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ON THE CHEMICAL NATURE OF VITAMIN D

Etsuo TAKAMIYA

HULDSCHINSKY (1919) confirmed that ultra-violet rays exert a curative action on rickets when the rickety rats are directly irradiated. McCOLLUM and co-workers (1922) reported that cod liver oil contains in abundance some calcium-depositing substance which is present in butter fat in but very slight amounts and which exerts a directive influence on bone development. This substance[1] is apparently distinct from fat-soluble vitamin A. STEENBOCK and BLACK (1924) demonstrated that rickets-producing diets after irradiation with a mercury vapour quartz lamp exert a curative action on rickets, being activated antirachitically. STEENBOCK and co-workers (1925) reported that purified proteins, carbohydrates, fats, salts, water, ether, hydrochinon, phloroglucin, or paraffin hydrocarbons could not be activated, but cholesterol purified by crystallization, then as benzoate, and finally as acetate, as well as phytosterols, could be photochemically activated after irradiation with a mercury vapour quartz lamp. Many investigators, thereafter, studied in detail on the activated cholesterol after irradiation, and for some time until the appearance of ROSENHEIM and WEBSTER's paper, it was generally held that cholesterol is the precursor of vitamin D. WINDAUS and HESS (1926) confirmed the fact that if cholesterol was purified by the formation of derivatives (e. g., dibromide), it could no longer be activated by ultra-violet rays, and then concluded that the mother substance of vitamin D is an impurity and not cholesterol, and suggested that ergosterol is probably the mother substance of vitamin D, since it cannot be separated from cholesterol by physical methods, and yet it is active in preventing rickets in mere traces after irradiation. ROSENHEIM and WEBSTER (1927) purified cholesterol as far as possible

[1] This is now called vitamin D.

by repeated crystallization, then converted it into the dibromide and regenerated the cholesterol by reduction. This purified cholesterol could not be activated after irradiation, which showed that it is not cholesterol, which is the precursor of vitamin D, but some impurity which is destroyed by bromination. They announced that ergosterol is the parent substance of vitamin D. ROSENHEIM and WEBSTER (1928) presented evidence that in ergosterol not only a typical ring structure but also the specific position of the three unsaturated bonds is essential for photochemical conversion into vitamin D.

Studies on vitamin D, therefore, have been advanced from the relation "pro-vitamin + ultra-violet rays → vitamin D" to the relation of "ergosterol + ultra-violet rays → vitamin D". Nevertheless, the significance of the action of ultra-violet rays on the photochemical conversion of ergosterol into vitamin D still remains unexplained. If this problem could be solved, the chemical nature of vitamin D would be elucidated. I present evidence in this paper from my experimental results that the action of ultra-violet rays in all respects, especially concerning problems on the formation of vitamin D is closely analogous to that of ozone, and then suggest that vitamin D is imperfectly ozonized ergosterol.

My best thanks is due to Prof. Dr. Y. Okuda and Prof. Dr. M. Yukawa for their kind guidance.

I. ENZYMIC HYDROLYSIS-VELOCITY OF VEGETABLE OIL BEFORE AND AFTER IRRADIATION WITH ULTRA-VIOLET RAYS

Six vegetable oils, differing in iodine value - coconut oil, olive oil, almond oil, cottonseed oil, soy-bean oil, linseed oil - were employed.

The castor-bean lipase preparation applied for this experiment was obtained as fine white powder, from castor-beans after removing their husks by hand, extracting the kernels with petroleum-ether.

The method of estimating for hydrolysis-velocity of oil by the lipase was similar to that of R. WILSTÄTTER (1924) except that a cylindrical bottle with a glass-stopper, a definite diameter and a flat bottom was used, for the reason stated in my paper (1926). The lipase preparation (0.2 g) was weighed into the estimating bottle (diameter 3.6 cm.), then 2.8 c.c.[2] of each vegetable oil added as substrate, and shaken for one

[2] The same volume of each substrate must be used in the mutual comparison for enzymic hydrolysis-velocity for the reason stated in my paper (1926).

minute, then 2 c.c. of acetic-acetate buffer solution (PH = 4.7) added and shaken well for 3 minutes, then placed standing in a constant water-bath (30°C) for 3 hours. The reaction mixture was washed cautiously into a flask with 30 c.c. of 95 per cent ethyl-alcohol and 15 c.c. of ether, and titrated with 0.1 N potasium hydroxide solution, using phenolphthalein as indicator. The enzymic hydrolysis percentage of each oil was calculated from the quantity of liberated fatty acids to each ester value.

A. 10 c.c. of each vegetable oil taken in a crystallization dish (diameter 9.5 cm.) were directly irradiated for 3 hours at a distance of about 19 cm. from a mercury vapour quartz lamp in a water-jacketed box, the temperature during irradiation rising up to about 60°C. The enzymic hydrolysis-velocity of each original vegetable oil and of its irradiated form was as follows:

Table 1

Substrate	Hydrolysis-velocity (%)		Decreasing ratio (b/a × 100)
	original (a)	irradiated (b)	
Coconut oil	52.1	36.6	70.2
Olive oil	44.3	27.0	60.9
Almond oil	41.0	23.7	57.8
Cottonseed oil	40.7	20.0	49.1
Soy-bean oil	41.5	19.5	46.9
Linseed oil	35.4	16.1	45.5

B. Cottonseed oil (10 c.c) taken in a crystallization dish (diameter 9.5 cm.) was directly irradiated for a definite time at a distance of about 18 cm. from the lamp in a water-jacketed box, the temperature during irradiation rising up to about 45°C. The enzymic hydrolysis-velocity of the original oil and of its irradiated form for a definite time was as shown in table 2.

From these experimental results, it became evident that after irradiation with ultra-violet rays, the irradiated vegetable oil was less readily hydrolysed by the enzyme than was the original, and the greater the iodine value the greater is the influence of irradiation, and that the

decrease in enzymic hydrolysis-velocity of oil is directly proportional to the length of time of irradiation.

Table 2

Time for irradiation (hr.)	Hydrolysis velocity (%)
0.0	40.7
0.5	32.3
1.0	24.3
2.0	22.3
21.0	0.0

II. ACTION OF ULTRA-VIOLET RAYS UPON VEGETABLE OIL SEEN FROM THE VIEWPOINT OF ENZYMOLOGY

The action of ultra-violet rays upon vegetable oil was investigated, the change in enzymic hydrolysis-velocity of oil caused by irradiation being the criterion.

A. ACID VALUE, SAPONIFICATION VALUE AND ESTER VALUE OF VEGETABLE OIL AND OF ITS IRRADIATED FORM

The irradiated vegetable oil was prepared similarly to that of the experiment I. A.

