LXXI. THE ANTI-RACHITIC PROPERTIES OF IRRADIATED STEROLS.

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IN a previous communication [1925] we have described the reasons which led us to the investigation of the action of ultra-violet light on sterols and brought evidence to show that irradiated cholesterol acquires anti-rachitic properties. We have since abundantly confirmed this discovery, which was made independently and almost simultaneously by the American investigators, Hess, Weinstock and Helman [1925], and Steenbock and Black [1925]. The investigation was extended mainly in two directions. After determining the best conditions for the maximum activation we made many attempts, up till now not entirely successful, to isolate, or at least concentrate, the active fraction of irradiated cholesterol. We further subjected a number of suitably selected derivatives of cholesterol to irradiation in order to determine which of the known molecular groupings of the cholesterol molecule is essential for successful activation.

EXPERIMENTAL.

Methods. A quartz mercury-vapour lamp (Cooper-Hewitt's 210 volt, 3.5 amp.) at a distance of 40 cm. was used. An exceptionally pure specimen of cholesterol (M.P. 149° uncorr.), prepared from ox brain by Rosenheim's method [1906], was employed. It had been subjected to repeated treatment with alcoholic potash and recrystallised at least twenty times from a variety of solvents. In our previous work we irradiated the substance dissolved in liquid paraffin and used this solution for feeding experiments. To avoid the danger of incomplete absorption from this medium we now irradiate thin films of solid cholesterol spread on the inner surface of a spherical quartz flask and use its solution in an inactive oil (linseed) for feeding purposes. A weighed quantity of cholesterol, not more than 1 g., is dissolved in a little anhydrous ether, free from peroxides. It is transferred to a quartz flask of 400 cc. capacity and the ether removed by a current of air, drawn gently through the flask, which is kept rotating horizontally. The solid is left evenly distributed as a thin film adhering firmly to the sides. The flask is now evacuated and afterwards filled with pure nitrogen, this process being repeated several times. When the irradiation was carried out in air, the flask was closed so as not to expose the material to the risk of oxidation from ozone produced

by the lamp. The neck of the flask is then attached to the axle of a slow-running motor, exposing the bulb to the light of two lamps, each being 40 cm. from the centre of the rotating flask.

The anti-rachitic activity of the material was tested by feeding experiments on rats, under the licence held by Dr Leonard Hill, F.R.S., to whom we are greatly indebted. The well-known low-phosphorus diet No. 84 of Sherman and Pappenheimer was used, modified in a slight, but essential detail. In our experience, shared by other workers, control rats fed on the original diet at times fail unaccountably to develop rickets. This failure to give constant controls, which may occasionally invalidate a whole series of valuable experiments, was traced to the fat content of the "Patent Flour" forming the bulk of the diet. Since the "Patent Flour" itself can be easily rendered anti-rachitic by ultra-violet light [Webster and Hill, 1925] and as the fat, amounting to 1 % of the flour, contains sitosterol, variable seasonal exposures to sunlight of the raw product may account for the occasional slight anti-rachitic activity of the flour. As soon as we commenced to employ a fat-free "Patent Flour" the control animals invariably developed rickets in a short period on the modified diet. For the removal of the fat we shake the flour with an equal volume of ether at room temperature for 15 minutes, filter through a Buchner funnel and wash twice with ether. The material is then spread in a thin layer on filter paper and left overnight at room temperature until the ether has completely evaporated. Compared with the original flour the product is perfectly white, having lost its "bloom," whilst the ether extract leaves a bright yellow oil. We convinced ourselves by a separate experiment that this oil is easily rendered anti-rachitic by short exposures to ultra-violet light.

The material to be tested is not incorporated with the diet, but administered daily to each individual rat in a solution of linseed oil, measured by means of a Wright's capillary micro-pipette [Wright and Colebrook, 1921].

For the diagnosis of rickets we relied on the evidence of the radiograph and the estimation of the content of inorganic phosphates in the blood. For the preparation of the former the hind limbs and ribs of the animal were placed on black paper and photographed with a "soft" X-ray tube. Typical photographs are shown (see Plate II). These demonstrate clearly the most characteristic radiographic evidence of rickets, *i.e.* the condition of the epiphyseal junction at the knee.

