XCV. THE SPECIFICITY OF ERGOSTEROL AS PARENT SUBSTANCE OF VITAMIN D.

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THE study of the antirachitic activity induced in various sterols by irradiation, which led to the discovery of ergosterol as the most potent source of vitamin D [Rosenheim and Webster, 1926; 1927, 1], has subsequently brought to light an interesting relationship between chemical structure and potential biological activity.

The most characteristic general features of the sterol structure are the presence in the molecule of (1) a hydroxyl group, (2) one or more unsaturated carbon linkages, and (3) a system of four reduced rings. An analysis of these molecular structures in their relation to induced antirachitic potency has so far failed to clarify the position with regard to the function of the hydroxyl group. We have shown [Rosenheim and Webster, 1927, 2] that two esters of ergosterol, the acetate and the benzoate, are rendered as highly antirachitic as ergosterol itself by irradiation. This activation may, however, be feasibly preceded by a photochemical hydrolysis, and since the removal or replacement of the hydroxyl group in ergosterol cannot be effected without destruction of the molecule [Reindel, Walter and Rauch, 1927] the bearing of the OH group on the vitamin formation cannot at present be experimentally studied from this direction.

On the other hand, it has become evident that not only the presence of the unsaturated carbon linkages, but also their number, is a deciding factor in the photochemical production of vitamin D. For it was found that the naturally occurring saturated sterols, coprosterol and α -amyrol, as well as the artificially reduced sterols, dihydrocholesterol and dihydrositosterol, remain inactive after irradiation. Further, neither cholesterol nor sitosterol¹, with one double bond, nor stigmasterol, cholesterylene and oxycholesterylene,

¹ The activity of irradiated α -sitosterol [Hess and Anderson, 1927], when administered in large doses, seems to have been due to an admixture of ergosterol, or a similar labile sterol. Since α -sitosterol cannot be purified by bromination, the removal of ergosterol from this admittedly non-homogeneous substance is not easily effected. The slight activity after irradiation observed in some specimens of cholesterol, purified by the bromine treatment [Jendrassik and Keményfi, 1927; Bills, Honeywell and MacNair, 1928], would seem to show that even this treatment may fail occasionally to remove the last traces of ergosterol. This is evident by the latter observers' spectroscopic findings, which show the absorption bands of ergosterol besides those due to cholesterylene [see also Heilbron, Morton and Sexton, 1928].

with two such linkages, can be activated. On the other hand ergosterol, with three such linkages, possesses this property to a high degree.

It became therefore of interest to investigate, in the first instance, whether the photochemical change connected with vitamin formation was specific for ergosterol, or whether other substances with three or more double bonds, but not possessing the sterol ring-structure, would also yield the vitamin on irradiation. We had already previously established that squalene, $C_{30}H_{50}$, the open-chain dihydrotriterpene with six double linkages occurring in the livers of elasmobranchs, cannot be activated and the same holds for nerolidol and *pseudo-ionone*, olefinic oxygenated terpenes with three double bonds [Windaus and Holtz, 1927]. In this connection we also investigated the behaviour on irradiation of the unsaturated open-chain amino-alcohol sphingosine, $C_{17}H_{35}NO_2$, a cleavage product of the cerebrosides. According to Zellner and his collaborators, similar nitrogenous substances, the cerebrins, constantly occur in fungi together with ergosterol, from which they can only be separated with difficulty [Rosenthal, 1922]. Neither the cerebroside phrenosin, to which antirachitic properties had been ascribed by Stepp and Woenckhaus [1926], nor sphingosine itself possessed, however, any antirachitic action either before or after irradiation. Accepting from these negative results the tentative conclusion that unsaturated open-chain compounds cannot be activated, there remains the possibility that isomers of ergosterol, differing from it in the relative position of the three double linkages, might be activable.