Table 3

Sample	Acid value*		Saponification value		Ester value	
	I	II	I	II	I	II
Coconut oil	2.0	2.6	242.9	243.7	240.9	241.1
Olive oil	4.5	5.5	193.5	194.8	189.0	189.3
Almond oil	1.3	2.8	194.7	195.6	193.4	192.8
Cottonseed oil	0.2	2.0	195.9	199.1	195.7	197.1
Soy bean oil	0.8	2.2	193.0	194.4	192.2	192.2
Linseed oil	2.5	4.8	194.2	197.4	191.7	192.6

I: Original oils II. Irradiated oils

* This acid value was estimated after the oil in about 5 times distilled water was cautiously warmed on a water bath for 3 hours.

After irradiation with ultra-violet rays, acid value and saponification value of each oil increased moderately, but its ester value remained unchanged. The increase in saponification value, therefore, is evidently due to the increase in acid value.

B. IODINE VALUE OF VEGETABLE OIL AND ITS ENZYMIC HYDROLYSIS-VELOCITY

Table 4

Substrate	Iodine value (Wijs' method)	Hydrolysis-velocity (%)
Coconut oil	7.3	52.1
Olive oil	84.3	44.3
Almond oil	96.3	41.0
Cottonseed oil	109.4	40.7
Soy-bean oil	133.8	41.5
Linseed oil	183.4	35.4

These experimental results indicate that there exists a certain correlation between the iodine value of oil and its enzymic hydrolysis-velocity; namely, the greater the iodine value the less is the velocity. From this fact, the nutritive value of fat and oil which was already made evident to be directly proportional to its enzymic hydrolysis velocity by Y. MATSUYAMA and M. YOSHIDA (1927), can be assumed to be inversely proportional to its iodine value. This assumption will also be confirmed by the results of feeding experiments which were carried out by other investigators such as J. OZAKI (1927) and S. UENO (1927).

C. IODINE VALUE OF IRRADIATED OIL AND ITS ENZYMIC HYDROLYSIS-VELOCITY

The irradiated vegetable oil was prepared similarly to that in the experiment I. A.

From the experimental results given in table 5, it became evident that after irradiation with ultra-violet rays, the vegetable oil is less readily hydrolysed by the enzyme than is the original, and the greater the iodine value the greater is the influence of irradiation, and that the

iodine value of irradiated oil became less than that of the original, and the greater the iodine value the greater is the influence of irradiation. These relations are readily seen in table 6, in which the figures given in table 4 and 5 are combined, and the decreasing ratios are compared.

Table 5

Substrate (irradiated oil)	Iodine value (Wijs' method)	Hydrolysis-velocity (%)
Coconut oil	7.0	36.6
Olive oil	80.5	27.0
Almond oil	90.7	23.7
Cottonseed oil	99.5	20.0
Soy bean oil	121.8	19.5
Linseed oil	163.8	16.1

Table 6

Substrate	Hydrolysis-velocity (%)		Decreasing ratio (b/a × 100)	Iodine value		Decreasing ratio (b/a × 100)
	I (a)	II (b)		I (a)	II (b)	
Coconut oil	52.1	36.6	70.2	7.3	7.0	95.9
Olive oil	44.3	27.0	60.9	84.3	80.5	95.5
Almond oil	41.0	23.7	57.8	96.3	90.7	94.2
Cottonseed oil	40.7	20.0	49.1	109.4	99.5	90.9
Soy bean oil	41.5	19.5	46.9	133.8	121.8	91.0
Linseed oil	35.4	16.1	45.5	183.4	163.8	89.3

These experimental results indicate that the decrease in iodine value of irradiated oil is in an intimate relation to that in enzymic hydrolysis-velocity. But on the other hand, it was already made evident in the experiment **II. B** that the enzymic hydrolysis-velocity of non-irradiated oil is inversely proportional to the iodine value, and the greater the iodine value the less is the velocity. Therefore, in other modes except the simple saturation, the iodine value must be decreased. What is

here meant by "other modes" was afterwards confirmed on investigation (see the experiment **II.** E and **II.** F) to be the formation of ozonide.

And it is also evident from table 6 that the decreasing ratio in enzymic hydrolysis-velocity of oil is far greater than that in iodine value. We must conclude, therefore, that the decrease in enzymic hydrolysis-velocity of oil after irradiation is more probably due to the fact that the presence of oil altered by irradiation retards in some way the enzymic activity, rather than to the fact that such altered oils are no longer hydrolysed by the enzyme. (Of course, what is here meant by altered oil corresponds to the ozonized oil as seen from the brief note above.)

D. VISCOSITY OF IRRADIATED OIL AND ITS ENZYMIC HYDROLYSIS-VELOCITY

Vegetable oil became strongly viscous after irradiation with ultraviolet rays for a long period. This made it essential to study the correlation between the viscosity of oil and its enzymic hydrolysis-velocity. For this purpose, OSTWALD's viscositometer was employed, the temperature being 30°C, sample oil measuring 5 c.c., and time (seconds) spent in dropping off of oil measured, and the viscosity of oil was calculated according to the following formula.

$$\eta = dt$$

η: viscosity d: sp. gr. t: time (second)

Table 7

Sample	Viscosity (η)	Hydrolysis-velocity (%)
Olive oil	369.8	44.3
Almond oil	332.8	41.0
Cottonseed oil	300.7	40.7
Soy-bean oil	266.7	41.5
Linseed oil	228.4	35.4

It became evident from the experimental results that the viscosity of oil runs parallel with its enzymic hydrolysis-velocity; namely, the greater the viscosity the greater is the velocity.

Table 8

Time for irradiation (min)	Viscosity (η)
0	300.7
30	315.6
60	342.5
180	490.4

Cottonseed oil was used and irradiated similarly to that in the experiment **I.** A.

From the experimental results obtained above, it became evident that the greater the viscosity the greater is the velocity, and that the viscosity of oil increases gradually according to the elongation of time of irradiation. If seen from the point of view of viscosity, it would appear the enzymic hydrolysis-velocity of oil should be increased through irradiation with ultra-violet rays, but, in point of fact, the enzymic hydrolysis-velocity of oil was strongly decreased after irradiation (see the experiment **I.** A and **I.** B). Therefore, the increase in viscosity of irradiated oil is seen to be independent of the decrease in its enzymic hydrolysis-velocity.

E. ACTION OF OZONE UPON OIL

The SIEMENS' ozone-generator with ten-tubes was employed as an ozone-generator and ozone was generated by passing oxygen gas in at 4 l/h velocity, voltage being raised first from 110 volts to 8,000 volts and after 5 minutes 10,000 volts. Under this condition, 20 c.c. of cottonseed oil were ozonized for 30 minutes.