(1) *Optimum conditions for activation.*

We have previously considered the question of dosage [1925] and found that 0.2 mg. of irradiated cholesterol per day represents the limit of protection against rickets, whilst 4 mg. are completely protective. We have therefore chosen a dose of 4-5 mg. for routine purposes and have now attempted to determine (1) whether the somewhat tedious procedure of irradiation in a closed flask in nitrogen (or in air) may be replaced by the simpler

(1) Diet No. 84:
Rickets

(2) Diet No. 84
+ 5 mg. cholesterol:
Rickets

(3) Diet No. 84
+ 5 mg. irradiated cholesterol:
Protection

(4) 1/100 mg. active fraction
(non-precipitable by digitonin):
Protection

(5) Control:
Rickets

(6) 0.1 cc. cod-liver oil:
Protection

direct irradiation in an open dish, and (2) to fix incidentally the optimum period necessary for activation. The following table gives a summary of the experiments.

Table I.

	Exp.	No. of rats	Time hrs.	Dose mg.	Inorganic blood-phosphate mg. %	Radiograph
Irradiated in nitrogen	1	4	$\frac{1}{2}$	5	4.0	Complete protection
	2	4	5	5	4.5	"
	3	3	40	5	2.7	Partial protection
Irradiated in air, in closed flask	4	4	$\frac{1}{2}$	5	4.2	Complete protection
	5	4	5	5	3.3	"
	6	2	10	4	4.0	"
Irradiated in open dish	7	4	1	5	4.0	"
	8	2	10	5	—	Rickets
Controls:						
Diet 84 only	9	6	—	—	2.1	"
Diet 84 + non-irradiated cholesterol	10	6	—	5	2.0	"

Whilst these results show apparently no difference as regards anti-rachitic power between irradiation in nitrogen and air (open dish or closed vessel) for short periods from $\frac{1}{2}$ to 5 hours, they are strikingly in favour of the closed flask during prolonged exposures. A period of 10 hours' irradiation in air under these conditions confers complete protection, whereas the same exposure in an open dish results in complete inactivity. The destruction of the initially formed "activated cholesterol" cannot be due entirely to the prolonged action of ultra-violet rays, since even after 40 hours' irradiation in nitrogen the material still possesses activity. We suggest tentatively that the effect may be due to the destructive action of organic "peroxides" formed from sterols by the action of light which is in this instance intensified by ozone. These oxidative changes are manifest by the yellow colour and the drop in melting point of the various sterols examined by us [1925], whilst they are absent in the nitrogen-irradiated material. This fact is also indirectly proved by the experiments of Hamano [1925], who found that the action on the photographic plate of irradiated cholesterol (now generally explained by the presence of hydrogen peroxide) is not demonstrable when the substance has been irradiated in a vacuum or in hydrogen.

As an outcome of this work we have now adopted a period of 1 hour's irradiation for routine purposes, although a much shorter time may be sufficient. In view of the additional evidence with regard to the instability of the air-irradiated material, clearly demonstrated by the fractionation experiments to be described later, we carry out the irradiation in an atmosphere of nitrogen.

Effect of sunlight. The following experiments are of interest in regard to the possible relationship of vitamin D to irradiated sterols and its formation in nature under the influence of sunlight.

Cholesterol was spread in a layer of 1 mm. on a glass plate, covered with a thin quartz disc and the cell made air-tight with a rubber washer. It was

then exposed to daylight from 10 a.m. to 5 p.m. for 30 days during the month of July, 1925. During this period there were many days of continuous bright sunshine, the total of ultra-violet light units measured by Webster, Hill and Eidinow's method [1924] being 149 units. Under the same conditions a sample of cholesterol was exposed in a similar all-glass cell. A third specimen was exposed between two glass plates for six months to white light (on sunless days to the rays of a carbon arc). Its melting point dropped from 149° to 139° .

