In the previous paper it was shown that highly purified spinal cord cholesterol is still activatable to antirachitic activity when irradiated in the solid form by a Cooper Hewitt mercury vapor quartz lamp. Although the activatability is decreased by the purification methods employed, nevertheless these purified products can again be rendered at least 25 times more activatable if heated under the proper conditions slightly above the melting point of cholesterol. Inasmuch as provitamin D activity has been claimed to be due specifically to ergosterol (1-4) our results might be interpreted as due to the presence of or formation of ergosterol in our preparations and as it is well known that the absorption spectrum of ergosterol is a very characteristic one we considered it desirable to conduct absorption spectra studies on our products in order to obtain physical evidence for or against this interpretation.

As a source of light the continuous radiation from hydrogen in the violet and ultra-violet region was utilized (5-7). The gas was contained in a Geissler tube of simple design (Text-fig. 1) so arranged that the luminous thread of the capillary portion was projected end on through a quartz window upon the slit of the spectrograph. The source tube contained pure electrolytic hydrogen at a pressure of about 15 mm. and was rendered luminous by excitation from the secondary of an open core transformer, the primary of which through a current-limiting rheostat was con-
Absorption Spectra Studies

connected to 110 volt A.C. mains. Between the source and the spectrograph was a simple quartz condensing lens and adjacent to the slit the absorption cell (Baly tube) containing the solutions being tested.

The spectrograph was one by Adam Hilger, Type E3, recording the spectrum from 2100 to 7000 Å.units well within the extent of a 10 inch plate. Wratten panchromatic plates were used, developed for 4 minutes with contrast glycine developer containing bromide.

The materials examined were dissolved in ether and placed in the Baly tube having quartz windows. The depth of liquid through which the light passed could be varied at will by adjusting the position of the plunger. Unless otherwise specified 2 minute exposures through a column of liquid 20 mm. in depth were employed.

The preparations studied in the previous paper and in this were the following: (1) A commercially pure cholesterol from the Wilson Laboratories (Cholesterol W); (2) this when purified by boiling with alkali and extraction with ether gave two fractions, one the main final recrystallized product (Cholesterol A) and another in the emulsion layer (cholesterol Fraction II); (3) "cholesterol Br-treated" was obtained by purifying Cholesterol W by means of bromine treatment; (4) cholesterol KMnO₄-treated"

was obtained from "Cholesterol W by destruction of ergosterol by KMnO₄. For more detailed information see the previous paper (8).

In Fig. 1 we made a preliminary comparison of the spectra of our cholesterol preparations with those of ergosterol. As we expected, the purified cholesterol was more transparent to ultraviolet than the original commercial product, Cholesterol W. The very striking change in the absorption of the purified cholesterol when it had been heated and also the great similarity in the spectra of the heated cholesterol and of Fraction II, both highly activatable preparations, to that of ergosterol, at least offered some confirmation of our biological reactions, described in the previous paper. In making these exposures the slit of the spectroscope was very narrow and the concentrations were relatively high, 5 per cent cholesterol and 2 per cent ergosterol. We next used a slightly wider slit and varied the concentrations of the various preparations, diminishing each to the point where no absorption different from that of the pure solvent occurred.

Fig. 2, in which diminishing concentrations of Cholesterol W were examined is self-explanatory. In dilutions of 3, 2, and 1 per cent, the typical ergosterol bands at approximately 2930, 2800, 2720, and 2600 Å units became evident. From this picture we might assume, like Rosenheim and Webster, that the provitamin D potency of Cholesterol W resided entirely in the ergosterol present as a contaminant.