Table 9

Cottonseed oil	Hydrolysis-velocity (%)	Iodine value	Viscosity (η)
Original oil	40.5	110.1	300.7
Ozonized oil	24.7	98.0	408.1

As these experimental results indicate, when compared with the original oil, the ozonized form decreased strongly in its enzymic hydrolysis-velocity, decreased in its iodine value and increased in its viscosity. This phenomena caused by the action of ozone are readily seen to be quite in line with those caused by irradiation with ultra-violet rays.

Thus, the action of ultra-violet rays upon vegetable oil is readily seen to be merely the action of ozone which is generated during irradiation. From this the facts now became evident that the acid value of oil increases after irradiation, and also (as described in the experiment **II.** C.) that the presence of ozonized oil retards the enzymic activity in some way.

F. REAL ACTION OF ULTRA-VIOLET RAYS UPON OIL

The action of ultra-violet rays upon vegetable oil can be regarded as equivalent to the action of ozone. (see the experiment **II.** E.) Whether ultra-violet rays in themselves exert the action upon oil which has been generally considered as the action of ultra-violet rays or ultra-violet rays in themselves produce no such result but play a rôle for the generation of ozone the results of which were generally supposed to be the action of ultra-violet rays formed a further problem which was investigated by means of the following experimental method.

Two crystallization dishes of the same diameter (3 8 cm) and content (11.4 c.c.) were filled to the brim with cottonseed oil. On one of these a quartz plate was cautiously slid in such a way as not to form bubbles, and the other dish was covered over at a distance of 2 cm. with a millboard which had been inlaid with the same quartz plate. These two portions of cottonseed oil in the two dishes were irradiated under the same conditions in a water-jacketed box at a distance of about 18 cm. from a mercury vapour quartz lamp, the temperature during irradiation rising up to about 50°C. In the former case, the oil was irradiated with ultra-violet rays but without the action of ozone produced during irradiation (designated as "a" in table 10) and in the latter case, the oil was affected by both the irradiation of ultra-violet rays and the action of ozone produced during irradiation (designated as "b" in table 10).

The experimental results indicated that in the former case (a), there was no change in its enzymic hydrolysis-velocity within the experimental error but in the latter case (b), it apparently decreased.

Therefore, it became evident that ultra-violet rays in themselves have no such affects as were hitherto attributed to the action of ultra-violet rays but plays a rôle of generating an ozone, which action was in practice recognized as the action of ultra-violet rays.

Table 10

Cottonseed oil	Time for irradiation (hr.)	Hydrolysis-velocity (%)
Original oil	0.0	41.0
a oil	1.5	40.8
a oil	5.0	40.4
b oil	1.5	39.3
b oil	5.0	36.6

It may be summarized from experiment **II.** that *the action of ultra-violet rays upon vegetable oil seen from the viewpoint of enzymology is nothing but the action of ozone,* and *the ultra-violet rays correspond to an ozone-generator.*

III. COMPARISON BETWEEN THE ACTION OF OZONE AND THAT OF ULTRA-VIOLET RAYS UPON CHOLESTEROL AND OIL

The fact was made evident from the experiment **II.** that the action of ultra-violet rays seen from the viewpoint of enzymology upon vegetable oil is no more than the action of ozone, and the ultra-violet rays correspond to an ozone-generator. Consequently the effects of ozone and ultra-violet rays upon cholesterol and oil were compared in this experiment by means of the criteria of photoactivity, colour reactions, etc.

A. PHOTOACTIVITY

K. TAKAHASHI and S. HAMANO first discovered the fact that vitamin A preparation is photoactive, and S. HAMANO (1925) reported that cholesterol and oil became photoactive after irradiation with ultra-violet rays. This problem was, thereafter, deeply investigated by H. VOLLMER

(1926), J. STRITESKY (1927) and N. S. LUCAS (1926). And it became evident that photoactivity is not due to any secondary radiation given out by the photoactive substance but seems to be due to the emanation of some substance from the photoactive substance. Above all, photoactivity is being observed with interest in relation to vitamins.

Photoactivity was determined as follows. A crystallization dish possessing a diameter (3 cm.) and a height (1.6 cm.) which was served with 1 gr. of sample was taken to a dark room and a photographic plate (Lion Eclipse Ortho was used throughout these experiments) was laid sentitive side downwards on the sample. The whole was then placed in a light-tight box, wrapped in a photographer's black cloth, and kept in a dark room for 24 hours. The development of the plate was carried out as carefully as possible to be uniform throughout the experiment.

First, by repeating the experiment I confirmed the fact that after irradiation with ultra-violet rays, cholesterol and oil become photoactive and their photoactivity become more intense, and oleic acid became strongly photoactive, but palmitic and stearic acid did not. From this fact, it became clear to be due to the unsaturated fatty acids of which the oil consists, that the oil became photoactive after irradiation with ultra-violet rays.

Next, the photoactivity of cholesterol and oil after the action of ozone was investigated. The SIEMENS' ozone-generator with ten-tubes was employed as an ozone-generator, and ozone was generated by passing oxygen gas in at 4 l/h velocity, voltage being first raised from 110 volts to 8,000 volts and after 5 minutes to 10,000 volts. Under this condition, 4 c.c. of olive oil were ozonized for 1 hour, 1.5 gr. of cholesterol for 40 minutes (this called "ozonized cholesterol I"), and white crystalline powder separated out by ozonizing cholesterol (2 gr.) dissolved in 160 c.c. of hexane was dried in vacuum over calcium chloride and paraffin (this called "ozonized cholesterol II")[3]. The photoactivity of these ozonized substances was as follows. Ozonized olive oil (see Fig. 1. B) was photoactive as well as irradiated olive oil and ozonized cholesterol I (see Fig. 2. C.) was strongly photoactive and also ozonized cholesterol II (see Fig. 2. D) violently. It became evident that cholesterol and oil become photoactive as well by the action of ozone as by that of ultra-violet rays.

3 See C. D. Harries; Untersuchungen über das Ozone und seine Einwirkung auf organische Verbindung, S 374.

B. COLOUR REACTIONS

The fact has been generally admitted that cholesterol after irradiation gives different and specific colour reactions from that of the original one. In this experiment, some comparison of colour reaction between irradiated cholesterol and the ozonized one was carried out by means of the colour reactions of irradiated cholesterol proposed by many investigators.

SHEAR's colour reaction for vitamin D (1926).