The samples were kept in the laboratory for one month before being tested. Two rats, which received 5 mg. each per day of cholesterol exposed to sunlight through quartz, were completely protected from rickets, whilst those fed on the specimens exposed through glass, as well as the control animals, developed typical rickets.

The failure of a similar experiment performed simultaneously in New York and since recorded by Hess, Weinstock and Sherman [1925], was clearly due to the admitted lack of sunshine.

(2) *Fractionation experiments.*

Our first attempts to separate an active fraction from irradiated cholesterol were made on both the nitrogen- and air-irradiated material. Since a preliminary examination showed that the bulk of this consisted of chemically unchanged cholesterol, it was intended to remove this as far as possible by recrystallisation from alcohol, thus effecting a possible concentration of the active substance in the mother-liquors, which were to be treated subsequently with digitonin in order to free them completely from cholesterol.

(a) *Fractionation with alcohol and light petroleum.* One g. cholesterol¹ was irradiated in nitrogen for 40 hours, dissolved in 10 cc. hot absolute alcohol and allowed to stand at room temperature for 24 hours. The crystals were filtered off, washed with alcohol and again recrystallised from 5 cc. alcohol: Fraction I.

The combined mother-liquors were evaporated *in vacuo* and the remaining solids treated with 5 cc. light petroleum at room temperature. The undissolved material was labelled: Fraction II, and the residue from the light petroleum extract: Fraction III. This fraction weighed 115 mg. and was somewhat yellowish, but consisted mainly of cholesterol, giving the usual colour reactions in a somewhat modified form. The melting points of the various fractions did not differ materially from that of the non-irradiated substance.

A second experiment was carried out in precisely the same way, except that the cholesterol was irradiated in air (closed flask) for 1 hour. The operations were carried out rapidly and with great care, the loss of material amounting only to 2 %.

¹ The specimen of cholesterol had been subjected to a light petroleum treatment before irradiation.

The results of the feeding experiments are given in an abbreviated form in the following table (Table II).

Table II.

Control		Air (1 hour)	Nitrogen (40 hours)
Fraction I		Rickets	Rickets
" II		"	Rickets*
" III		"	Partial protection
			"

* The inactive Fraction I was easily reactivated by a subsequent exposure to the lamp.

The surprising result of this experiment is not so much that the last fractions of the nitrogen-irradiated cholesterol remained active, but the fact that the whole of the activity was lost by the same treatment of the air-irradiated specimen. The incompleteness of the protection afforded by the last fractions in the nitrogen experiment may be partially explained by the fact that the original product had already, after 40 hours' irradiation, lost a considerable part of its activity (see Exp. 3, Table I), a fact unknown at the commencement of the fractionation.

(b) *Digitonin experiments.* One g. cholesterol was irradiated in nitrogen for 5 hours. It was subsequently recrystallised from 10 cc. absolute alcohol and the mother-liquor evaporated *in vacuo* at 40° to dryness. The residue (0.2120 g.) was dissolved in alcohol and precipitated with an excess of alcoholic digitonin solution. The filtrate and washings were evaporated *in vacuo* and the residue treated with pure ether. The ether extract was washed repeatedly with water for the complete removal of digitonin.

The ether left a yellow resinous solid residue which was unweighable, but was judged to weigh less than 1 mg. The amount of cholesterol recovered as digitonide was 99.9 %, *i.e.* well within the limits of error of the method.

The experiment was repeated with 5 g. cholesterol with the single modification of irradiating the substance for 1 hour in air (closed flask). The fraction to be treated with digitonin weighed 0.5355 g. and the amount not precipitated by digitonin rose to 56.2 mg. = 10.5 %.

The feeding experiments gave the following results (Table III):

Table III.

	Dose mg.	Nitrogen	Dose mg.	Air
Before fractionation	5	Complete protection	5	Complete protection
Controls	—	Rickets	—	Rickets
Fraction not precipitated by digitonin	1/100	Fairly active (1 normal, 1 mild rickets)	0.5	"

These results prove that at least a portion of the activity of the nitrogen-irradiated material may be recovered by the digitonin process. If we remember that the actual weight of the residue available for feeding purposes was less than 1 mg. and that each rat received on the highest estimate 1/100 mg. of this material per diem, its actual potency is of a high order.

