attached itself to the nitrogen atom. This demonstrates that neither weight nor acidity is the determining factor.

2. Several new acyl derivatives of *o*-aminophenol have been prepared and studied.

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A QUANTITATIVE STUDY OF THE PHOTOCHEMICAL ACTIVATION OF STEROLS IN THE CURE OF RICKETS

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Recent discoveries in chemical physiology, showing that rickets can be cured by sterols which have previously been exposed to ultraviolet radiation,[1] offer interesting possibilities for photochemical investigations. The study appears attractive because the severity of rickets and its cure can be measured fairly quantitatively and because the antirachitic activation of the curative agent—the sterols—can be effected by such small amounts of energy that monochromatic light obtained with a spectrometer may be used.

In the present investigation an attempt was made to determine quantitatively the amount of energy necessary to secure a demonstrable deposition of calcium in the bones of a rachitic rat. This determination not only gives an idea of the extreme sensitiveness of the physiological reaction but, together with spectrographic evidence, indicates also that while cholesterol of even more than ordinary purity can be activated, the effect is probably due to a contaminant. This, hypothesis is in harmony with the recent suggestions of other investigators.[2]

The absorption spectrum of cholesterol was determined first because it was not available in the literature when the investigation was started. Since it could not be taken for granted that all portions of the absorption region are necessarily concerned in the activation process, cholesterol was exposed in different parts of the spectrum and tested physiologically.

Experimental Procedure

The source of light was a 220-volt quartz mercury vapor lamp loaned by the Hanovia Chemical and Manufacturing Company. It was operated on a 500-volt D. C. current with suitable resistances and a reactance in the circuit. After bringing the lamp to equilibrium by running it for some time, the resistances were so adjusted as to give a current of 2.4 amperes and a potential drop of 130 volts across the lamp terminals. For

[1] (a) Steenbock, Black, Nelson, Nelson and Hoppert, *J. Biol. Chem.*, **63**, xxv (1925); (b) Hess and Weinstock, *ibid.*, **63**, xxv (1925); (c) Rosenheim and Webster, *Lancet*, **1925**, p. 1025.

[2] Rosenheim and Webster, *Biochem. J.*, **21**, 389 (1927).

purposes of stability the base of the lamp was fixed in position with a plaster of Paris cast. The entire lamp was enclosed in a large ventilated box in order to prevent stray radiations.

The radiations of the lamp were resolved by means of a Hilger quartz monochromator which was provided with a helical drum graduated from 200 to 800 millimicrons. Its graduation was checked with reference to the well-known lines of the emission spectrum of sodium and the mercury arc. The collimator slit was set at a width of 0.5 or 1 mm. and the instrument gave ample separation of the different lines as proved with photographic plates.

The intensity of irradiation was measured with a Coblenz vacuum thermopile connected with a Leeds and Northrup galvanometer. The thermopile was provided with a fluorite window and the 10 silver-bismuth junctions had a total area of 13.5 square mm. It was mounted on a sliding rack behind the slit so that it could be raised to allow the radiation to strike the sample of cholesterol.

The thermopile-galvanometer system was calibrated in absolute energy units with a standard filament lamp (No. C44, U. S. Bureau of Standards). A radiation intensity of 5.4×10^{-7} watts per square mm., after allowing for a slight correction for the fluorite window, produced a deflection of 28 mm. on the galvanometer scale. The galvanometer had a sensitivity of 15 mm. per microvolt.

It was found necessary to adjust the spectrometer and lamp very carefully, because any shift in their relative positions which might be hardly noticeable was sufficient to reduce the intensity of the emergent light greatly. This adjustment was made by setting the spectrometer with its slit 10 cm. from the end of the lamp and then turning it until the thermopile gave a maximum deflection.

Ordinary purified cholesterol which had been shown to be activatable[1a,b,c] was used as the absorbent for the ultraviolet radiations. All of the experiments were carried out with one preparation known as V-1-24. It represented cholesterol which had been obtained as a commercial product from the Wilson Laboratories. It was purified by further saponification with alcoholic potassium hydroxide, diluted with water and extracted with ether. After evaporation of the ether the residue was crystallized repeatedly from purified ethyl alcohol. The above preparation represented the crystallizations from the alcoholic mother liquors of the 28th to the 40th fractions. It was found in the course of other experiments to be more activatable than the ordinary material.