The SHEAR's reagent was prepared by adding 1 part of conc. HCl to about 15 parts of aniline which should be redistilled before using. Boiling a small quantity of purified cholesterol with the reagent in a test tube for about half a minute gives a clear pale yellow solution, but when irradiated cholesterol is used, it gives a red colour which becomes intense on standing. He announced that this colour reaction is characteristic for vitamin D. Against this announcement ROSENHEIM and WEBSTER (1926) published an antagonistic opinion that the SHEAR's colour reaction is not specific for vitamin D and appears to be due to the formation of organic peroxides. In reply to this antagonistic opinion of ROSENHEIM and WEBSTER, SHEAR and KRAMER (1926) answered and declaring that the question as to how close a connection exists between the chromogenic substance (or substances) and the antirachitic factor remains to be solved and the object was to obtain an antirachitically active fraction from irradiated cholesterol with aid of this colour reaction; and they separated actually "U. V. oil of cholesterol" as an atirachitically active fraction. MOORE and WILLIMOTT (1927) reported from their experimental data that the SHEAR's colour reaction is not specific for vitamin D. SEXTON (1928) studied the effects of the SHEAR's reagent upon a number of sterol derivatives and concluded that the SHEAR's colour reaction is by no means specific for vitamin D but on the other hand the positive reaction given by irradiated ergosterol and ketones is in harmony with the view expressed by HEILBRON, MORTON and SEXTON (1928) that the antirachitic vitamin is possibly ketonic in character. Above all, it is evident fact that after irradiation with ultraviolet rays, cholesterol, etc. for the first time give the SHEAR's colour reaction.

In my experiment ozonized cholesterol I and ozonized cholesterol II together positively gave the SHEAR's colour reaction.

STEIGMANN's colour reaction for vitamin D (1928).

When 5 c.c. of fuchsin solution (1 : 10,000) decolorized with hydrosulfit ($Na_2S_2O_4$) are added to an alcoholic solution of sample, ordinary fuchsin colour appears again, and it is diluted with some amounts of distilled water, and then again decolorized by adding of a little excess of hydrosulfit. After standing for 1-2 hours, a specific blue-violet colouration appears when sample containing vitamin D are used. STEIGMANN announced that this fuchsin aldehyde colour reaction is specific for vitamin D and concluded from his colour reaction experiment that vitamin D will probably be aldehyde or unsaturated ketone and that vitamin D will have a atomic group $>C:C:O-$ in its molecule.

I investigated the question as to whether cholesterol gives the STEIGMANN's colour reaction after the action of ozone or not. Cholesterols ozonized for 5, 10 and 40 minutes, and ozonized cholesterol I respectively positively gave the STEIGMANN's colour reaction and among them, that for 40 minutes was the strongest. When cholesterol (2 gr.) dissolved in 50 c.c. of carbon tetrachloride was ozonized for 2 hours and petroleum-ether added to it, white crystalline powder was precipitated which was dried in vacuum over calcium chloride and paraffin. This white crystalline powder also gave the STEIGMANN's colour reaction strongly.

EULER, MYRBÄCK and KARLSSON (1926).

reported that irradiated cholesterol gave different colour reactions from that of the original when compared by means of the four cholesterol colour reactions (e.g., A-reaction (Whitby's method), B-reaction (Whitby's method), C-reaction (Whitby's method), D-reaction (Harden's method); namely, at least among the four colour reactions, first C- and A- reaction, and next B-reaction were eliminated.

I confirmed experimentally the fact that ozonized cholesterol II gave no A- and C- reactions at all and coloured a pale yellowish-pink colour emitting a green fluorescence in case of B-reaction, and gave no colouration on the addition of furfurol and conc. sulphuric acid, and coloured a yellowish-brown colour on the addition of anhydrous acetic acid; namely, ozonized cholesterol II did not give the A-, B-, C-, and D- cholesterol colour reaction.

C. OTHER PROPERTIES

It has been generally recognized that the melting point of cholesterol depresses after irradiation with ultra-violet rays. For example,

EULER, MYRBÄCK and KARLSSON (1926) reported that when exposed to a mercury vapour quartz lamp at a distance of 4 cm., the temperature during irradiation rising up to 150°C, the cholesterol melted and became a porous, yellowish-brown coloured glassy substance after cooling and the melting point of these substances first depressed and again rose according to the elongation of time of irradiation.

I confirmed the fact experimentally that the melting point of cholesterol depressed after the action of ozone; namely, the melting point of original cholesterol was 146°C, that of ozonized cholesterol I 138°C and that of ozonized cholesterol II 113-121°C.

It has been also generally recognized that cholesterol becomes incapable of precipitation with digitonin after irradiation with ultra-violet rays. I further confirmed experimentally the fact that cholesterol became incapable of precipitation with digitonin after the action of ozone.

It may be summarized from experiment **III** that *the action of ozone upon cholesterol and oil as regards photoactivity, colour reactions, etc., is closely analogous to that of ultra-violet rays.*

IV. BEHAVIOUR OF OZONE FOR THE FORMATION OF VITAMIN D AND FOR THE HEALING OF RICKETS

It was shown in the preceding experiments that when seen from the viewpoint of enzymology, the action of ultra-violet rays upon vegetable oil is no more than the action of ozone and the ultra-violet rays correspond to an ozone-generator, and also that the action of ozone is closely analogous to that of ultra-violet rays as regards photoactivity, colour reactions, etc.

If these several phenomena caused by the irradiation with ultra-violet rays in photoactivity, colour reactions, etc. could be shown to stand in some relation to the photochemical conversion of pro-vitamin into vitamin D, it would be readily seen that ergosterol could be converted into vitamin D after the action of ozone, without irradiation with ultra-violet rays. And if ergosterol would not become an antirachitically active substance after the action of ozone, it would be seen that the phenomena obtained above would stand in no relation to the photochemical conversion of ergosterol into vitamin D, and it would be a pseudo-phenomena caused by the accessory action of ultra-violet rays.

Above all, it will be suggested in the extent of my experiments that a correlation such as exists between vitamin D and ultra-violet

rays will also exist between vitamin D and ozone. In this experiment, consequently, I attempted the feeding experiments to ascertain whether ergosterol becomes an antirachitically active substance after the action of ozone or not, and whether ozone exerts a curative action on rickets when the rickety rats fed in an ozonized atmosphere or not.

A. RELATION BETWEEN THE PHOTOCHEMICAL FORMATION OF VITAMIN D AND OXIDATION PROCESSES

Many investigators have hitherto attempted to ascertain whether oxidation does or does not play a rôle in the activating process of irradiation with ultra-violet rays. This problem was here discussed in detail in the literature.

SCHULTZ, ZIEGLER and MORSE (1927) suggested that it seems probable that the ultra-violet rays might cause a reaction to take place at the double bond, possibly an oxidation, and if irradiation does cause an oxidation of cholesterol, one would not expect a very deep seated change, and the milder the oxidizing agent, the simpler is the resulting product and the more likely that such a compound could be obtained by the irradiation of cholesterol with ultra-violet rays. But α- and β-cholesteryl-oxide, hydrocholesterol, and α-cholestantriol were shown to be the antirachitically inactive substances.

HESS and WINDAUS (1926) investigated the antirachitic potency on a number of cholesterol derivatives both when irradiated and when not, and announced that cholesterol ozonide is antirachitically inactive in either case. But they did not describe how they prepared the cholesterol ozonide.

