XII. THE ABSORPTION SPECTRUM OF CHOLESTEROL AND ITS BIOLOGICAL SIGNIFICANCE WITH REFERENCE TO VITAMIN D. PART I. PRELIMINARY OBSERVATIONS.

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It has been fully proved by various investigators that although cholesterol itself exhibits no antirachitic properties prior to irradiation with ultra-violet light, these are developed on comparatively short exposure to these rays. This brings the reaction within the sphere of photochemistry and since photochemical changes do not occur without the absorption of light and since it follows from the Grotthus-Draper law that only the rays absorbed are active, it would immediately become a matter of urgency to ascertain whether cholesterol showed well-defined absorption bands in the ultra-violet. The hitherto published results on this aspect of the problem are essentially preliminary in character. Hess and Weinstock [1925] observed that when cholesterol was irradiated by ultra-violet rays a change in the absorption spectrum occurred. The activated material absorbed light of certain wave-lengths to a less degree than ordinary cholesterol, a difference in the absorption over the entire range of wave-lengths (integrated) being detected by the use of a thermopile and galvanometer set. Schlutz and Ziegler [1926], carrying the matter further, observed that carefully recrystallised cholesterol, melting at 148·5° or very near that point, showed selective absorption "of wave-lengths between 294–296 $\mu\mu$ and 279–294 $\mu\mu$ with a great deal of general absorption beyond 294 $\mu\mu$." The absorption bands, which were evidently very shallow, could not be detected in alcoholic solution but showed up in ether or chloroform. Moreover, the bands were only observed in the first crop (five fractions collected) from a fractional crystallisation from alcohol of cholesterol (M.P. 148·5°) which had previously been recrystallised seven times from the same solvent.

As these results are not wholly consistent with the idea of ready photochemical change in the cholesterol molecule, we deemed it advisable to undertake a still more extensive and quantitative study of the absorption spectrum of this compound.

The results now to be described were obtained using a Hilger quartz spectrograph, rotating sector-photometer, iron-nickel arc arrangement. The arc (5 amps., 105 volts) was situated at a distance of 132 cm. from the absorbing solution. In our first experiments a sample of carefully purified cholesterol (from brain), M.P. 148·5°, kindly supplied by Prof. Drummond,

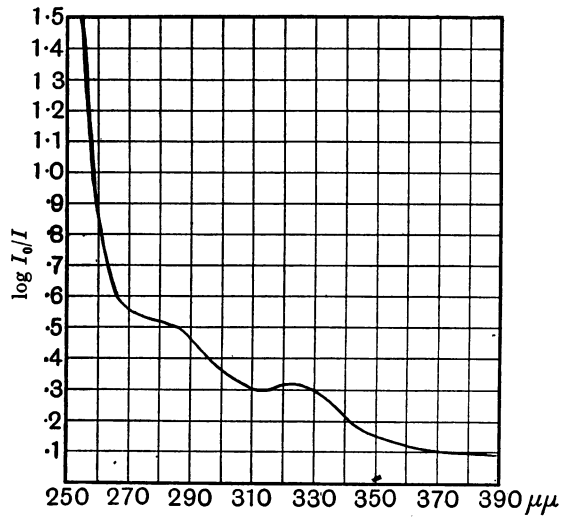

Fig. 1. Cod-liver oil film approx. $\frac{1}{10}$ mm. thickness.

