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Anti-Rachitic Activity of Vitamin-D_3-Precursors in the Rat.

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In a preceding paper[1] we have found that subcutaneous injection of 7-dehydro-cholesterol shows an anti-rachitic activity only if the previously depleted rats also receive ample irradiation. Further studies of the anti-rachitic activity of other cholesterol derivatives are submitted in this report.

In the synthesis of 7-dehydro-cholesterol from cholesterol, 7-keto-cholesterol and 7-hydroxy-cholesterol* are important intermediaries. It was, therefore, considered of interest to investigate whether or not any evidence could be found for the ability of the animal body to form 7-dehydro-cholesterol from its *in vitro* precursors, namely, 7-keto-cholesterol and 7-hydroxy-cholesterol. Such a possibility has been indicated by Wintersteiner and Ritzman[2] and Rosenberg.[3] Furthermore, Hazlewood[4] and McPhillamy[5] isolated 7-hydroxy-cholesterol from liver and from serum. Wintersteiner and Ritzman,[2] and Wintersteiner and Bergstrom[6] thought that the 7-hydroxy-cholesterol isolated by the above workers might possibly be an artefact produced by accidental oxidation during its isolation. This possibility seems to be eliminated by the more recent work of Hazlewood.[7]

It was thought that the injection of solutions of 7-keto-cholesterol and 7-hydroxy-cholesterol into rachitic rats on a depletion diet followed by exposure of the rats to ultraviolet irradiation might indicate whether the animal body is capable of converting these two precursors into 7-dehydro-cholesterol.

The following experiments were therefore undertaken:

7-dehydro-cholesterol was prepared from cholesterol by the method of Windaus[8] and the product was repeatedly recrystallized from methyl alcohol in darkness. The α-epimer of 7-hydroxy-cholesterol was prepared by saponification from the dibenzoate according to Windaus[8] and was recrystallized several times from petrol ether. The 7-keto-cholesterol was prepared by saponification from the keto-cholesterol-acetate and recrystallized from methyl alcohol (m.p. 166 to 167° C $\Sigma_{238} = 12760$). These 3 sterol compounds were dissolved in propylene-glycol U. S. P., using 5.3 mg of the purified substance for 50 cc of propylene-glycol. The solutions were kept in a dark refrigerator.

For each group of experiments 7 fully depleted rats (see U.S.P. XII, p. 640) were used.

Group 1 was injected subcutaneously every other day with 0.2 cc of 7-dehydro-cholesterol solution.

Group 2 was injected subcutaneously every other day with 0.2 cc of 7-hydroxy-cholesterol.

Group 3 received subcutaneously every other day 0.2 cc of 7-keto-cholesterol solution.

All 3 groups were irradiated daily for 4 minutes with a 2 arc therapeutic lamp at a

1 Geiger, E., and Lassen, S., Proc. Soc. Exp. Biol. and Med., 1942, **52**, 11.

* This compound is usually not regarded as a pro-vitamin in a strict sense as irradiation of this substance (Bills and MacDonald[10]) shows only a very slight anti-rachitic activity and there is, furthermore, no indication that the material used for irradiation in those experiments did not contain 7-dehydro-cholesterol as impurity.

2 Wintersteiner and Ritzman, *J. Biol. Chem.*, 1943, **136**, 697.

3 Rosenberg, H. R., *Chemistry and Physiology of the Vitamins*, Interscience Publ., 1942.

4 Hazlewood, G. A. D., *Biochem. J.*, 1939, **33**, 709.

5 McPhillamy, H. B., *J. Am. Chem. Soc.*, 1940, **62**, 3518.

6 Wintersteiner and Bergstrom, *J. Biol. Chem.*, 1941, **141**, 597.

7 Hazlewood, G. A. D., *Biochem. J.*, 1942, **36**, 389.

8 Windaus, A., Lettre, H., and Schenck, F., *Ann.*, 1935, **98**, 520.

TABLE I.

Test No.	Group of 7 rats	Injected	Avg Healing
Test A	I	7-dehydro-cholesterol	1.2
	II	7-hydroxy- "	2.0
	III	7-keto- "	1.1
Test B	I	7-dehydro-cholesterol	0.86
	II	7-hydroxy- "	1.8
	III	7-keto- "	1.75

distance of 12 inches. The animals were also daily exposed to daylight for 3 hours. Otherwise the rats were kept during the whole experiment on a rachitogenic diet in a dark room. The injection of the sterols was always performed after the irradiation. After 8 days, the rats were killed and the degree of calcification in the tibiæ was evaluated by the line-test. (Table I).

The results of Table I, Test A, show that the injection of 7-hydroxy-cholesterol gave greater calcification than the injection of 7-dehydro-cholesterol did and that the 7-keto-cholesterol had about the same activity as 7-dehydro-cholesterol. In order to reduce any experimental error, we repeated this experiment using newly prepared sterol solutions. The results of this series are listed in Table I under Test B. The repetition of the experiments confirmed the results of Test A, showing the 7-hydroxy-cholesterol and 7-keto-cholesterol injected subcutaneously into rats subsequently irradiated was more effective than the injection of 7-dehydro-cholesterol, but this time the 7-keto-cholesterol was as effective as 7-hydroxy-cholesterol.