Further investigation, however, leads us to believe that there are at least two substances in Cholesterol W which influence the picture, one ergosterol and the other a modified form of cholesterol which produces general absorption and that both of these substances impart to Cholesterol W its antirachitic activatability. When, for example, we compare the spectra of Cholesterol W with those of ergosterol (Figs. 2 and 3) it appears that a 2 per cent solution of Cholesterol W was equivalent to a 0.002 per cent solution of ergosterol except for a difference in end absorption and that in antirachitic activatability this ergosterol should have been 1000 times as potent as Cholesterol W. This particular lot of ergosterol, however, when tested biologically proved to be only 100 times as activatable as the cholesterol. The end absorption in the case of Cholesterol W then appears to be of fundamental significance.
Again we always observed that the four bands were never as clearly defined in the spectra of the three samples of Cholesterol W examined as in the case of ergosterol (compare Figs 2 and 3). It appears as though in Cholesterol W a general absorption in this region obscured the bands. We mixed cholesterol of low antirachitic activatability, which had been purified by bromine treatment and which showed no ergosterol bands, with ergosterol in the proportion of 1000:1. This mixture should have the same degree of antirachitic potency when irradiated as Cholesterol W. Yet when compared with the spectrum of Cholesterol W in the same concentrations the picture is quite different (Fig. 4). In the higher concentrations of Cholesterol A + ergosterol (5 to 3 per cent Cholesterol A + 0.005 to 0.003 per cent ergosterol) there is absorption between 2600 and 2800 Å units due to a merging of three of the bands, while beyond 2600 the light comes through. The band at 2930 appears distinctly, but the other three are only faintly suggested. At the concentration of 2 per cent Cholesterol A + 0.002 per cent ergosterol, the four bands become clear. This picture is comparable to pure ergosterol in the same concentrations.

In the 5 to 3 per cent solutions of Cholesterol W on the other hand, there is complete absorption beginning sharply at 2980 Å units. In the 2 per cent solution the first ergosterol band at 2930 appears and in the 1 per cent solution all four bands become apparent. This is what we might expect if in Cholesterol W there are two substances present—one causing general absorption, and the other ergosterol—and it is only after the substance causing general absorption is sufficiently diluted that the weaker ergosterol bands can be seen.

The substance in Cholesterol W, which causes general absorption and which has antirachitic potency when irradiated, was concentrated, we believe, in our Fraction II. This fraction separated in an emulsion layer at the second stage of purification of our original Cholesterol W. After the cholesterol had been boiled in a solution of sodium hydroxide in alcohol, the recovered crystals were dissolved in ether and the ether solution was washed several times with water. With every washing an emulsion layer formed which it was impossible to break either by adding ether or by long standing. The cholesterol in these emulsion layers was recovered as Fraction II.
When irradiated and fed to rats on a rachitic diet Fraction II had about the same antirachitic potency as the original Cholesterol W and yet it showed no ergosterol bands (Fig. 5). There was only general absorption beginning at about 3050 Å-units and diminishing with decreasing concentrations until a 0.1 per cent solution gave essentially the same picture as the pure solvent.

This finding that Fraction II, although of the same order of provitamin potency as Cholesterol W, showed no ergosterol bands was given added importance when we found that our heated samples of Cholesterol A behaved in precisely the same manner (Fig. 6). Instead of bands there was general absorption beginning at this particular concentration (3 per cent) at 3250 Å-units and gradually fading out with diminishing concentrations. We have some evidence of a similar change in cholesterol when it is heated with alkali.

This change in cholesterol which caused general absorption was always correlated with the increase in antirachitic activatability. On the other hand those samples of heated cholesterol which when irradiated did not show increased curative effect likewise showed little or no change in their spectra. For example, we introduced portions of Cholesterol A into tubes and passed nitrogen through the tubes for 1 hour. The tubes were then sealed and heated for periods of time varying from 2 hours to 72 hours and at different temperatures varying from 200° to 260°. These specimens showed only slight increases in activatability (8). When examined spectrographically they showed very little change from the original Cholesterol A.

