradiated in an all-quartz apparatus, containing an electromagnetic stirrer and bearing a device for withdrawing samples. The working of the latter will be understood from Fig. 1 b: a small part of the solution to be examined can be decanted into one of the sample-tubes, *C*; the tube is then sealed off at the capillary part, *D*, and can be used for further examination. A polarimeter-tube was directly sealed to the apparatus.

The ergosterol had been recrystallised from alcohol-benzene [Bills and Honeywell, 1928] and proved to be quite pure, $[\alpha]_D^{18}$ being -134.0° in chloroform. Pure peroxide-free ether was used as a solvent. The whole apparatus was thoroughly evacuated until the pressure of non-condensable gas was less than 0.001 mm., and then sealed off. The solution was irradiated with a quartz mercury arc through a filter of xylene-alcohol, cutting off all radiation below 284 $m\mu$. After a suitable period of irradiation, several readings of the rotation for the yellow sodium line were taken with a Schmidt and Haensch polarimeter; then one of the sample tubes was partially filled with solution and carefully sealed off from the rest of the apparatus. The degree of transformation of the ergosterol was estimated by the digitonin method in the sample withdrawn. With the rest of the solution the irradiation was continued, *etc.*

EXPERIMENTAL RESULTS.

(a) *Rotation.*

The results of three different series of experiments are shown in Table I, in which the first column gives the time of irradiation in hours, the second the % quantity of unchanged ergosterol as found with the digitonin method, and the third the specific rotation of the irradiated preparation.

Table I.

Time of irradiation in hours	% ergosterol	Rotation measured	Rotation calculated
0	100.0	-105.2°	.
1	93.9	-95.8	-94.5°
2	90.7	-87.5	-89.2
4	79.6	-71.3	-70.1
6	70.9	-56.9	-55.1
8½	60.2	-40.5	-36.8
0	100.0	-105.2	.
¼	96.8	-100.4	-99.5
¾	77.7	-68.7	-66.9
0	100.0	-105.2	.
1	94.0	-94.3	-94.8
2	90.1	-85.2	-88.1
4	78.6	-70.0	-68.4
6	72.0	-55.3	-57.0
9	62.2	-38.4	-40.2
13	52.9	-21.5	-24.2

More illuminating is Fig. 2, where the measured values of the rotation from the three series of experiments are plotted against the relative amounts of unchanged ergosterol. This figure indicates clearly, that the points fall on

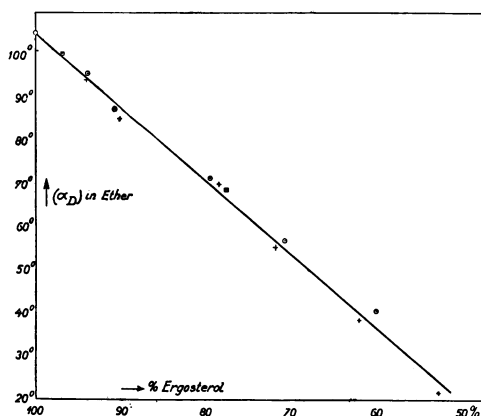


Fig. 2.

a straight line, the deviation being of the order of magnitude of the experimental errors. Now this behaviour is exactly what must be expected if the reaction-scheme of p. 1001 holds true.

If α_E and α_L are the specific rotations of ergosterol and of the reaction product, then, if x % of the ergosterol have been left unchanged, the measured rotation must be

$$\alpha_m = x \cdot \alpha_E + (100-x) \alpha_L,$$

or

$$\alpha_m = x \cdot (\alpha_E - \alpha_L) + 100 \cdot \alpha_L,$$

giving a linear relation between α_m and x .

Putting the experimental parameters of the line in Fig. 2, equal to the theoretical parameters of the above formula, we obtain for $\alpha_L = +66.7^\circ \pm 2.7^\circ$ (in ether).