If we assume that the 7-keto-cholesterol and 7-hydroxy-cholesterol were transformed in the body of the experimental animals to 7-dehydro-cholesterol in the same general way as *in vitro* and that this substance is finally activated by our irradiation procedure, it is difficult to explain why the precursor is more active than the 7-dehydro-cholesterol itself. We are, therefore, forced to conclude that either the keto and hydroxy compounds are more easily absorbed from the place of injection (subcutis) than the 7-dehydro-cholesterol and are conveyed faster to the specific tissues (liver, skin, or bones) where they exert their activity or that the keto and the hydroxy compounds in the body are transformed to Vitamin D or pro-vitamin D in a way which is entirely different from the *in vitro* procedure. Further experiments are in progress to determine which one of the two assumptions is correct.

In the *second* group of experiments we compared the anti-rachitic activity of the above mentioned 3 sterols when given *per os* and by injection. The mode of procedure is that used above. Seven or 14 D-depleted rats were used in each group and the animals were irradiated daily as in the experiments mentioned before. 0.1 cc of the propylene glycol solution of the same concentration as used for the subcutaneous injection was administered daily *per os* following the irradiation of the animals, so that the total amount of the sterols administered to each rat during the experiment was the same as in the earlier experiments using the injection method.

TABLE II.

Group No.	Rats in Group	Treatment	Avg Healing
I	14	Irradiation and *per os* daily 0.1 cc 7-dehydro-cholesterol	0.39
II	7	Irradiation and *per os* daily 0.1 cc 7-hydroxy-cholesterol	0.64
III	7	Irradiation and *per os* daily 0.1 cc 7-keto-cholesterol	0.29

The experiments of Table II show that *per os* administration of the 3 sterols investigated was less effective than the subcutaneous injection of the same sterols. This may be attributed to a diminished resorption of these compounds from the intestinal tract. In the foregoing paper[1] we have proved that there is no essential difference in the curative effect of *activated* 7-dehydro-cholesterol whether it is given the rats subcutaneously or *per os*. This seems to indicate that the intestinal resorption of the so-called D_3-pro-vitamins is very limited in comparison with that of the irradiated product.

This result is in conformity with the observations of Schoenheimer *et al.*[9] who showed that ergosterol is likewise less readily

[9] Schoenheimer, Behring and Gottberg, *Z. Physiol. Chem.*, 1932, **208**, 77.

[10] Bills, C. E., *Cold Spring Harbor Sym. Quant. Biol.*, 1935, **3**, 328.

absorbed from the intestinal tract than Vitamin D_2, produced from this pro-vitamin by irradiation.

Summary. 1. 7-hydroxy-cholesterol when given subcutaneously to rats, that are subsequently exposed to ultra-violet irradiation has a higher anti-rachitic effect than 7-dehydro-cholesterol injected into rats under identical conditions. 2. 7-keto-cholesterol when given subcutaneously to rats that are subsequently exposed to ultra-violet irradiation has equal or higher-anti-rachitic effect than 7-dehydro-cholesterol. 3. The *per os* introduction of 7-keto-cholesterol, 7-hydroxy-cholesterol and 7-dehydro-cholesterol into rats subsequently irradiated produces a lesser anti-rachitic effect than the subcutaneous injection of similar amounts of the sterols under otherwise identical conditions. Activated 7-dehydro-cholesterol forming an exception as shown in a previous report.

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A Sensitive Test for Fox Encephalitis Virus.

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Work with the virus of fox encephalitis has been hampered by the failure of this virus to cause visible illness or death in all infected animals. In a small series of from 5 to 10 foxes or dogs, evidence of infection may be observable in from 0 to 100% of animals after the intramuscular or intracerebral injection of adequate doses of living virus. Such inconstant results make accurate titrations of virus or antiserum, or the test of the efficacy of a vaccine, so costly as to be impracticable.

The following experiments have been conducted in developing a sensitive test for fox encephalitis virus. It was believed that foxes that failed to show symptoms of infection after the injection of virus underwent inapparent infections.[1] Inasmuch as cells infected with the fox encephalitis virus develop characteristic, large, intranuclear inclusions, an attempt was made to place the virus in contact with susceptible cells that could be subsequently examined histologically. It is known that this virus attacks vascular endothelium, reticulo-endothelium of the liver, spleen, and lymph nodes, histiocytes, and certain specialized cells such as ependymal and hepatic cord cells.

In our laboratory, fox encephalitis virus is routinely preserved as a 20% suspension of homogenized fox brains in 50% neutral glycerin. One such suspension, Lot 94, mixed with an equal volume of a similar suspension of liver from infected foxes was used in the first experiment. Just before injection, the tissue suspensions were diluted with 9 volumes of isotonic NaCl solution.

A 3-fold approach to the problem was made by searching for inclusion bodies in (1) tissues at the site of subcutaneous and intramuscular injections, (2) lymph nodes draining such regions, and (3) cells of the eye after intra-ocular injection. Virus was injected into each of the 4 legs and into the floor of the mouth of 5 foxes. Tissue from the sites of subcutaneous and intramuscular injections (1 or 2 cc of 2% virus suspension) was removed at intervals of from 1 to 5 days and examined for inclusion bodies. The results were consistently negative. It is probable that inclusions develop in such areas but are too hard to find to be of value in detecting small amounts of virus.

From the same animals, lymph nodes draining the regions in which the inoculations had been made were removed at intervals of from 1 to 5 days. Grossly, a few of the lymph nodes were enlarged, edematous and hyperemic; others were normal. As shown

[1] Green, R. G., Katter, M. S., Shillinger, J. E., and Hanson, K. B., *Am. J. Hyg.*, 1933, **18**, 462.