CLII. NOTES ON THE IRRADIATION OF ERGOSTEROL.

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It was recorded by Rosenheim and Webster [1927] that the exposure of ergosterol to ultra-violet radiation produced a mixture of substances including vitamin D, and evidence was given suggesting that, for moderate periods of irradiation, the amount of vitamin present in the mixture was nearly independent of the period of irradiation, apparently owing to simultaneous formation and destruction of the vitamin. This work has been continued in search of possible means of isolating vitamin D.

I. *The effect of using "filtered" light at various temperatures.*

Attempts were made to produce more concentrated preparations of vitamin D by the irradiation of ergosterol by selected portions only of the ultra-violet spectrum. If, as was suggested by Morton, Heilbron and Kamm [1927, 1] and also by Pohl [1926], vitamin D shows absorption chiefly between 230 and 260 $\mu\mu$, while ergosterol shows it chiefly between 260 and 290 $\mu\mu$, then the use of a filter cutting off all rays of wave-length shorter than 265 $\mu\mu$ should increase the quantity of vitamin D formed during irradiation by reducing the rate of destruction of the vitamin without seriously affecting that of ergosterol. The use of "vitaglass" for this purpose was suggested by Morton *et al.* in the above paper, and has since been tested by them [1927, 2], and by Rosenheim and Webster, with negative results. Alcoholic cobalt chloride is, however, even more suitable for such a test. A 5 % solution of thickness 10 mm. in a cell with plane polished silica walls, transmits between 20 % and 30 % of radiations between 400 $\mu\mu$ and 280 $\mu\mu$ and about 17 % of 280 $\mu\mu$. The transmission then falls rapidly to 3 % at 265 $\mu\mu$ and then very rapidly indeed to less than one part in a million at 254 $\mu\mu$. By multiplying the exposures used without a filter by about 4.5, the use of this filter thus secures an undiminished exposure to radiation above 280 $\mu\mu$ and only slight diminution of radiation from 280 to 270 $\mu\mu$, with freedom from any disturbance at all by radiation of wave-lengths shorter than 265 $\mu\mu$. Preliminary trials with this filter showed no effect on the

equilibrium, and, as it seemed possible that temperature changes during radiation were affecting the results, the trials were extended over a wide range of temperature.

Experimental details.

The ergosterol was a highly purified crystalline specimen of M.P. 162° prepared from ergot, presented to Dr Rosenheim by Dr H. D. Jowett of Messrs Burroughs Wellcome and Co. and kindly placed at our disposal by Dr Rosenheim. The solvents used were pure ether dried over sodium, and absolute alcohol, these being chosen as the most stable solvents available without marked ultra-violet absorption. Solutions were freshly made before use, and in these experiments were always of concentration 0.1 g. per litre.

For all except the lowest temperatures the source of light was a K.B.B. silica mercury arc worked at atmospheric pressure with 2.5 amp. and 125 volts across the arc. An opaque screen with a circular hole 5 cm. diameter was placed 5 cm. from the lamp and 10 cm. from the ergosterol solution. This solution was contained in a fused silica cell 10 mm. thick, with sides 2.5×2.5 cm. of optically plane polished silica. It was immersed in an outer vessel of fused silica containing a fluid kept at constant temperature and a thermometer. For the tests at + 1° and - 18° an air chamber dried by calcium chloride and fitted with a silica window was attached to the side of the bath facing the lamp to avoid deposition of dew. At each temperature exposures were made both to unfiltered radiation and to radiation filtered through the cobalt chloride filter described above. (This filter was circular in section and 2 inches in diameter.) A uniform exposure of 20 minutes for unfiltered light and 90 minutes for filtered light was given in order to secure equal amounts of radiation of wave-lengths between 280 and 350 μ .

For the tests made at very low temperatures the above cell was immersed in liquid oxygen or nitrogen and a Hewittic vacuum silica mercury arc was placed horizontally 10 inches above the cell, and run at 3 amp. and 130–140 volts after a preliminary test with a photoelectric cell to show that these conditions gave radiation of intensity roughly similar to that used at other temperatures. No part of the body of the cell was above the surface of the liquid, and no exposure to ultra-violet radiation occurred before complete cooling. Alcoholic solutions only were used at these temperatures since they froze to a transparent glass without separation of solute, in contrast to ethereal solutions which solidified as an opaque crystalline mass. The silica cell was occasionally broken by a sudden shattering of the contents, due apparently to further contraction of the glassy mass after it had become rigid. Unfiltered light only was used, and exposure varied from 5 minutes to 5½ hours. All solutions were photographed through a Hilger quartz spectrograph before and after irradiation.