Likewise when Cholesterol A was heated under very low pressures of 1 to 2 mm., in which case most of the cholesterol sublimed within 15 minutes, the sublimate was not changed from the original Cholesterol A either in activatability or in its spectrographic picture. Whenever we heated cholesterol alone and obtained increased activatability there was always a marked general absorption in the ultra-violet beginning at 3100 to 3300 Å-units. If no such increase in activatability occurred we obtained no general absorption. On the other hand, if we heated cholesterol with reagents such as P₂O₅ and CuSO₄ which destroyed considerable cholesterol, then we frequently had strong general absorption with no increased activatability. These facts are illustrated in Fig. 7.
Here the strong similarity between Fraction II and an activatable heated preparation is shown in D, E, F, and G. Cholesterol A heated in the sealed tube, on the other hand, shows very little difference from the original Cholesterol A. See J and K.

When CuSO₄ and soda-lime were added the cholesterol underwent more profound changes, and strong general absorption was observed. But these preparations were of low potency when irradiated. Probably this is the type of oxidation products described by Kon, Daniels, and Steenbock (4) as causing general absorption.

Schlutz and Ziegler (9) in 1926 obviously observed the same change in the spectrum of cholesterol when it was heated. They found that: “Apparently these absorption bands are characteristic of the hydrated cholesterol while the anhydrous form shows no bands but general absorption.” Treating the anhydrous form with water and evaporating to dryness on the water bath yielded the hydrated form again. Undoubtedly the bands described by them are ergosterol bands. We have not found such a reversion of the heated material to take place readily. A heated sample of cholesterol was dissolved in hot 95 per cent alcohol and slowly evaporated to dryness. This when irradiated and tested biologically was not reduced in antirachitic potency and also still showed the same degree of general absorption.

Since our first purified Cholesterol A was not subjected to rigorous treatment it seemed important to employ some of the methods described by other workers as completely destroying all antirachitic activatability such as bromination or boiling with KMnO₄ in acetone. Kon, Daniels, and Steenbock (4) have claimed that cholesterol treated three times with KMnO₄ has no ergosterol and cannot be rendered antirachitic. Rosenheim (3), Windaus (1), and Pohl (2) agree that cholesterol which has been treated with bromine likewise loses all activatability. We purified one lot of Cholesterol W by bromine treatment as described by Bills and another by a triple treatment with KMnO₄ in boiling acetone. When irradiated both of these samples produced calcification in dosages 70 to 30 times that required of the original Cholesterol W. These results confirm those reported by Bills, Honeywell, and MacNair (10). We do not assert that this low activatability may not be due to a trace of ergosterol, although we do not believe
that this is the fact since we have other evidence of highly potent cholesterol preparations which show no bands.

Again, we heated both of these purified preparations in an atmosphere of Nz at 185° and increased the activatability so that a 0.1 mg. dose was adequate for a cure of ++ when administered for 10 days after a 14 day preliminary period on the rachitic diet. Fig. 8 shows the spectra of Cholesterol W, of the dibromide, of the reduced dibromide cholesterol, and of the heated reduced dibromide cholesterol. Tested biologically, Cholesterol W produced a cure of ++ in 0.1 mg. daily dose; the dibromide was entirely inactive in 15 mg. daily dosages; the reduced dibromide was potent in 7 mg. daily dosage; the heated reduced product was again potent in 0.1 mg. dosage. The same increased activatability was demonstrated in the case of the KMnO₄-treated cholesterol after heating. In fact these results have been repeated so many times by two different workers that we can have no doubt of their accuracy.

SUMMARY

The commercial preparation of spinal cord cholesterol studied shows the four typical absorption bands of ergosterol. This product appears to possess provitamin activity due to the presence of ergosterol and of another substance or substances, probably in part cholesterol itself and in part a modified cholesterol of unknown chemical character.

Purification of the commercial cholesterol by repeated washings and recrystallizations, by bromine treatment, or by boiling with potassium permanganate in each case yields a product which has no absorption bands corresponding to ergosterol and which shows no general absorption in the ultra-violet region. These products are nevertheless activatable although only one-seventieth to one-thirtieth as potent as the original commercial cholesterol.