HESS and WEINSTOCK (1925) irradiated a linseed oil in an atmosphere of nitrogen, in order to ascertain whether oxidation plays a rôle in the activating process or not. To this end a very small amount of linseed oil was placed in a quartz tube and the air removed from the oil by means of suction. Nitrogen was then run into the tube, after it had been passed through pyrogallol and over soda-lime. The quartz tube containing the oil was evacuated and flushed with nitrogen several times. The oil was then irradiated for 1/2 hour at a distance of 6 inches. And they announced that the lack of oxygen did not prevented the activation and that as far as could be judged this oil prevented rickets as well as that which was irradiated in air.

ROSENHEIM and WEBSTER (1926) evacuated the flask containing cholesterol which had been left evenly distributed as a thin film adhering firmly to the sides, and afterwards filled it with pure nitrogen and repeated this process several times, and then carried out the irradiation of the flask. And their experimental data showed apparently no difference for the photochemical formation of an antirachitic substance from the cholesterol whether irradiated in nitrogen or in air.

On the contrary, YODER (1926) reported that there was no noticeable difference in the response to the test for peroxidation whether cholesterol in a quartz flask was irradiated in air or in nitrogen purified according to the precautions observed by HESS and WEINSTOCK (1925), and that examinations on a number of vegetable oils, cod liver oil and cholesterol for peroxidation showed (1) correlation between apparent antirachitic potency and peroxidation in untreated samples, (2) correlation between apparent potency and peroxidation in irradiated samples and (3) no correlation between apparent potency and peroxidation in excessively irradiated samples.

VOLLMER and SEREBRIJSKI (1926) suggested from their experimental data on photoactivity that the antirachitic activation of inactive substances through ultra-violet radiation is regarded as oxidation, peroxidation or ozonization of easily oxidable organic compounds.

As readily seen from the above-described literature, the question whether oxidation plays a rôle in the activating process or not, has not yet been decisively settled.

On the other hand, from the quantity of radiant energy necessary to form an amount of vitamin D sufficient to cause a demonstrable deposition of calcium in the bones of a rachitic rat, the weight of vitamin D synthesized is calculated to be 2×10^{-8} gr. on cholesterol by FOSBINDER, DANIELS and STEENBOCK (1928) (it should be noted that this is the whole dose during the 10 days' test period, not a daily dose.) From the feeding experiments, ROSENHEIM and WEBSTER (1927) confirmed the fact that irradiated ergosterol in daily dose of 1×10^{-7} gr. cures and prevents rickets in rat on a rachitogenic diet and COWARD (1928) showed that the daily dose of irradiated ergosterol necessary to give a positive result is 2×10^{-8} gr.

From this literature, it is now evident that vitamin D in very small quantity is capable of curing and preventing rickets in rats. If something was assumed to be involved into the chemical change which might be caused in the photochemical formation of vitamin D by

irradiation, that substance, consequently, would be readily seen to be sufficient in surprisingly small quantities. For example, if calculated from my theory that Δ_1 mono-ozonide ergosterol is vitamin D, the amount of ozone necessary to form the whole dose of vitamin D proposed by KON, DANIELS and STEENBOCK (1928) is sufficient in such a surprisingly small quantity as 5×10^{-10} gr.

Above all, the question as to whether oxidation plays a rôle in the activating process by irradiation with ultra-violet rays or not will remain unsolved in the future until the experimental demonstration in an ideal absence of oxygen shall have been achieved.

B. CURATIVE ACTION OF OZONE ON RICKETS WHEN THE RICKETY RATS ARE FED IN AN OZONIZED ATMOSPHERE

In the present case I attempted a feeding experiment to ascertain whether ozone exerts a curative action on rickets when the rickety rats are fed in an ozonized atmosphere or not, in the same way as ultra-violet rays exert a curative action on rickets when the rickety rats are directly irradiated.

METHOD OF THE FEEDING EXPERIMENT

Albino rats weighing about 30 gr. were put on the McCOLLUM rachitogenic diet No. 3143 (whole wheat 33 %, Maize 33 % wheat gluten 15 %, gelatine 15 %, NaCl 1 %, $CaCO_3$ 3 %) and distilled water in a dark room after they had been fed for 25 days on the SHERMANN basal diet B (whole dried milk 33.3 %, whole wheat 65.4 %, NaCl 1.3 %) and some vegetables 2-3 times per week. They became rickety on this diet after 35 days and then the curative action of ozone was tested on them, they being fed in an ozonized atmosphere for some days. Each litter of rats was used only throughout one experiment and the grade of rickets disease was compared separately in male and female by means of the histological examination (line test) before and after the test period. The food intake of each rat was recorded. And all rats which lost in body weights during the test period or ate less than 2 grs. of food per day were discarded.

TECHNIQUE OF LINE TEST PROCEDURE

The tibiae were dissected from the tissue after the rats had been anesthetized with chloroform and killed, and their longitudinal sections were prepared by means of a freezing microtome, the sections being

immersed in 1 % silver nitrate solution. Light from a carbon arc lamp was focused for 1.5 minutes upon these immersed surfaces and then the histological examination of the bones was carried out.

ANIMAL FEEDING ROOM

In this experiment in place of a dark animal feeding room was used a large light-tight box (1.4 × 0.7 × 1.0 m) with a special structure to insulate from light but to permit a ventilation in which the usual animal feeding boxes were placed. The animal feeding room was made dark by drawing a photographer's black cloth curtain over the windows of frosted glass when feeding or other work was attempted.

OZONE-GENERATOR

KODAMA's ozone-generator was especially employed in this experiment and placed on the shelf made above in the large animal feeding box which covered fully inclosed the usual animal feeding boxes. Ozone was generated for some minutes per day in the large animal feeding box by means of the ozone-generator and thus the tested rats were fed in an ozonized atmosphere on the rachitogenic diet.

Curative action of ozone on rickets

Table 11

Rat No.	Total length of time for the generation of ozone (min.)	Test period (day)	Average amount of diet eaten per rat per day (gr.)	Body weight (gr.) test-period before	Body weight (gr.) test-period after	Line test
1 (♂)	150.0	20	7.6	74	106	++
2 (♂)	150.0	20	8.1	74	118	++
3 (♂)	150.0	20	7.7	64	103	++
4 (♂)	95.0	14	7.7	58	72	++
5 (♀)	150.0	20	7.8	64	100	++
6 (♀)	87.5	13	8.2	64	86	+

The feeding experiments in table 11 and table 12 were quite analogous with each other in their experimental method and object but differed respectively as to the mother rat and the experimental date.