The following table shows the temperatures used and the fluid surrounding the cell at each temperature.

+ 77.8°	Alcohol (boiling)
+ 30.6°	Ether (boiling)
+ 1°	Water
- 18°	Ether (boiling at low pressure)
- 183° approx.	Liquid oxygen
- 195° „	„ nitrogen

Biological testing of antirachitic activity.

Immediately after irradiation an aliquot part of the fluid to be tested was dissolved in 5 cc. of olive oil and the alcohol or ether removed *in vacuo* at room temperature. This solution was then suitably diluted with olive oil so that the required amount of irradiated ergosterol was contained in 20 mm.³ of oil.

The test animals were young albino rats of about 40 g. weight and the diet used was the modified diet 84 [Rosenheim and Webster, 1926]. In most cases the protective method was used, the animals being kept on the diet for 21 days, during which period daily doses of the test solutions were given to all except the control animals. At the expiration of this time the animals were killed and the left hind leg was dissected out and photographed with a soft X-ray tube. The limbs from each litter of rats were photographed on one plate, and the degree of rickets in each rat was carefully estimated.

Although litter mates react fairly consistently to rachitic diets and anti-rachitic treatment, different litters show much less consistency. To overcome this variation each litter of rats was divided into two groups; one group received graded doses of the solution under test and the other graded doses of a standard solution. Comparison of corresponding members of the two groups then gave a measure of the activity of the solution under test in terms of the standard.

Each rat received one dose per day, Sundays excepted, administered with Wright's capillary pipette. The use of a standard preparation for the assay of vitamins has also been described by Coward [1928].

The standard solution of irradiated ergosterol was prepared as follows. A 0.1 % solution of ergosterol in dry ether was exposed to the radiations of a K.B.B. silica mercury vapour lamp taking 2.5 amp. at 125 volts for 40 minutes. The ether solution was contained in the standard silica cell 1 cm. thick at a distance from the lamp of 8 inches. After exposure the ether was rapidly removed on the steam-bath in a current of nitrogen and the residue dissolved in olive oil, the concentration being 0.01 %. This solution was kept frozen solid at about 0° and dilutions were made when required. Fresh dilutions were made for every set of experiments, so that no solution was kept at room temperature longer than 3 weeks.

Results of antirachitic tests.

All products appeared of approximately equal activity except those obtained in liquid oxygen or nitrogen, which were markedly less active. The results obtained from $+78^{\circ}$ to -18° are based on tests on about 170 rats, and are probably fairly reliable for this class of work. A difference of 100 % between any two fluids would probably have been evident, and a regular difference of this magnitude between filtered and unfiltered light would be most unlikely to have escaped detection.

The products obtained at -183° and -195° are definitely less active. In a series of trials products have been obtained varying from one-tenth to one-half of the standard strength, but in some series both the antirachitic activity and the spectrographic changes in the solutions are less than in others. The cause of this variability is not yet certain, but may be turbidity of the liquid nitrogen or oxygen, caused by particles of ice with which it is liable to become contaminated.

A further test of the filtered light at 30° was made by prolonged irradiation of 0.1 % solutions of ergosterol in ether for periods of 1, 4, and 63 hours respectively.

The first two fluids showed a maximum standard activity, while that given 63 hours' exposure (equivalent to about $1\frac{1}{2}$ hours' exposure of 0.01 % solution to unfiltered light) was less than one-quarter as active.

It seems reasonable to conclude that the exclusion of wave-lengths shorter than 265μ does not seriously alter the ratio of rates of production and destruction of vitamin D. This suggests, but does not prove, that vitamin D either has strong absorption for wave-lengths longer than 265μ , or has not great absorption for wave-lengths of 230 to 250μ as suggested by previous workers.

The lack of marked effect of changes of temperature between $+78^{\circ}$ and -18° on the equilibrium, shows that the temperature coefficients of the changes causing production and destruction are not widely different, and the very moderate effect of lowering the temperature to -180° suggests that the temperature coefficient of both reactions is very small, and hence that both reactions are directly photochemical in nature.