For exposure to the monochromatic radiations the cholesterol was packed in a little rectangular box of sheet copper, held in a vertical position by a spring clamp directly behind the thermopile slit. The box was slightly larger in area than the slit and 2 mm. deep and it held approximately 50 mg. of cholesterol crystals packed tightly. The crystals were cemented together by the addition of a little ether, which evaporated rapidly. This quantity of cholesterol was vastly greater than the amount required for complete absorption of the radiations. Great care was taken that the cholesterol was exposed in the exact position previously occupied by the thermopile—a procedure which was made relatively easy by the mechanical arrangement.

The tests for activation and quantitative degree of activation were carried out with rats, using the line test as developed in the Johns Hopkins Laboratories.[3] Rickets was produced in the animals by feeding the Wisconsin 2965 ration[4] for a period of three to four weeks. When the rickets was of a severe type, as indicated by the peculiar shambling gait of the animal and the accompanying enlargements of the distal ends of the radii and ulnae, the exposed sample of cholesterol was taken up in ether and poured over 50 gm. of the ration and the ether evaporated. This material was fed to the

[3] McCollum, Simmonds, Shipley and Park, *J. Biol. Chem.*, **51**, 41 (1922).

[4] Steenbock and Black, *ibid.*, **64**, 263 (1925).

animal daily with consumption control and when completely consumed was followed with the original 2965 ration until a total of ten days had elapsed. The animals were then killed with ether, the distal ends of the radii and ulnae removed, split with a scalpel and stained with a silver nitrate solution. The amount of calcium deposition was described as negative, positive or very positive.

The first attempts in this work led to failure because it was not known how long an exposure to make nor what part of the absorption spectrum is the most efficient. The pioneering work, which was done some time before any publications appeared from other laboratories, was done by spraying a solution of cholesterol in ether on a glass plate with an ordinary atomizer. There was thus deposited a uniform film of cholesterol. This film was exposed in a large quartz spectrograph with a dispersion of 25 cm. for wave lengths between 200 and 800 millimicrons. After an exposure of ten hours the plate was lowered slightly to expose fresh areas and this procedure was repeated 8 times. A sheet metal mask with long vertical slots corresponding to various sections of the mercury arc spectrum was then fitted over the plates and the cholesterol in each of these slots was scraped out and tested separately by means of the feeding technique already described. It was found in this manner that only those wave lengths shorter than 313 millimicrons were effective in the process of activation. These results corresponded exactly with what had been reported by Hess and Weinstock[5] for the upper limits of the active region when ultraviolet light cures rickets by direct action upon the animal. In all the later experiments the monochromator and thermopile were used and the results will be published later in detail. For the present research most of the experiments were carried out using the 265-mercury vapor line. This line was chosen because it possesses a sharp maximum, well separated from other radiation and also because at this time a large number of experiments had definitely established that this region was particularly effective in the photochemical reaction.

Experimental Results

The experimental results are summarized in Table I.

TABLE I

INFLUENCE OF QUANTITY OF RADIATION OF 265 mμ WAVE LENGTH ON CURATIVE EFFICIENCY

Time of irradiation, seconds	Energy absorbed, ergs	Number of quanta absorbed·$nx10^{13}$	Antirachitic action
2400	24,960	350	Very positive
1200	12,480	175	Very positive
600	6,240	87.5	Very positive
300	3,120	43.7	Very positive
150	1,560	21.8	Positive
75	780	10.9	Positive
45	468	6.5	Positive
22.5	234	3.2	Positive
11	117	1.6	Negative
5	52	0.8	Negative
2.5	26	.4	Negative
1.25	13	.2	Negative

It is evident from these data that with the mercury line at 265 mμ, a minimum energy input of 234 ergs is necessary to give a positive test for

[5] Hess and Weinstock, *J. Amer. Med. Assoc.*, **80**, 687 (1923).

the deposition of calcium in a rat suffering with rickets. This energy input corresponds to an exposure of 22.5 seconds under the conditions of the experiment.

Similar experiments were carried out with three other lines and in a later research all the lines were carefully investigated. Single observations failed to give positive results with the 257 line at 350 ergs. The 280 line required 1170 ergs and the 302 line 2730 ergs.

Calculations

The following specific calculation shows how the number of quanta absorbed, as recorded in the third column of Table I can be calculated from the thermopile readings.