Table 12

Rat No.	Total length of time for the generation of ozone (min.)	Test-period (day)	Average amount of diet eaten per rat per day (gr.)	Body weight (gr.) test-period before	Body weight (gr.) test-period after	Line test
1 (♀)	100	20	6.2	69	90	++
2 (♂)	100	20	6.0	75	92	+++
3 (♂)	100	20	5.9	80	94	++
4 (♂)	100	20	5.7	70	86	++

From these experimental data it became evident that ozone exerts certainly some definite curative action on rickets in rats on the rachitogenic diet when the rickety rats are fed in an ozonized atmosphere.

C. ANTIRACHITIC ACTIVATION OF ERGOSTEROL BY MEANS OF THE ACTION OF OZONE

In the present experiment I attempted a feeding experiment to ascertain whether ergosterol becomes an antirachitically active substance after the action of ozone or not, in the same way as ergosterol becomes active after irradiation with ultra-violet rays.

BILLS, HONEYWELL and COX (1928) studied the rate of activation of ergosterol during irradiation with ultra-violet rays. In their experiment the maximum potency was reached in 22.5 minutes, at which time the ergosterol was 250,000 times as potent as average cod liver oil. From this time further irradiation caused a falling off in potency, the product becoming inactive in about 3 hours. It has not yet been explained clearly why the initially formed activated ergosterol changes again to an antirachitically inactive substance through over-irradiation, but the presence of this phenomenon is being clearly recognized by many investigators. Taking this phenomenon into consideration, I attempted here to demonstrate experimentally my suggestion that ergosterol will become an antirachitically active substance after the *appropriate* action of ozone. For this purpose, SHIMAZU's primitive ozone-generator with one-tube was especially employed in this experiment from which ozone was generated about the same quantity as in the case of irradiation with an ACME mercury vapour quartz lamp.

METHOD OF PREPARING ERGOSTEROL

Ergosterol was isolated from beer-yeast by means of the HEIDUSCHKA and LINDNER's modification (1929) of the WINDAUS and GROSSKOPF's method (1922) and was recrystallized two times from ethyl-alcohol, once from acetone. The melting point of the ergosterol was 154°C (uncorrected). The ergosterol in itself was antirachitically inactive on a feeding experiment.

OZONE-GENERATOR AND CONDITIONS FOR THE ACTION OF OZONE

The SIMAZU's ozone-generator with one-tube (see Fig. 3.) was especially employed in this experiment for the above-mentioned reason. An induction coil having 3.5 cm. length of spark and dry cells (4.3 volts) which were arranged serially with three dry cells, were attached to the ozone-generator. Each ozone-generator and small cylindrical bottle served with sample was deeply placed separately in a large porcelain bottle. Ozone was generated by passing in at 20-25 l/h velocity oxygen gas which had been purified through conc. sulphuric acid and over soda-lime.

OZONIZED ERGOSTEROLS AND METHODS OF PREPARING THESE

Ergosterol (20 mg.) was dissolved in 10 c.c. of olive oil and then ozonized for a definite time under the above-described conditions. One drop of this olive oil solution corresponds to 0.1 mg. of ergosterol. The olive oil solution ozonized under the above-described conditions was, if necessary, diluted with olive oil so that one drop of the olive oil solution corresponds to 0.05 mg. or to 0.015 mg. of ergosterol.

Now under the above-described conditions, ergosterol (20 mg.) in olive oil (10 c.c.) ozonized for 0.5 minutes was designated as "Oz. Erg. I" and those for 3, 6 minutes respectively as "Oz. Erg. II" and "Oz. Erg. III." Oz. Erg. II was diluted with olive oil so that one drop of the olive oil solution corresponds to 0.05 mg. or to 0.015 mg. of ergosterol. The former was designated as "Oz. Erg. IV" and the latter as "Oz. Erg. V."

METHODS FOR FEEDING EXPERIMENT

The methods of this feeding experiment were nearly similar to those of the preceding feeding experiment. The samples to be tested were not incorporated in the diet, but one drop of the olive oil solutions was administered daily per rat by means of a glass-spatula.

Antirachitic potency of ozonized ergosterol

Table 13

Rat No.	Days of the feeding		Body weight (gr.)		No. of samples to be tested	Line test
	1st period. (on rickets producing diet)	2nd period (on test diet)	2nd period before	2nd period after		
1 (♂)	21	14	64	92	Oz. Erg. I	— —
2 (♂)	21	14	42	57	Oz. Erg. I	—
3 (♂)	21	14	51	60	Oz. Erg. II	+ + +
4 (♂)	21	14	43	54	Oz. Erg. II	+ + +
5 (♀)	21	14	40	54	Oz. Erg. II	+ + +
6 (♀)	21	14	45	61	Oz. Erg. II	+ +
7 (♂)	21	14	57	82	Oz. Erg. III	±
8 (♂)	21	14	58	87	Oz. Erg. III	±
9 (♂)	25	18	41	57	Oz. Erg. II	+ + +
10 (♂)	25	18	46	66	Oz. Erg. II	+ +
11 (♂)	25	18	42	49	Oz. Erg. IV	+ + +
12 (♂)	25	18	32	40	Oz. Erg. IV	+ + +
13 (♂)	25	18	35	45	Oz. Erg. V	+

In line test, +, + + and + + + signs express respectively moderate healing, advanced healing and complete healing, ± sign prevention and — sign expresses as deterioration.

From the results of the feeding experiments as recorded above it becomes experimentally certain that ergosterol becomes an antirachitically active substance after the action of ozone. And it became evident also that the rate of activation of ergosterol increased after the action of ozone and then reached a maximum potency and from this time further action caused a falling off in potency, the product becoming completely inactive (one can readily see the presence of such a phenomenon also in the case of the activation of ergosterol through the irradiation with ultra-violet rays). And it further became clear that under my experimental conditions, a daily dose 0.1 mg. and 0.05 mg. of the ergosterol ozonized for 3 minutes was sufficient to heal rickets

completely and a daily dose 0.015 mg. was somewhat efficaious, while ergosterol ozonized for 0.5 minutes was no efficacious and ergosterol ozonized for 6 minutes prevented even in a daily dose 0.1 mg. in both cases.

It may be summarized from experiment IV that (1) *ozone exerted certainly some definite curative action on rickets in rats on the rachitogenic diet when the rickety rats were fed in an ozonized atmosphere* (2) *ergosterol was activated antirachitically by the appropriate action of ozone.*

DISCUSSION

One cannot have now any accurate knowledge on the chemical nature of vitamin D except the fact that the antirachically inactive ergosterol is photochemically converted into vitamin D after irradiation with a mercury vapour quartz lamp. Many investigators almost attempt to make clear the chemical nature of vitamin D by means of the isolation of a pure active fraction from irradiated ergosterol, but they do not succeed in it. On the other hand I attempt here to elucidate the chemical nature of vitamin D by making evident the significance of the action of ultra-violet rays on the photochemical conversion of ergosterol into vitamin D.