By means of this value for the specific rotation of the reaction-product, and of that of ergosterol, the corresponding rotation can be calculated for every degree of transformation (second column in Table I). These calculated rotations are shown in the fourth column of Table I; the deviation of their values from the experimental values prove to be within the limit of experimental error.

It may be concluded from the foregoing, that, just as in the experiments on the absorption spectrum, the experimental facts are in complete harmony with the assumption that the first long-wave reaction-product of ergosterol is a single substance. Still, as was already pointed out in an earlier paper, both series of experiments may also be explained by a more complex hypothesis, *viz.*, that several substances are formed simultaneously in a constant proportion. As up to the present in our experiments we have never found irrefutable facts that could not be explained by the former assumption, which moreover has the advantage of its very simplicity, we are inclined to consider the individuality of the substance L as practically certain.

(b) *Rate of the photochemical reaction.*

From the results of these experiments, an interesting calculation can be made concerning the velocity of the reaction: Ergosterol $\rightarrow$ L, under our experimental conditions.

Since the concentration of the solution is so high that practically all active radiation is absorbed, the rate of disappearance of the ergosterol would be constant, if the intensity of the radiation were kept constant and if the ergosterol were the only absorbent. Now for practical purposes the intensity of the radiation remains constant in one series of experiments, but the ergosterol is not the only absorbent, as the vitamin D, which is formed, also shows an absorption in the same range of wave-lengths. Under these circumstances the incident radiation is divided between the ergosterol and the vitamin, and we may safely assume that the ratio in which the radiation is absorbed by the separate substances is

$$I_{\text{erg}}/I_{\text{vit}} = (\epsilon_{\text{erg}} C_{\text{erg}})/(\epsilon_{\text{vit}} C_{\text{vit}}),$$

where I_{erg} and I_{vit} are the intensities of the radiation absorbed by ergosterol and the vitamin respectively, ϵ_{erg} and ϵ_{vit} are the absorption indices and

C_{erg} and C_{vit} the concentrations. Strictly, this equation holds for each wave-length present in the radiation used. From the absorption curves, given in our previous paper [1929], we can deduce, however, that for our purpose as an approximation, the average of ϵ_{erg} may be put equal to the average of ϵ_{vit} in the range of wave-lengths under consideration. Our equation then becomes:

$$I_{\text{erg}}/I_{\text{vit}} = C_{\text{erg}}/C_{\text{vit}}.$$

Since now

$$I_{\text{total}} = I_{\text{erg}} + I_{\text{vit}}$$

we have all the necessary data to calculate the rate of disappearance of the ergosterol:

$$-(dC_{\text{erg}})/dt = k \cdot I_{\text{erg}}, \text{ in which } I_{\text{erg}} = C_{\text{erg}}/(C_{\text{erg}} + C_{\text{vit}}) \cdot I_{\text{total}},$$

hence $-(dC_{\text{erg}})/dt = k \cdot I_{\text{total}} \cdot C_{\text{erg}}/C_{0\text{erg}}^1$ or $-(dC_{\text{erg}})/dt = k' C_{\text{erg}}.$

After integration, this equation leads to

$$\log C_{\text{erg}}/C_{0\text{erg}} = k'' \cdot t, \text{ where } k'' = 2.303 k'.$$

We can now put this equation to the test with the data from Table I: this is done in Table II, where k'' is calculated for the two longer series of

Table II.

1	2	3	4	5	6
t	C_E/C_{E0}	k''	t	C_E/C_{E0}	k''
0	1.000	—	0	1.000	—
1	0.939	0.027 ⁵	1	0.940	0.027
2	0.907	0.021	2	0.901	0.023
4	0.796	0.025	4	0.786	0.026
6	0.709	0.025	6	0.720	0.024
8½	0.602	0.026	9	0.622	0.023
			13	0.529	0.021

measurements. From an inspection of the figures in the third and sixth columns of this table it can be concluded that the calculated factor k'' is remarkably constant in each series of experiments. This proves that our assumptions must have been fairly correct.