The completely negative result yielded by the fractionation of the air-irradiated cholesterol supplements our previous evidence for the instability

of this material and is in agreement with an experiment since published by Hess, Weinstock and Sherman [1926], who failed to recover any activity by the digitonin method from activated cholesterol (presumably air-irradiated).

In this connection the analytical figures are of considerable interest, as showing that as much as 10 % of air-irradiated cholesterol is not precipitable by digitonin, whilst the corresponding figure is less than 0.1 % when the irradiation is carried out in nitrogen. Beumer [1925] has also reported a loss of 8-9.5 % on precipitating cholesterol, irradiated in air, with digitonin. It might further be mentioned that according to Lifschütz [1914] the amorphous oxidation products of cholesterol ("oxycholesterol") are only partially precipitated by digitonin. Beumer suggests, without experimental evidence, that the non-precipitable fraction represents the active substance, whilst our negative results lead us to the contrary suggestion that these clearly oxidative products are concerned in the inactivation of the material.

These experiments emphasise the importance of carrying out the irradiations in an atmosphere free from oxygen. Further work is necessary to test the question whether irradiation of cholesterol in a high vacuum and in the absence of even traces of moisture will increase the stability of the active product. This question, as well as the selection of the most suitable solvent for extraction, is at present engaging our attention. We have accumulated evidence indicating that by the use of light petroleum instead of alcohol in the fractionation, the loss of activity may be considerably reduced.

It may be recalled that an unknown amount of vitamin D of cod-liver oil escapes precipitation by digitonin. The above result therefore supplies further evidence for the suggested identity of irradiated cholesterol and vitamin D.

(3) *Action of ultra-violet light on cholesterol derivatives.*

The following substances were investigated:

Cholesteryl chloride, $C_{27}H_{45}Cl$, prepared from cholesterol and thionyl chloride [Diels and Blumberg, 1911].

Cholestene, $C_{27}H_{46}$, the unsaturated hydrocarbon, prepared from cholesteryl chloride [Mauthner and Suida, 1894].

Cholestenone, $C_{27}H_{44}O$, the ketone of cholesterol, prepared from dibromocholesterol [Windaus, 1906].

Cholesteryl acetate
Cholesteryl palmitate } prepared by the well-known methods.

Coprosterol, $C_{27}H_{48}O$, the naturally occurring dihydrocholesterol, prepared from dog's faeces.

It seemed further of interest to include in this series a typical myco-sterol, as a representative of which we chose the highly unsaturated and unstable *ergosterol*, $C_{27}H_{42}O$, prepared from ergot. The saturated resin alcohol α -amyrol (amyrin) $C_{30}H_{50}O$ was selected as a representative of the sterols from resins. *Squalene*, $C_{30}H_{50}$, the unsaturated hydrocarbon of certain liver-oils was tested, since recent work suggests that this substance plays a rôle in cholesterol

metabolism. Finally, the bile acids, *cholalic* and *desoxycholic acids*, possessing the same ring system as cholesterol, were also subjected to irradiation.

The cholesterol derivatives were prepared from cholesterol by the methods referred to above, and purified by many recrystallisations. They showed the characteristic properties and melting points as described in the literature. We were enabled to employ a freshly prepared specimen of ergosterol (m.p. 162° [$\alpha_{5461}^{19} = -127^{\circ}$) kindly put at our disposal by Dr H. A. D. Jowett. We are indebted to Mr J. A. Gardner for a specimen of coprosterol prepared from dog's faeces by Dorée and Gardner [1908], and Mr H. J. Channon kindly supplied us with a sample of squalene isolated by him from the liver-oil of *Spinax niger*. Our colleague, Dr P. P. Laidlaw, kindly put at our disposal a well-crystallised specimen of α -amyrol prepared by himself from elemi resin.