From my experimental results, it has now become evident that vitamin D is formed from ergosterol after the *appropriate* action of ozone. Then it should be considered what chemical changes take place to form vitamin D from ergosterol by the action of ozone.

Those which are formed by the chemical action of ozone are in general the ozonide and in special case the ozonide-peroxide and the polymer. Let us now here examine closely by means of the criteria of my experimental results which of these products of ergosterol produced by the action of ozone is in connection with vitamin D. The ozonide-peroxide will be readily seen to be in no connection with vitamin D from the fact that this product is produced when strongly ozonized. The polymer may be produced by such a weak action of ozone as was employed in my experiment, but it is also being generally admitted that the polymer is produced in addititition to the ozonide when moderate strongly ozonized. Therefore, the polymer will also be readily seen to have no connection with vitamin D from the fact that vitamin D is formed from ergosterol by the appropriate action of ozone but the

initially formed active substance becomes again an inactive product by the excessive action of ozone. Consequently the ozonide of ergosterol must be vitamin D.

But on the other hand, it was seen in my experiment as in SUMI's paper (1930) that ergosterol ozonide obtained by passing ozone completely or strongly upon ergosterol was antitachitically inactive, and in my experiment it also became clear that ergosterol became antirachitically active after the appropriate action of ozone. Therefore, it cannot be immediately decided that ergosterol ozonide is vitamin D.

Hereupon such a hypothesis was assumed as follows; namely, every three double bonds in ergosterol differs, respectively in the power to combine with ozone according to its position in the structure of ergosterol. From this hypothesis, the most common ozonide formative double bond among them was expressed with $\triangle_{I}$, in order $\triangle_{II}$ and $\triangle_{III}$. Ergosterol having one ozonide only at $\triangle_{I}$ and none at $\triangle_{II}$, $\triangle_{III}$ is now designated as "$\triangle_{I}$ mono-ozonide ergostetol" and ergosterol having two ozonides at $\triangle_{I}$, $\triangle_{II}$ and not at $\triangle_{III}$ is designated as "$\triangle_{I, II}$ di-ozonide ergosterol" and ergosterol having three ozonides at $\triangle_{I}$, $\triangle_{II}$ and $\triangle_{I.I}$ is designated as "$\triangle_{I, II, III}$ tri-ozonide ergosterol."

We must next examine closely which of these three ozonides of ergosterol corresponds to vitamin D. What one means in general by ergosterol ozonide is $\triangle_{I, II, III}$ tri-ozonide ergosterol and it has been made clear above that this substance is antirachitically inactive. Therefore, vitamin D must be $\triangle_{I}$ mono-ozonide ergosterol or $\triangle_{I, II}$ di-ozonide ergosterol.

On consideration of a quantity of ozone necessary to the activation of ergosterol, a concentration of ozone to be used in the reaction and of a shape of the activation curve of ergosterol by irradiation with ultra-violet rays or by the action of ozone, it appears to be more reasonable that vitamin D is $\triangle_{I}$ mono-ozonide ergosterol rather than $\triangle_{I, II}$ di-ozonide ergosterol. The rate of the activation of ergosterol in point of fact increased with a rapid curve and reached to the maximum potency and then decreased with a slow curve. If $\triangle_{I, II}$ di-ozonide ergosterol was assumed to be vitamin D, the rate of the activation should increase with a slow curve and reach to the maximum potency and then decrease with a rapid curve. If $\triangle_{I}$ mono-ozonide ergosterol was assumed to be vitamin D, the presence of maximum point in the activation curve, and also of the fact that initially formed active sub-

stance becomes again inactive, would be readily understood if this phenomenon is explained as follows; namely, the initially formed active $\triangle_{I}$ mono-ozonide ergosterol alters gradually to the inactive $\triangle_{I, II}$ di-ozonide ergosterol and $\triangle_{I, II, III}$ tri-ozonide ergosterol through over-irradiation or excessive action of ozone. BOURDILLON, FISCHMANN, JENKINS and WEBSTER (1929) reported recently that on stirring the liquid during radiation by a steam of nitrogen, the three substances "A," "B," "C" were isolated from the irradiated ergosterol among which "A" substance was very strongly antirachitically active and "B," "C" substances inactive. They suggested that "A" substance would be vitamin D and explained the fact that the initially formed active substance becomes gradually an antirachitically inactive substance after over-irradiation with ultra-violet rays as follows; namely, "A" active substance alters gradually to the "B," "C" inactive substances through over-irradiation. If "A" substance was assumed to be $\triangle_{I}$ mono-ozonide ergosterol and "B," "C" substances respectively to be $\triangle_{I, II}$ di-ozonide ergosterol and $\triangle_{I, II, III}$ tri-ozonide ergosterol, their experimental results would be readily seen to be in harmony with my theory that $\triangle_{I}$ mono-ozonide ergosterol is vitamin D.

It will require a determination of the chemical structure of ergosterol, to demonstrate experimentally my theory on the chemical nature of vitamin D, but I believe from the experimental results obtained above that $\triangle_{I}$ mono-ozonide ergosterol will possibly prove to be vitamin D.

But if my theory is roughly expressed, it is concluded such that the imperfectly ozonized ergosterol is vitamin D.

Vitamin A was formerly supposed to be probably lipochrome pigments or substances in their intimate relation but on investigation it was experimentally demonstrated that these pigments have none of the properties of vitamin A. On the other hand, it became recently evident that what was regarded as vitamin A had been in reality the mixture of vitamin A and vitamin D. Thereafter, having examined again the behaviours of these lipochrome pigments in the presence of vitamin D, EULER, EULER and HELLSTRÖM (1928) demonstrated the fact that carotin has the properties of vitamin A in the presence of vitamin D (the irradiated ergosterol was used). Thereafter, MOORE (1929), COLLISON, HUME, SMEDLEY-MCLEAN and SMITH (1929), and KAWAKAMI and KIN

(1929) confirmed this fact experimentally. And recently KAWAKAMI and KIN (1930) demonstrated that vitamin A in cod liver oil is not carotin and proposed that there are possibly several kinds of vitamin A. Certainly, at the present time vitamin A can be considered to be a mixture of vitamin D + carotin or x substance.

On the other hand, it was formerly made evident by ZILBA (1922) that vitamin A loses its physiological action through the action of ozone. And the cause of this phenomenon still remains unexplained. But if it was seen from my theory that $\triangle_{I}$ mono-ozonide ergosterol is vitamin D, this phenomenon could be readily explained as follows; namely, because vitamin D ($\triangle_{I}$ mono-ozonide ergosterol) loses its physiological action by altering to the inactive $\triangle_{I,\ II}$ di-ozonide ergosterol or $\triangle_{I,\ II,\ III}$ tri-ozonide ergosterol through the action of ozone, vitamin A (vitamin D + carotin or x snbstance) results to be carotin or x substance and to lose the physiological action of vitamin A as if lipochrom pigments have no physiological action of vitamin A in the absence of vitamin D.