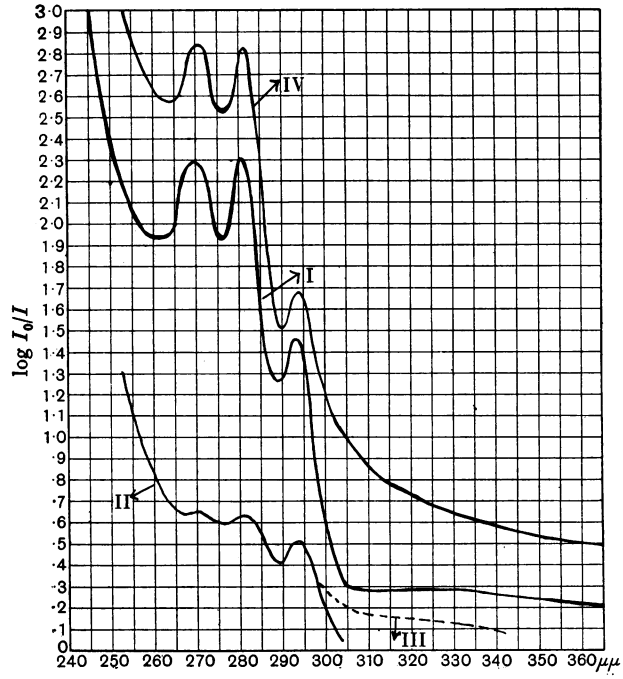

Fig. 2. I. Cholesterol (2½ g. from 200) 2 g. in 50 cc. ether, 4 cm. cell, fresh.
II. Drummond's cholesterol from brain—our ordinary purified 148·5° cholesterol.
III. Solution II after taking 1 plate (*i.e.* after standing 1 hour).
IV. Cholesterol (2½ g. from 200) 2 g. in alcohol, 4 cm. cell, fresh.

was employed and showed clearly the very shallow bands recorded by Schlutz and Ziegler [1926]. In addition it gave indications of an extended region of absorption on the ultra-violet side (Fig. 2, curve II). We next employed a sample of cholesterol obtained from cod-liver oil (M.P. 148·5°) and recrystallised by us three times. The absorption curve coincided exactly with that obtained in the previous case. An examination of the curves seemed to us to indicate that the observed curve was made up of two separate curves, one of which we regard as being due to cholesterol itself and the other to the material which becomes active after absorbing light energy.

With the idea of developing and testing this point of view, the unused portion of this cholesterol was once more recrystallised from ethyl acetate, the least soluble 2 g. being again examined spectrographically. It was found that this fraction gave a higher extinction, a higher definition for the bands and made the existence of a third band near 269 $\mu\mu$ certain (Fig. 3, curve III).

As these preliminary results indicated the presence of some foreign material in cholesterol to which the selective absorption could be attributed, we next undertook fractional crystallisation of 200 g. of our crude material. After one recrystallisation from ethyl acetate (1500 cc.) 50 g. were obtained, M.P. 147·5–148° (Charlottenberg certificated thermometer), and this portion was again dissolved in the same volume of ethyl acetate and the solution allowed to crystallise slowly. The portion which crystallised out (25 g.) melted sharply at 148·5°, the melting point being, if anything, fractionally higher than that of the pure specimen of brain cholesterol obtained from Prof. Drummond, which was at the same time re-determined as a check. Two further crystallisations of this material were carried out:

(*a*) the 25 g. were crystallised from 600 cc. ethyl acetate, yielding 5 g. of a less soluble portion;

(*b*) this latter portion was again dissolved in ethyl acetate (120 cc.), yielding after cooling 2·5 g. of material melting at 148·5°.

It will be noted that as the fractional crystallisation proceeded, the solubility of each successive fraction of residual substance decreased to a marked degree and that whereas the crude product was soluble to an extent of 10 % in ethyl acetate at room temperature, the final fraction which represents approximately 1 % of the original material was only soluble in this solvent under the same conditions to an extent of 2 %. It is thus clear that a substance differing in solubility from ordinary cholesterol may be concentrated by fractional crystallisation, but these results are only attained if an adequate quantity of material is originally taken.

A spectrographic examination of this highly concentrated product now showed that in this material the extinction coefficient had increased to approximately four times that of the purest material previously obtained[1] and

[1] We desire to record our thanks to Dr O. Rosenheim and Mr T. A. Webster who have now tested biologically this preparation and report that it is three to four times as active as a sample of the non-concentrated product. This shows remarkable agreement with the spectrographic results. [February 2, 1927. I. M. H.]

that the definition of all three bands was very much improved (Fig. 2, curves I, IV). Consistent results were obtained when the spectrograms were taken in either alcohol or ether solution.