(c) Crystalline preparation.

There is still another indication for the purity of the preparations of the reaction-product obtained in this way, that is, the remarkable ease with which it is obtained in the crystalline state when the excess of unchanged ergosterol is removed by recrystallisation from ether and alcohol *in vacuo*. The procedure (including the precipitation of the residual ergosterol with digitonin) may be described somewhat more in detail.

An ethereal solution of ergosterol, *e.g.* 5 g. in 600 cc. is irradiated in the quartz apparatus during some hours, till a proper degree (*e.g.* 25 %) of transformation, as measured by the rotation, has been reached. The solution is

¹ In agreement with the assumption that only one substance is formed from the ergosterol, $C_{\text{erg}} + C_{\text{vit}} = C_{0\text{erg}}$, the ergosterol concentration at $t=0$.

then transferred into another vessel, from which part of the ether is distilled. A large part of the unchanged ergosterol is then removed by cooling on ice and decanting the supernatant liquid into another vessel through a sintered glass-filter. The ether is then distilled off and replaced by about 75 cc. alcohol. By shaking vigorously the reaction-product is extracted from the crystalline mass along with only about 0.15 g. of ergosterol; then the solution is filtered into a new vessel, containing an alcoholic solution of digitonin, which precipitates the rest of the ergosterol. After filtering the liquid into a new vessel the alcohol is evaporated, and the reaction-product is extracted with ether. The ethereal solution again being filtered into another vessel the ether is distilled off; the ergosterol-free reaction-product is then obtained as a white crystalline mass, consisting of rosettes of fine needles. These crystals are very soluble in all ordinary organic solvents, but can be crystallised from strongly cooled alcohol or from diluted methyl or ethyl alcohol. Fig. 3 shows the crystals as they are obtained by evaporation of an ethereal solution.

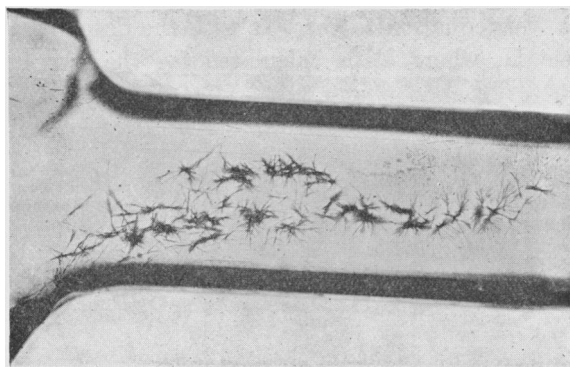


Fig. 3.

There is, however, one very interesting complication concerning this crystalline preparation, *viz.* the specific rotation is not 67° , as would be expected from the above measurements of the optical rotation of the primary reaction-product. In various experiments we have always obtained preparations with a rotation more or less exceeding the theoretical value. The longer the time between irradiation and final measurement of the rotation, and the higher the temperature used in the series of operations of recrystallisation and precipitation with digitonin or evaporation of the solvent, the higher was the value found for the rotation. For example, in one instance we found $[\alpha]_D = +94^\circ$, in an experiment in which 20 days elapsed between irradiation and the final measurement, whilst the temperature had several times been as high as 50° . In another experiment, in which all operations had been performed as expeditiously as possible, and in which the temperature was kept as low as possible, the rotation was found to be $+68.1^\circ$.

This increase of the rotation even takes place when the solution in ether

is preserved on ice, as is shown in Table III, giving in 14 days an increase of about 7 %.

Table III.

Time	Rotation
0 days	+ 68.1°
3 "	+ 69.7
5 "	+ 71.1
14 "	+ 72.6

This shows that the crystalline substance is very unstable even in absence of oxygen and at a low temperature when it is dissolved in ether.

From the foregoing it follows that, though some of the physical properties of the pure vitamin can be derived comparatively easily from the properties of mixtures of vitamin D and ergosterol, other properties, for the measurement of which the vitamin D must be separated from the ergosterol, we cannot for the moment give with so much certainty.