SUMMARY

1. Vegetable oils after irradiation with ultra-violet rays were less readily hydrolysed by the castor-bean lipase than were the original and the greater the iodine value the greater was the influence of irradiation, and the decrease in the enzymic hydrolysis-velocity of oil is directly proportional to the length of time of irradiation.

There existed a certain corretation between the iodine value of vegetable oil and its enzymic hydrolysis-velocity; namely, the greater the iodine value the less was the velocity and from this fact the nutritive value of fat and oil was assumed to be inversely proportional to its iodine value. The viscosity of vegetable oil ran parallel with its enzymic hydrolysis-velocity; namely, the greater the viscosity the greater was the velocity.

2. The action of ultra-violet rays which decrease the enzymic hydrolysis-velocity of vegetable oils, increase the acid value and the viscosity, and decrease the iodine value of the oils, was nothing but the action of ozone, and the ultra-violet rays corresponded to an ozone-generator when seen from the viewpoint of enzymology.

3 The action of ozone upon cholesterol and oil was closely

analogous to that of ultra-violet rays as regards photoactivity, colour reactions, etc.

4. Ozone exerted some definite curative action on rickets in rats on the rachitogenic diet when the rickety rats were fed in an ozonized atmosphere.

5. Ergosterol was activated antirachitically after the appropriate action of ozone.

6. The rate of activation of ergosterol after the action of ozone increased gradually and then a maximum point of potency was reached but thereafter the further action caused a falling off in potency, the product becoming inactive at last.

7. Under my experimental conditions, a daily dose 0.1 mg. and 0.05 mg. of ergosterol ozonized for 3 minutes was sufficient to heal rickets completely and a daily dose 0.015 mg. was somewhat efficacious. Ergosterol ozonized for 0.5 minutes was not efficacious and ergosterol ozonized for 6 minutes prevented even in a daily dose 0.1 mg. in both cases.

8. It was suggested from my experimental results that Δ_1 monoozonide ergosterol will possibly be vitamin D. And also it was roughly concluded such that the imperfectly ozonized ergosterol is vitamin D.

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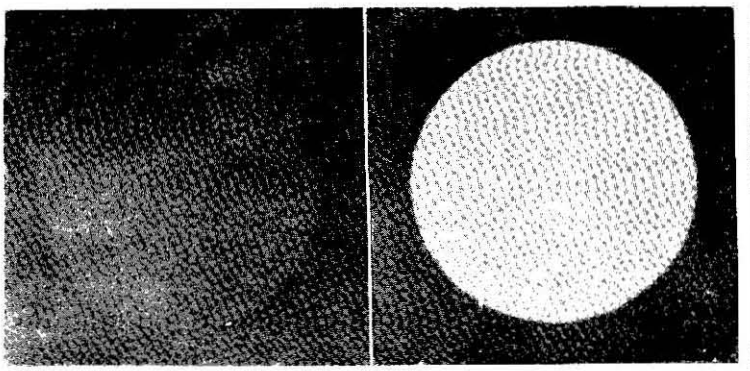
EXPLANATION OF PLATE I

Fig. 1 A: Original olive oil.
B: Ozonized olive oil.

Fig. 2 A: Original cholesterol.
B: Irradiated cholesterol.
C: Ozonized cholesterol I.
D: Ozonized cholesterol II.

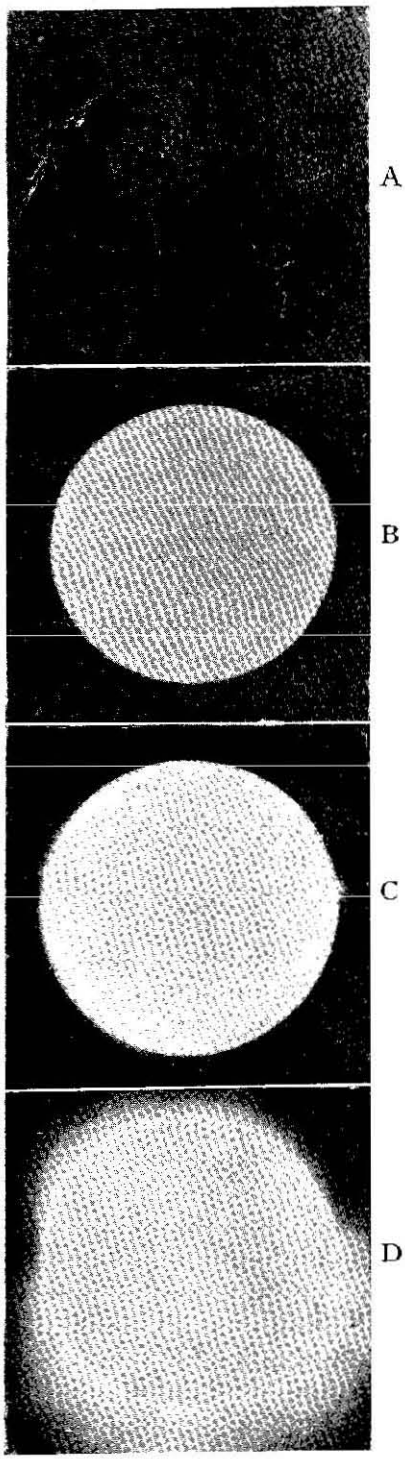
Fig. 3 Shimazu's ozone-generator.

Fig. 1.



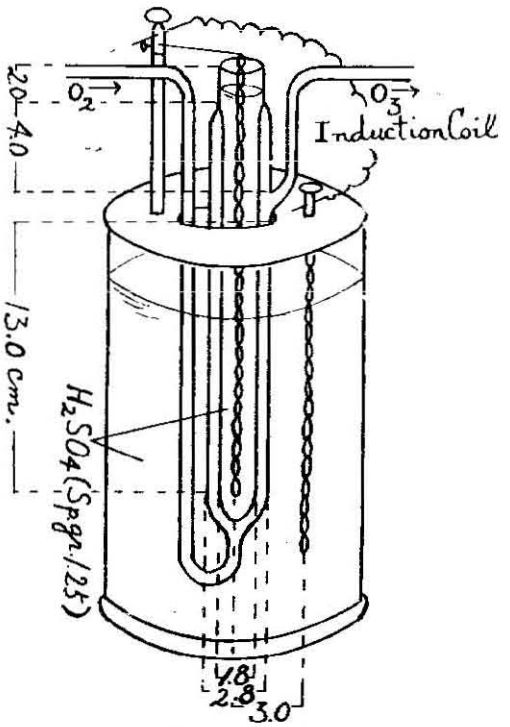
A B

Fig. 2.



A B C D

Fig. 3.



Shimazu's Ozone-generator