II. *Some properties of the products of irradiation after removal of unchanged ergosterol.*

The products remaining after the removal of unchanged ergosterol from solutions irradiated for short periods only are being investigated. By this means it was hoped to secure potent preparations of vitamin D free from ergosterol and containing a reduced proportion of inactive decomposition products. The technique was as follows. A 0.1 % or 0.15 % solution of ergosterol in alcohol was irradiated for periods of $\frac{1}{2}$ minute to 20 minutes in a layer 10 mm. thick at 3 to 6 inches from a mercury vapour lamp. The irradiated solution was treated (either with or without previous concentration by evaporation

in vacuo) with a 1 % solution of digitonin in 95 % alcohol, using an amount of digitonin equal to 5 times the weight of ergosterol present before irradiation, *i.e.* an excess of more than 25 % over the quantity theoretically needed for precipitation. Enough water was added to bring the total concentration of water up to 10 %. The mixture was allowed to stand overnight and then filtered. The precipitate of ergosterol digitonide was rejected. The filtrate was in some cases used for spectrographic and biological tests, but always contained small traces of ergosterol. To remove these the solution was evaporated to dryness *in vacuo* at about 40° (thus ensuring the complete precipitation of ergosterol during concentration), and the residue was then extracted with dry ether, in which digitonin and ergosterol digitonide are practically insoluble. The solution was used as such, or evaporated to dryness in order to weigh the residue. All these operations were carried out as far as possible in an atmosphere of nitrogen and at temperatures never exceeding 50°. The application of this process to non-irradiated ergosterol yields only traces of oily substances which are without appreciable absorption.

The products so obtained formed a transparent glassy hard solid of indefinite M.P. beginning about 30°, at times colourless, but more often contaminated with a yellow pigment which appeared during the original solution in alcohol of certain samples of ergosterol [Tanret, 1908]. The product is soluble in approximately its own weight of alcohol at 30°. This is in striking contrast to the small (0.2 %) solubility of ergosterol in alcohol, and is a proof of the efficiency of the process described above for removing ergosterol. The product is also very soluble in ether, chloroform and light petroleum, but insoluble in water. It is moderately soluble in methyl alcohol containing 10 % of water, and this mixture was used in attempts to fractionate the mass. It shows high antirachitic activity. This appears to vary in different samples prepared on different occasions. The highest observed activity appears to have been about twice that of the standard described above. The absorption spectrum varied considerably in different samples, and is discussed in the next section.

III. *Absorption spectra of products of irradiation.*

Preliminary studies of absorption have been made by a photographic method, using a modification of the technique described by Dobson, Griffith and Harrison [1926]. The results obtained were in general concordant to about 5 %, but difficulty was found in controlling the voltage on the lamp used, and at times a larger error occurred. Greater sources of error were found in the presence of traces of the yellow pigment referred to above, which showed strong absorption between 300 and 400 μ . For these reasons the curves shown are only rough approximations, and no attempt has been made to locate exactly the heads of the individual bands.

Curve 1 (Fig. 7) and (Plate III, fig. 1) show the absorption of the ergosterol used.

Curve 2 (Fig. 7) and (Plate III, fig. 2) show the absorption of a practically colourless sample of the product obtained after the removal of ergosterol as described above.

Curve 3 (Fig. 7) shows the absorption of another sample of the product.

Fig. 7.

These products show strong absorption for wave-lengths shorter than $290\mu\mu$ and in these samples show no maximum between 265 and $280\mu\mu$ in contrast to ergosterol and to certain other samples of products in which a maximum occurred between 300 and $330\mu\mu$. The absorption is, however, much greater than that of ergosterol. The above features can be seen by inspection of Plate III, figs. 1 and 2, and are of much interest in conjunction with the following phenomena, which can be observed during the earliest stages of the irradiation of ergosterol.

If a solution of ergosterol be examined after short periods of irradiation, an increase in absorption is noteworthy, in contrast to the decrease recorded by many workers after longer periods of radiation. In a 10 mm. layer of 0.2 % solution this increase is very striking at $313\mu\mu$ (Plate IV, fig. 3), while in 0.1 % solution it is obvious at $302\mu\mu$ (Plate IV, fig. 4), and in 0.01 % solution it is evident at 290 and $265\mu\mu$ and visible at $253\mu\mu$ (Plate IV, fig. 5) after 4 minutes' irradiation. In 0.05 % solution it is just visible in the intervening wave-lengths between 290 and $265\mu\mu$ after about 2 minutes' irradiation. The increase is at its maximum after a period depending on the concentration

of solution as well as on the intensity of radiation. Under our conditions this maximum occurs after about 6 minutes in 0.01 % solution, or an hour in 0.1 % solution (the exact times have not been measured). On further irradiation the absorption decreases steadily to the almost complete disappearance often recorded. In fig. 5 (Plate IV) a marked decrease in absorption is seen after 20 minutes' irradiation.