All these substances were irradiated in an open dish in air for 30 minutes and fed to two rats in each experiment. The doses were 5 mg., except in the case of the bile acids of which only 2.5 mg. were fed, on account of their possible toxicity. Two control animals received 5 mg. of the non-irradiated substance and in each case two control rats were fed on the rachitic diet.

The results need not be given in detail, since with the exception of ergosterol and the two cholesteryl esters (acetate and palmitate) all the irradiated substances were completely inactive. Irradiated ergosterol was found to be highly protective even in doses of 1 mg. The activity of the irradiated esters was confirmed in a subsequent set of experiments.

These results enable us to draw conclusions which throw some light on the relationship between chemical constitution and anti-rachitic activity induced by ultra-violet light.

(1) Only members of the three groups of "protoplasmic" sterols (sterols occurring in animal or plant tissues) can be activated, *i.e.* cholesterol (a zoo-sterol), sitosterol (a phyto-sterol) and ergosterol (a myco-sterol).

(2) Coprosterol and amyrol, members of the group of "excretory" sterols (meta-sterols of Dorée [1913]) cannot be activated.

(3) Both the secondary alcohol group and the unsaturated carbon linkage contained in the sterol molecule must be intact for successful activation.

An interesting parallelism may be pointed out in this connection between the power to acquire anti-rachitic properties by ultra-violet light and the anti-haemolytic properties of sterols [Hausmann, 1905] in relation to their chemical constitution. Only the "protoplasmic" sterols share the two properties, which are both lost when either the (OH) group is occupied or removed (cholesteryl chloride, cholestene, cholestenone) or when the unsaturated bond is reduced (coprosterol, dihydrocholesterol, amyrol). The apparent exception in the case of the two cholesteryl esters may be explained by the relative ease with which these organic acid esters are hydrolysed in comparison with cholesteryl chloride. The production of even a trace of free cholesterol under the influence of ultra-violet light would account for their activation.

The only significance which can be attached to this parallelism consists in the suggestion that the same molecular groupings are concerned in both instances.

SUMMARY.

(1) Cholesterol is rendered anti-rachitic by exposure to sunlight as well as to other sources of ultra-violet light.

(2) By irradiating cholesterol in the absence of oxygen, the stability of the "activated" cholesterol is considerably increased.

(3) An amorphous substance which prevents rickets in rats in doses of 1/100 mg. per day has been separated from "activated" cholesterol by precipitating the cholesterol with digitonin.

(4) Both the secondary alcohol group and the unsaturated carbon linkage of the sterol molecule must be intact for the production of anti-rachitic properties by ultra-violet light.

EXPLANATION OF PLATE II.

The large number of radiographs necessary for this work were kindly taken for us in the department of Mr J. E. Barnard, F.R.S., to whom we wish to express our best thanks. Nos. 1, 2 and 3 are typical direct photographs (slightly reduced) taken from experimental animals, whilst Nos. 4, 5 and 6 have been enlarged for the purpose of reproduction.

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Note added 1st June, 1926. We are able to confirm the recent statement of Shears [*Proc. Soc. Exp. Biol. Med.* 1926, **23**, 546] that irradiated cholesterol gives the colour reaction with aniline, which he suggested to be characteristic for vitamin D. We found, however, that the reaction is negative with cholesterol which had become anti-rachitic by irradiation in nitrogen. On the other hand, cholesterol exposed to sunlight through glass for six months, although inactive in the animal test, gives an intense aniline reaction. Further, air-irradiated specimens of cholesterol, which have lost their anti-rachitic activity on being kept in the dry state for a year, react with the same intensity as freshly prepared specimens. These results, as well as other contradictory tests on vegetable oils before and after irradiation, prove that the reaction is not specific for vitamin D, and suggest that it is due to the formation of organic peroxides. This conclusion is confirmed by the fact that inactive olive oil after the addition of a trace of benzoyl peroxide (or turpentine) gives the reaction with the same intensity as aerated cod-liver oil. According to our experience the phospho-molybdo-tungstic acid reagent of Bezssonoff [*Compt. Rend. Acad. Sci.* (1924), **179**, 572] is equally non-specific.