We may, however, give some properties of one of our preparations from which the ergosterol had been removed as far as possible by means of several recrystallisations from alcohol.

Melting-point (in sealed evacuated tube) 115°–117°.

The ergosterol content of the preparation proved to be 3.7 %.

Specific rotation (corrected for ergosterol content): $[\alpha]_D = +100^\circ$ (ether).

Analysis: H, 11.30 %, C, 85.4 %; calculated for $C_{27}H_{42}O$: H, 11.07 %, C, 84.8 %.

From the results of the analysis it may be concluded, that, during the very short time the preparation has been in contact with the air, no appreciable oxidation has taken place.

The crystals seem to be only very slightly hygroscopic, but in contact with the air gain rapidly in weight by oxidation, as is shown by the following experiment:

43.6 mg. were taken;

after 1 hour	the weight was 43.7 mg.:	increase 0.2 %.
„ 42 hours	„ 45.5 mg.:	increase 4.4 %.
„ 66 „	„ 45.9 mg.:	increase 5.3 %.
„ 114 „	„ 46.4 mg.:	increase 6.4 %.

In the meantime the colour had changed from pure white to bright yellow.

That this gain in weight is not a result of absorption of water follows from the fact that it is not reversed in a high vacuum.

Even when the preparation is kept in a thoroughly evacuated desiccator and brought into contact with air only during some brief interval necessary for taking out parts of it, it acquires a slight yellow tinge within a week and becomes oxidised.

Analysis: crystals, kept *in vacuo* for 1 week: H, 10.98 %, C, 83.5 %. Crystals kept in air for 114 hours: H, 10.37 %, C, 80.1 %; calculated: H, 11.07 %, C, 84.8 %.

This proves that the crystals are very susceptible to oxidation.

The absorption spectrum of the vitamin D after preservation is not the same as that of the pure vitamin D; as we have not yet found a definite form of absorption spectrum that is common to all such preparations, we will not for the present go further into this question.

In our former paper [1929] we mentioned the fact that vitamin D can be obtained in a crystalline state. These crystals were obtained by rapidly evaporating and thus cooling an ethereal solution; they showed a melting-point below 0° . The same phenomenon can be observed if an ethereal solution of the crystalline preparation described in this paper is treated in the same way. Thus it may be concluded that the crystals mentioned in the former paper were of the same substance as the present ones, the difference in melting-point being caused by ether of crystallisation.

DISCUSSION.

(a) *Rotation.* The data which are found in the literature concerning the rotation of reaction-products of ergosterol show a wide variation. Whereas the earlier work of Windaus *et al.* [1929] and of Castille and Ruppel [1929] seems to indicate that the photochemical reaction-products show a relatively small rotation, Lahousse and Gonnard [1929] find for their most potent products a value for $[\alpha]_{578m\mu}$ of $+25^{\circ}$ (alcohol).

In a recent paper, Windaus [1930] gives the rotation of a large number of ergosterol-free reaction-products; he states that his most potent samples prepared by irradiation with the Mg-spark show a low negative rotation; a high positive rotation was found for samples prepared with long-wave irradiation (Hg-arc and Uviol-glass filter), and for aged, or heated and over-irradiated samples. The dextrorotatory, long-wave irradiation products, which we would expect to be most similar to our preparations, proved to be less active than those prepared by irradiation with the Mg-spark. The difference between the results of Windaus and our own may be due to the fact that his preparations were mostly long-time irradiation-products (*ca.* 80 % of ergosterol transformed) whereas in our experiments the degree of transformation did not exceed 50 %.