When these purified products are heated slightly above the melting point under conditions that avoid appreciable oxidation every one of the purified products changes to practically 25 to 70 times the activatability of the presumably purer cholesterol. The minimum curative dose of all heated cholesterol preparations by whatever means originally purified was 0.05 to 0.1 mg. Parallel to the increases in activatability a strong general absorption is
observed in the ultra-violet region. In the absorption spectra of these more activatable forms there are however no bands whatever and hence it is not probable that the increased activatability is due to ergosterol.

BIBLIOGRAPHY.

EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Unless otherwise specified the depth of solution is 20 mm. and the time of exposure 2 minutes. A, empty tube; B, solvent; C, 5 per cent Wilson cholesterol; D, 5 per cent Cholesterol B obtained therefrom; E, 5 per cent Cholesterol A obtained from Wilson product; F, 5 per cent of unsublimed residue from 15 hours heating at 225° of Cholesterol A; G, 5 per cent of Fraction II cholesterol obtained in purifying the Wilson product; H, 5 per cent of same but exposed for 4 minutes; I, 2 per cent ergosterol, 20 mm. for 4 minutes; J, same, but for 2 minutes; K, same, 10 mm. for 2 minutes; L, same, 5 mm. for 2 minutes.

FIG. 2. 5, 4, 3, 2, 1, and 0.5 per cent concentrations in anhydrous ether of our second lot from the Wilson Laboratories.

PLATE 2.

FIG. 3. A, empty Baly tube; B, anhydrous ether; C, D, E, F, G, H, I, and J are 0.01, 0.008, 0.005, 0.004, 0.003, 0.002, 0.001 and 0.0005 per cent concentrations of ergosterol in anhydrous ether.

FIG. 4. A, solvent; B, 5 per cent cholesterol from dibromide + 0.005 per cent ergosterol; C, same, but 3 per cent + 0.003 per cent; D, same but 2 per cent +0.002 per cent; E, same but 1 per cent + 0.001 per cent; F, 5 per cent Wilson cholesterol; G, 3 per cent same; H, 2 per cent same; I, 1 per cent same.
PLATE 3.

Fig. 5. A, 0.004 per cent ergosterol in anhydrous ether; B, same of ergosterol previously irradiated in the solid form; C, D, E, F, and G are 2, 1, 0.5, 0.25, and 0.12 per cent concentrations of our "Fraction II" cholesterol obtained in purifying the Wilson product; H, anhydrous ether alone. The slight irregularities observed in this plate are due to using alternately two different Baly tubes.

Fig. 6. A, B, C, D, E, and F are 3, 2, 1, 0.5, 0.3, and 0.1 per cent concentrations of our heated purified product, Cholesterol A. For the method of preparation of the product and the subsequent treatment see the text. G, ether.

PLATE 4.

Fig. 7. A, 3 per cent irradiated Wilson cholesterol; B, 3 per cent Cholesterol A (non-irradiated); C, same irradiated; D, 3 per cent Fraction II cholesterol (non-irradiated); E, same irradiated; F, 3 per cent Cholesterol A, previously heated at 7 to 10 mm. pressure (non-irradiated); G, same irradiated; H, 3 per cent Cholesterol A heated with CuSO₄; I, 3 per cent Cholesterol A heated with soda-lime; J, 2 per cent Cholesterol A heated at 200° at high pressure for 25 hours (non-irradiated); K, same irradiated; L, solvent.

Fig. 8. Unless otherwise stated the depth of solution used is 20 mm. A, solvent; B, 6 per cent Wilson cholesterol; C, same, but 10 mm. depth; D, 3 per cent cholesterol dibromide; E, same, but 10 mm. depth; F, 6 per cent cholesterol from the dibromide; G, same, but 10 mm. depth; H, 3 per cent cholesterol from the dibromide, and then heated for 2 hours at 165° before dissolving in ether; I, same, but at 15 mm. depth; J, same, but at 10 mm. depth.
(Koch, Koch, and Lemon: Absorption spectra studies.)
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