With the idea of still further concentrating our "*X*-material," a fresh crystallisation was carried out. In this case we started from 2000 g. of the crude cholesterol, obtaining after one crystallisation from ethyl acetate 450 g. of less soluble material (M.P. 147·5–148°). This was repeatedly crystallised from large volumes of ethyl acetate yielding successively the following less soluble fractions: 117 g., 43 g., 6 g. This last portion was further crystallised from dilute alcohol, yielding 5 g. of material the absorption curve of which is reproduced on Fig. 5, curve I. The extinction coefficient has again increased beyond the highest point previously recorded, but, on the other hand, the bands, rather contrary to our expectation, have lost somewhat in definition.

An important fact regarding this highly concentrated material is that its melting point is not only definitely lower than that of purified material isolated from the less concentrated portions, but is indefinite over a range 146·5–149°. This result is quite consistent with our assumption of an "*X*-compound" in cholesterol. Moreover, this substance is almost entirely accumulated in the least soluble portion, for, on working up the filtrate from which the 43 g. portion was crystallised, a product was obtained of definitely higher melting point (148·3°) which nevertheless only showed very faint selective absorption, while the material (M.P. 148·5°) obtained by concentration of the filtrate from the previous crystallisation (117 g.) followed by careful fractionation showed no selective absorption whatsoever (Fig. 5, curve II).

A full chemical investigation of this least soluble fraction has just been started. While still in the early stages and awaiting confirmation, the results so far obtained certainly indicate the presence of a substance which has a slightly higher carbon content, is not precipitated with digitonin and does not show the typical Liebermann reaction for cholesterol.

It is of some interest to record that the addition of sodium ethoxide to an alcoholic solution of cholesterol (brain cholesterol from Prof. Drummond) favours increased definition to the band at 269 $\mu\mu$ at the expense of the other bands, whereas addition of hydrogen chloride causes the appearance of a new band at 318 $\mu\mu$. The latter band is not far removed from the second band of cod-liver oil first recorded by Schlutz and Ziegler [1926] and now confirmed by ourselves (Fig. 1). The significance of these results is not at the moment ripe for discussion.

Irradiation of cholesterol.

When cholesterol is irradiated in the solid state between quartz plates for 10 minutes at a distance of 6 inches from a quartz mercury lamp which had been in use for some time, almost complete disappearance of selective absorption is noted (Fig. 4, curve III). A sample of the brain cholesterol irradiated by Prof. Drummond in an atmosphere of nitrogen gave a smooth curve

showing only general absorption (Fig. 3, curve I). Moreover, all samples of cholesterol irradiated for periods longer than 10 minutes in the solid state or in alcoholic or ethereal solution (Fig. 3, curve II) resulted in the formation of a product exhibiting no signs of selective absorption. In the case of the least soluble fraction obtained from the 2000 g. fractionation, the absorption spectrum after irradiation (Fig. 5, curve III) although showing no bands has nevertheless a very high extinction coefficient.

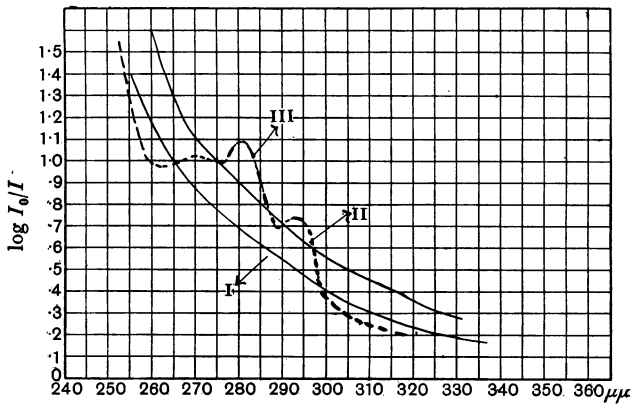

Fig. 3. I. Drummond's irradiated cholesterol 2 g. in 50 cc. ether solution, 4 cm. cell.
II. Cholesterol irradiated in ethereal solution 2 g., 50 cc., 4 cm. cell.
III. Ordinary 148·5° cholesterol (from cod-liver oil) before irradiation.