iso-Ergosterol is obtained from ergosterol by a relatively mild chemical treatment, that is, removal of hydrochloric acid from its hydrochloride [Reindel, Walter and Rauch, 1927]. Through the kindness of Prof. Windaus, we were enabled to investigate Reindel's own preparation. We found it inactive both before and after irradiation, and our results are in agreement with those of Windaus and Holtz [1927]. Another isomer of ergosterol, *neo-ergosterol*, has recently been obtained by distillation of ergopinacol and has also failed to be activated by irradiation [Windaus and Borgeaud, 1928]. Zymosterol, the only naturally occurring sterol of this class, which accompanies ergosterol in yeast [Smedley-MacLean, 1928], has not yet been biologically examined. In distinction from ergosterol, however, the new sterol possesses no selective absorption in the ultra-violet, and, in view of the correlation existing between antirachitic activity and ultra-violet absorption, it would be surprising if zymosterol, when completely freed from ergosterol, acquired antirachitic activity on irradiation¹.

The position of ergosterol as parent substance of vitamin D would, therefore, appear to be unique, except for the statement of Windaus [1927] that

¹ Hume, Smith and Smedley-MacLean [1928] have since established that irradiated zymosterol has no antirachitic properties. We have recently succeeded in isolating a sterol, associated with ergosterol in ergot (*Tanret's fungisterol*?) and found that it resembles zymosterol in not possessing any selective ultra-violet absorption. Preliminary experiments indicate that it also cannot be rendered antirachitic on irradiation (June 4th, 1928).

digitaligenin, $C_{23}H_{30}O_3$, a substance structurally related to cholic acid, and therefore to the sterols, could also be activated by irradiation [see also Windaus and Holtz, 1927]. Since digitaligenin, the anhydro-aglucone of the digitalis glucoside *digitalinum verum*, is a hydroxy-lactone containing three double linkages, its activation would not seem improbable. A statement coming from these authors deserves serious attention and would obviously be, if confirmed, of considerable theoretical importance. It has already been accepted in the literature and has led to the deduction of the existence of more than one vitamin D in nature.

A detailed consideration of Windaus and Holtz's experimental technique, however, leaves their conclusions open to doubt and we found on a critical repetition of their experiments that digitaligenin, irradiated under the usual conditions, was entirely devoid of antirachitic properties.

This fact, together with the evidence already available, strengthens the assumption that not only a typical ring-structure but also the specific relative positions in which the three double bonds are present in the molecule of ergosterol are essential for the photochemical conversion of a sterol into vitamin D.

EXPERIMENTAL.

The animal experiments were carried out by our usual technique. Except in the case of some of the earlier experiments, in which a thin film of the substance was irradiated, ethereal or alcoholic solutions of the substances were exposed to the radiations of a mercury vapour lamp (atmospheric type). After removal of the solvent in a current of nitrogen, the residue was dissolved in liquid paraffin or in inactive olive oil and a suitable dose administered separately to each rat. In every case control experiments with the non-irradiated substance gave negative results.

Sphingosine was prepared by hydrolysis of pure phrenosin [Rosenheim, 1916]. A thin film of the substance was irradiated for 30 minutes and dissolved in liquid paraffin. Daily doses of 2 and 4 mg. neither prevented nor cured rickets.

Oxycholesterylene, obtained by oxidation of cholesteryl acetate with chromic acid [Mauphner and Suida, 1896], was equally inactive under the same conditions in doses of 0.5 mg.

iso-Ergosterol [Reindel, Walter and Rauch, 1927] was tested in the same way in doses of 0.5 mg. and 1/1000 mg. and found to be devoid of antirachitic activity. The two last substances were examined with the same result by Windaus and Holtz [1927], who irradiated them in liquid paraffin solution instead of in a thin film. It is suggestive, in view of the criticism of their technique (see later), that the only positive result recorded by Windaus and Holtz with a substance other than ergosterol (or its acetate), namely, digitaligenin, was obtained by irradiation in olive oil as a solvent.