Galvanometer deflection on 265 mμ (g) = 4 mm.
Galvanometer deflection with standard lamp, (s) = 28 mm.
Radiation from standard lamp = 5.4 ergs per second per sq. mm. under conditions specified by the Bureau of Standards
Area of slit, (a) = 13.5 sq. mm.
Time of radiation, (t) = 22.5 seconds
Total radiation, (E), = $g/s\ a\ t$ (5.4 ergs) = $4/28 \times 13.5 \times 22.5 \times 5.4 = 234$ ergs

According to the quantum theory the total radiation (E) = $nh\nu$, where n = the number of quanta, h = Planck's constant of 6.54×10^{-27} erg seconds and ν = the frequency of the light. For light of 265 mμ, ν = $(3 \times 10^{10}$ cm. per sec.$)/(2.65 \times 10^{-5}$ cm.$) = 1.1 \times 10^{15}$ sec.$^{-1}$. Then $n = E/h\nu = 234/(6.54 \times 10^{-27} \times 1.1 \times 10^{15}) = 3.2 \times 10^{13}$ quanta.

According to Einstein's law of photochemistry each quantum of light produces a molecule of activated material, provided that all the light is used in the photochemical reaction. According to this assumption 3.2×10^{13} molecules of vitamin D were synthesized by the 3.2×10^{13} quanta during the exposure of 22.5 seconds.

The number of gram molecules may be obtained by dividing the number of molecules by the Avogadro number, as follows: $(3.2 \times 10^{13})/(6.06 \times 10^{23}) = 5 \times 10^{-11}$ g. molecule. Assuming that the molecular weight of the antirachitic material is essentially the same as that of cholesterol (385) the number of g. of vitamin D may be calculated as follows: weight of vitamin D synthesized = $5 \times 10^{-11} \times 385 = 2 \times 10^{-8}$ g. This calculation indicates that about twenty billionths of a gram of vitamin D is sufficient to produce calcium deposition in a rachitic rat.

Conclusions

The calculation just given indicates that a quantity of vitamin D so small as to defy any chemical test is sufficient to initiate a cure of rickets. It is difficult then to draw definite conclusions concerning the antirachitic properties of irradiated material from the gross chemical or physical properties.

The validity of the Einstein law may well be questioned and there are

many reactions in which it does not apply. The greatest discrepancies appear in highly polar systems such as in the photochemical combination of chlorine and hydrogen, where a chain mechanism is assumed to account for the large number of molecules produced by each quantum. In the photo-activation of a sterol it is not easy to imagine a mechanism which will *greatly* vitiate the Einstein relation, and the assumption of the validity of the Einstein law is certainly justified as a first approximation. The calculation indicates that about 2×10^{-8} g. of vitamin D is sufficient to give a positive effect on a 100 g. rat and this quantity is smaller than the quantity of adrenaline or other drugs which can be detected by the most delicate physiological tests.

This calculation though indirect is probably more accurate than any direct measurement now available, for even the greatest refinement in the purification of cod-liver oil still leaves a great excess of inactive material. It is probable, however, that in the future, pure vitamin D can be isolated which will have the sensitivity predicted in this research. Already in a recent investigation by Rosenheim and Webster[2] it has been found that 1×10^{-7} g. of a sample of irradiated ergosterol is sufficient to give a positive test.

It must be emphasized that the calculation of 3.2×10^{13} quanta given here is based on the assumption that all the light absorbed by the purified cholesterol (V-1-24) was converted into vitamin D. Probably some of the light was absorbed by material which is not converted into vitamin D and further purification of the pro-vitamin in future preparations should lead to greater efficiencies. It is to be expected then that the minimum quantity of 2×10^{-8} g. calculated here may be still further reduced.

The authors wish to acknowledge the help of Mr. Kenneth G. Bulley,[6] who carried out some of the preliminary work.

Summary

1. A quantitative photochemical investigation using monochromatic light from a spectrometer has been made possible by utilizing a sensitive physiological test for the cure of rickets.

2. 3.2×10^{13} quanta of light of 265 mμ produced sufficient vitamin D from purified cholesterol to affect a rat suffering with rickets.

3. Assuming the Einstein photochemical relation it has been calculated that 3.2×10^{13} molecules or 2×10^{-8} g. of vitamin D is sufficient to give a detectable deposition of calcium.

4. Similar experiments with purer pro-vitamin may show that this minimum quantity may be still further reduced.

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[6] B. S. *Thesis*, University of Wisconsin, June, 1925.