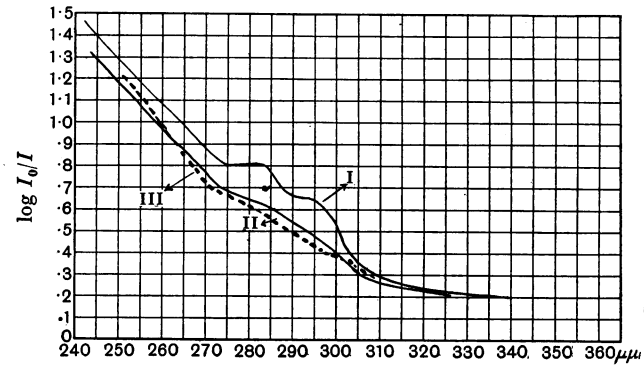

Fig. 4. I. 4 g. ordinary 148·5° cholesterol in 250 cc. alcoholic solution, 10 cm. cell, fresh.
II. Ditto after 2 hours.
III. Same cholesterol after 10 minutes' irradiation in solid state.

Finally we have found it impossible again to observe any of the characteristic bands in irradiated material which has subsequently been recrystallised. We have also confirmed the observation of Schlutz and Ziegler [1926] that a solution of cholesterol in alcohol or ether after standing four hours no longer showed the bands (Fig. 4, curves I and II).

Discussion.

The facts that purified cholesterol exhibits selective absorption before irradiation, and that the bands gain in definition and show increased extinction when the material is fractionally crystallised, all point to the presence of an "*X*-substance" in the original compound. Since the bands disappear on irradiation, with concomitant appearance of vitamin potency, it would seem that there is definite evidence for a correlation between absorption bands and a vitamin precursor. Various lines of investigation combine to show that the precursor is different from cholesterol itself. In the first place, the quantity of antirachitic material produced does not increase beyond a definite limit by prolonging the period of irradiation. This would not be expected if the cholesterol itself were being transformed.

Secondly, as already mentioned, cholesterol recovered from irradiated material no longer shows selective absorption.

Thirdly, Hess, Weinstock and Sherman [1925] failed to prove that deactivated cholesterol could acquire antirachitic potency by renewed irradiation.

Cholesterol becomes active when irradiated in solution—only in those solvents transparent to ultra-violet radiation of the wave-lengths covered by the bands. In acetone (Fig. 6, curve *d*), which effectively absorbs these rays, no activation should occur. This inference is strikingly confirmed by the fact recorded without comment by Hess, Weinstock and Sherman [1925], that irradiation in acetone confers no antirachitic potency.

Two possibilities must not be neglected in interpreting these results: firstly, it is quite conceivable that a material very rich in the vitamin precursor should undergo photochemical change very readily, even to the point of militating against its detection by spectrographic methods. Secondly, cholesterol itself may consist of a mixture of inactive stereoisomerides (compare Anderson and co-workers [1926] on sitosterol) which might differ in absorption spectra. Stereoisomerides frequently exhibit parallel curves differing only in extinction. Either of these alternatives would account for the apparent loss of definition in the bands shown by the end-fraction of the most concentrated material (Fig. 5, curve I).

We must accept, therefore, that cholesterol itself shows only general absorption and that in those fractions which exhibit selective absorption another substance is present. Fig. 6 is a purely geometrical illustration of the type of curve which would result in such a case. It will be seen (*a*) that the resulting summation curve is not dissimilar to the curves actually obtained in our concentrated cholesterol; (*b*) that the ultra-violet component of the band showing a triplet structure necessarily appears in a less well-defined manner than the longer wave component.

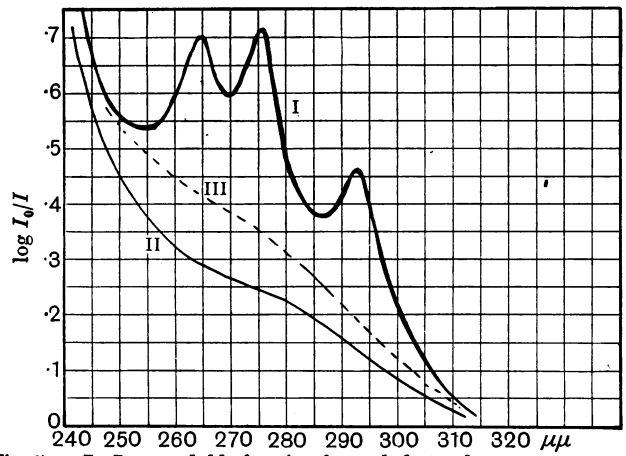

Fig. 5. I. Least soluble fraction from cholesterol.
II. Cholesterol after removal of active compound.
III. Irradiated fraction.