Digitaligenin was prepared by hydrolysis from *digitalinum verum* according to Kiliani [1914] and had the melting point 212° , as stated for the pure substance by Windaus and Bandte [1923]. We are greatly indebted to our friend, Dr M. Guggenheim, Basle, through whose offices a supply of this valuable substance was kindly put at our disposal by Messrs Hoffmann, La Roche, Basle.

(a) *Biological evidence.*

The lowest active dose of irradiated digitaligenin had not been determined by Windaus and Holtz, who stated that 1/100 and 1/200 mg. were active. We therefore investigated its action in smaller as well as in larger doses. The substance was irradiated in 0.01 and 0.2 % alcoholic solution, in a quartz cell of 10 mm. thickness, for periods, varying in different experiments, of 30 minutes, 1 hour and 2 hours. One animal in each set of experiments, in which rats of the same litter were employed, received a daily dose of 1/10,000 mg. of irradiated ergosterol, prepared under conditions similar to those employed in irradiating digitaligenin. The results need not be given in detail since all the doses, 1/10,000, 1/5000, 1/250, 1/100 up to 1 mg., of irradiated digitaligenin gave entirely negative results, whilst the rats receiving 1/10,000 mg. of irradiated ergosterol were free from rickets. It follows from these results that irradiated digitaligenin, even when given in doses 10,000 times larger than those which are effective in the case of irradiated ergosterol, is devoid of antirachitic activity.

(b) *Spectroscopic evidence.*

The above conclusion is in harmony with the evidence obtained by the spectroscopic examination of digitaligenin. We found, as was to be expected in the case of a highly unsaturated substance, that digitaligenin showed in alcoholic solution an intense absorption in the ultra-violet, differing entirely in character and position, however, from that of ergosterol. Instead of the three well-defined absorption bands of ergosterol, of which that at $280\mu\mu$ is the most intense and characteristic, digitaligenin shows only one band with a maximum at $340\mu\mu$, extending from $270\mu\mu$ to $390\mu\mu$. In addition, there is general absorption, and possibly secondary bands, in the region below $250\mu\mu$. Moreover, the broad absorption band of digitaligenin remains practically unchanged after 2 hours' irradiation, whereas the absorption spectrum of ergosterol rapidly changes under these conditions.

It may be suggested that the antirachitic action of irradiated digitaligenin, reported by Windaus and Holtz, was due to the fact that the actual irradiation of the substance was carried out on a 0.01 % solution in olive oil (see above). The obvious criticism arises that the choice of olive oil as a solvent for this type of experiment is an unsuitable one. It is an established fact, which was recognised early in the history of the subject [Steenbock and Black,

1925], that olive oil itself, in common with other vegetable oils, acquires anti-rachitic properties on irradiation. Further, Windaus and Holtz obtained their positive results in experiments in which relatively large doses (100 mg.) of irradiated olive oil were administered daily when testing the action of digitaligenin. We found that much smaller doses (40–50 mg.) of such oils, three different specimens of which were tested¹, completely prevented rickets in every case. In the single negative control experiment with irradiated olive oil, mentioned by the above authors, the oil was subjected to the intense irradiation of the magnesium spark for 3 hours. Since it is well known that prolonged irradiation destroys the antirachitic activity of vegetable oils and vitamin D, it may be suggested that, for such a control experiment to be convincing, a substance of equally powerful protective absorption in the ultra-violet as digitaligenin should have been added to the olive oil.

SUMMARY.

Evidence is presented which strengthens the assumption that only a molecular structure such as that possessed by ergosterol enables a sterol to be photochemically converted into vitamin D, and confirms the evidence already available for the view that ergosterol is the specific parent substance of vitamin D.

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¹ One of these specimens was kindly procured for us as guaranteed pure "virgin oil" directly from the grower by Mr L. Erles, in Malaga, Spain.