These changes do not occur if a filter of thick window-glass is interposed between the lamp and solution, but do occur if filters of chlorine or alcoholic cobalt chloride are used, although the latter modifies the changes. They appear unaffected by bubbling either oxygen or nitrogen through the solution during irradiation, or by the use of ether as solvent instead of alcohol. The most simple explanation of them is the hypothesis that ultra-violet irradiation has as an initial (or early) effect the formation from ergosterol of products showing strong absorption between 313 and $250\mu\mu$. The intensity of this absorption markedly exceeds that of ergosterol between 320 and $290\mu\mu$, and between 265 and $250\mu\mu$, and slightly exceeds it between 290 and $270\mu\mu$. (We have only worked with a mercury vapour spectrum and so have not excluded the possibility of narrow troughs between bands, in which the absorption may be less than that of ergosterol.) This hypothesis is strongly supported by the absorption of the products described above which remain after the removal of ergosterol from an irradiated solution. For, although these products show an absorption which only exceeds that of ergosterol between 300 and $330\mu\mu$, if it is assumed that the products are a pure absorbing substance, it is much more probable that the products are a mixture of absorbing and non-absorbing substances. If such non-absorbing substances form 40 % of the sample whose absorption is given in curve 2 (Fig. 7), the true absorption curve of the absorbing product would be that shown in curve 4 (Fig. 7). Such an absorption would satisfactorily account for the increase in absorption shown in the first few minutes of the irradiation of ergosterol.

IV. *Possible absorption spectrum of vitamin D.*

We have made numerous attempts to find a quantitative relation between the magnitude of the absorption coefficients of the products described above and their antirachitic activity. Owing to the difficulties of such tests, no exact relationship has been proved or disproved, but the following conclusions can be stated.

1. If a 0.1 % solution of ergosterol in alcohol or ether is irradiated at room temperature under the conditions described in Section I, products having an antirachitic activity and absorption of the type described are present to a small extent after 30 seconds' irradiation, and to a marked extent after 1 minute's irradiation.
2. After 10 minutes' irradiation both properties are increased to something like 10 times the former values (Plate IV, fig. 6).

3. Irradiation for periods of between 10 and 60 minutes increases both properties somewhat further, to about 2 or 4 times their value after 10 minutes' irradiation.

4. Thus there is evidence that both antirachitic activity and absorption are produced at approximately the same rate, and that both begin in the earliest stages.

5. If the products of irradiation after removal of ergosterol are exposed to ultra-violet light, both antirachitic activity and absorption decrease, and both disappear almost entirely after between 3 and 5 hours' irradiation under the above conditions.

6. We have not yet succeeded in obtaining a product showing either antirachitic activity without the type of absorption described, or *vice versa*. Each property has always been accompanied by the other. There is thus some reason for thinking that absorption of the type shown in curve 4 (Fig. 7) may be a property of vitamin D. This conclusion is, however, only one of several possible explanations of the observed phenomena, and is not easy to reconcile with the observations of Morton *et al.* or of Pohl [1926]. A more probable explanation, which we hope to test shortly, is that the irradiation of ergosterol produces two substances in succession, of which the first has an absorption maximum at about 280 or 290 $\mu\mu$, and the second a maximum at about 230 $\mu\mu$, and that the former is vitamin D. This hypothesis would explain the variable absorption of the products obtained after removing ergosterol from irradiated mixtures, and would also fit the absorption changes found by us in the early stages of the irradiation of ergosterol and the changes found by the other authors quoted in the later stages. On any hypothesis the products of irradiation must show considerable antirachitic activity until some time after the practical disappearance of ergosterol, and this makes the evidence attributing absorption at 240 $\mu\mu$ to vitamin D open to question unless a quantitative relation between such absorption and antirachitic activity can be shown. As arguments suggesting a connection between vitamin D and absorption at 280 $\mu\mu$ may be quoted the very early production of marked antirachitic activity on irradiation of ergosterol, and the fact that in our experiments we have always found marked absorption at 290 and 300 $\mu\mu$ in products showing antirachitic activity.

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Fig. 1. Ergosterol in alcohol.

Fig. 2. Products of irradiation after removal of ergosterol.

In Figs. 1 and 2 it should be noted (a) that in Fig. 1 (ergosterol) absorption is still marked at 270 to 290 in a concentration of 0.0062 % although line 254 is clearly visible, whereas in Fig. 2 at concentration 0.0084 % the absorption between 270 and 290 is no greater than that at 254; (b) that in Fig. 2 at concentration 0.067 % the line 302 has entirely disappeared, although in Fig. 1 this line is still prominent even at 0.1 % concentration.

Fig. 3. Ergosterol 0.2 % in ether.

Fig. 4. Ergosterol 0.1 % in ether.

Fig. 5. Ergosterol 0.01 % in alcohol.

Fig. 6. Products of irradiation after removal of ergosterol