These curves are not strictly comparable with Fig. 1 but curve I in this figure corresponds with greater absorption than curve IV, Fig. 2.

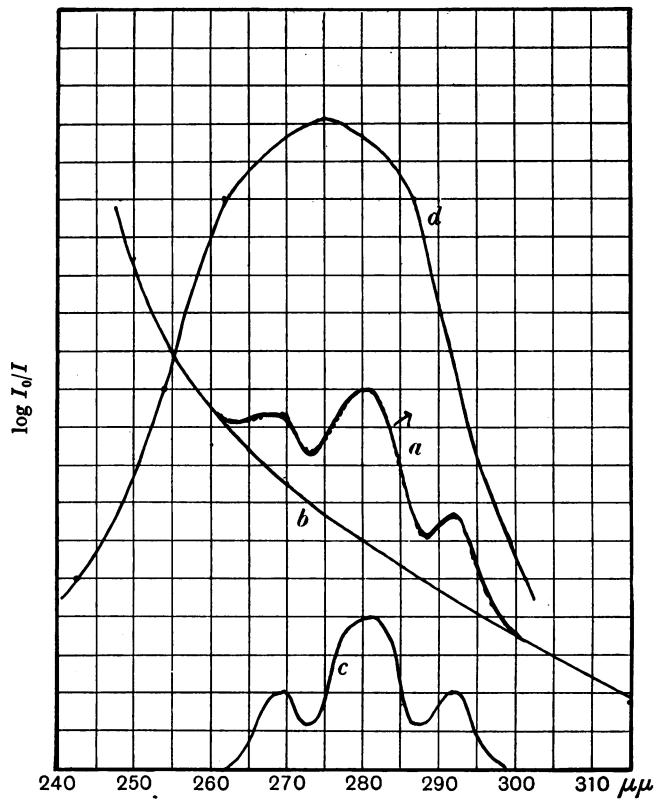

Fig. 6. (*a*) is a purely geometrical illustration of the effect of superposing selective absorption (*c*) on general absorption (*b*). (*d*) shows the absorption band of acetone on an arbitrary scale.

SUMMARY.

1. Ordinary purified cholesterol contains another compound in small quantity which can be accumulated in the least-soluble fraction.

2. This substance shows well-defined absorption bands at 293 $\mu\mu$, 280 $\mu\mu$, and 269 $\mu\mu$, while cholesterol itself has only general absorption.

3. These bands disappear on irradiation with ultra-violet light with concomitant appearance of antirachitic potency.

4. It is obvious that the unknown substance is closely connected with the vitamin D precursor.

We desire to express our thanks to Prof. J. C. Drummond and Mr H. J. Channon, of the Biochemical Department of University College, London, for providing us with brain cholesterol, to Messrs. Joseph Nathan and Co., Ltd. (Proprietors of Glaxo), for the large quantities of cod-liver oil cholesterol necessary for these experiments, and to the Food Investigation Board of the Department of Scientific and Industrial Research for a grant which has enabled the work to be carried out.

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[Note added February 2, 1927.] R. Pohl (*Nachrichten der Gesellschaft der Wissenschaften zu Göttingen, Mathematisch-Physikalische Klasse*, 1926) communicated on December 10, 1926, a paper on the absorption spectrum of antirachitic cholesterol. A monochromator with double spectral resolution was employed in conjunction with a photoelectric photometer. The presence of three absorption bands which disappear on irradiation is confirmed and the absorption curve for inactive cholesterol is found to be quite smooth. The photoelectric method, using monochromatic light, thus establishes our view that no change occurs during the measurement of absorption spectra by the photographic method, and our results are fully confirmed.