(b) *The crystals.* Besides the crystals mentioned in our former publication, crystalline reaction-products from irradiated ergosterol have been described by Windaus [1930; Windaus *et al.* 1930] and by Askew *et al.* [1930, 1, 2]. Whereas the crystals described by Windaus are produced by over-irradiation and consequently do not show antirachitic activity, the crystalline preparation of Askew *et al.* shows a very intense activity and therefore consists at least partly of vitamin D. The melting point as well as the form of the crystals and the absorption spectrum suggest a similarity between these crystals and ours.

Since writing the above, we have had the privilege of discussing the subject with the English authors, who kindly informed us that their further purified crystalline preparation shows a much higher dextrorotation than ours; this

proves that the substance must be different. As to the nature of our crystals, we already pointed out that they probably do not consist of pure vitamin D, since the rotation of the crystalline preparation does not as a rule tally with the calculated rotation for pure vitamin D.

The way in which Askew *et al.* have obtained their crystalline preparation, *viz.* by distillation *in vacuo* of the crude reaction-product, implies the necessity of heating the vitamin D to temperatures above 100°; therefore in these experiments too the vitamin D is likely to undergo a change of the same nature as that occurring in our method of preparation.

The instability of vitamin D on storage or on heating has been described by Windaus [1930; Windaus and Auhagen, 1931] and by Askew *et al.* [1930, 1]. Though in some respects our results are not very different from those of these authors, there still exist a good many points in which they differ. Since, however, the experiments have been performed with reaction-products which may show a good deal of dissimilarity, the difference in the results does not at the present time provide a useful starting-point for a discussion. We wish to emphasise however one point of difference, *viz.* that, contrary to the experience of the other authors, we have never found an increase in absorption if the ergosterol-free reaction-product was irradiated with short-wave ultra-violet rays [Reerink and v. Wijk, 1931].

SUMMARY.

Experiments are described in which the change of the rotation of ergosterol solutions on irradiation with long-wave ultra-violet rays is measured.

If the necessary precautions are taken, this change is very simple, the rotation showing a linear dependence on the degree of transformation of the ergosterol. These experiments provide further proof for the hypothesis, put forward in our previous publication, *viz.* that under definite conditions long-wave irradiation of ergosterol gives rise to one reaction-product only, which, on account of its intense antirachitic activity, must be vitamin D.

From these experiments the rotation of vitamin D can be calculated to be

$$[\alpha]_D = + 66.7^\circ \pm 2.7^\circ \text{ (in ether).}$$

From correctly irradiated solutions, crystalline ergosterol-free preparations of vitamin D may easily be produced by recrystallisation and treatment with digitonin *in vacuo*. The properties of these preparations vary with the time and temperature used during the performance of the necessary operations, as a result of the instability of the vitamin D.

In conclusion we wish to express our thanks to Mr W. Breen and Mr J. Freen for their valuable assistance in the performance of the experiments.

REFERENCES.

- Askew, Bourdillon, Bruce, Jenkins and Webster (1930, 1). *Proc. Roy. Soc. Lond.* B **107**, 77.
—— ——— ——— (1930, 2). *Proc. Roy. Soc. Lond.* B **107**, 91.
Bills and Honeywell (1928). *J. Biol. Chem.* **80**, 15.
Castille and Ruppel (1929). *Bull. Acad. Roy. Med. Belg.* 799.
Everse and v. Niekerk (1931, 1). *Ned. Tijdschr. Geneesk.* **75**, 1101.
—— ——— (1931, 2). *Strahlentherapie*, **40**, 728.
Lahousse and Gonnard (1929). *J. Soc. Franç. Physique*, **280**, 114.
Reerink and v. Wijk (1929). *Biochem. J.* **23**, 1294.
—— ——— (1931). *Strahlentherapie*, **40**, 728.
Windaus, (1930). *Nachr. ges. Wiss.* (Göttingen), 36.
—— and Auhagen (1931). *Z. physiol. Chem.* **196**, 108.
—— Gaede, Köser and Stein (1930). *Liebig's Ann.* **483**, 17.
—— Westphal, v. Werder and Rugh (1929). *Nachr. ges. Wiss.* (Göttingen), 45.