

ANTIRICKETIC SUBSTANCES.

II. THE ACTION OF *n*-BUTYL NITRITE ON ACTIVATED CHOLESTEROL AND THE ANTIRICKETIC VITAMIN.

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Following somewhat separate lines of inquiry, three groups of investigators have recently associated the antiricketic¹ vitamin with a derivative of cholesterol produced by ultra-violet radiations. Hess, Weinstock, and Helman (1), Rosenheim and Webster (2), and Steenbock and Black (3) have in turn produced the antiricketic derivative by exposing cholesterol to the rays of the mercury vapor quartz lamp. Although the suggested hypothesis that this derivative is identical with the natural vitamin is not without foundation, further data are required for its confirmation or confutation.

If it can be demonstrated that a substance, X, is destroyed by a substance, Y, and a substance, Z, under similar conditions is not destroyed by Y, then it follows that X and Z are not identical; but if both X and Z are destroyed by Y, they may or may not be identical, and the reaction merely suggests their *similarity*. I have previously shown (4) that the antiricketic vitamin in cod liver oil is destroyed by nitrous fumes; in the present communication this reaction is applied to activated cholesterol.

¹ Since the English noun "rickets" affords a better connotation than does the etymologically unrelated term "rachitis" it would seem to the author that "antiricketic" or "antiricketics" is better usage than "antirachitic." This detail of orthography appears to have been generally overlooked.

Methods.

The facilities of a laboratory especially designed for nutritional studies made it possible to overcome certain of the difficulties in attaining uniform experimental conditions. In a future paper a more extensive discussion of technique will be given, but the present experiments were performed as follows: Young rats were fed the McCollum rickets-producing diet, No. 3143. This diet appears to be sensitively constituted, but when prepared and employed as described it produced satisfactory rickets. All the

Diet 3143.

Whole wheat flour (Pillsbury's "100 per cent").....	33
Yellow maize (seed quality, finely ground).....	33
Wheat gluten (Pure Gluten Food Company).....	15
Bacteriological gelatin ("Difco," powdered form).....	15
Calcium carbonate (Baker "analyzed," heavy powder).....	3
Sodium chloride (Eimer and Amend "tested purity," powder).	1

rats developing rickets, and those receiving curative preparations, were kept in metal cages in a sanitary room without windows. The only light employed was from ruby glass bulbs, the illumination being that of a photographic dark room. The temperature was maintained at about 30°C. Under these conditions the rats developed severe rickets in 18 days.

Curative preparations were incorporated at a definite per cent by weight by trituration with Diet 3143. In all cases the period of administration was 7 days, and five rats weighing at least 50 gm. were used for each test. Experience has shown that to secure statistical reliability with this technique it is necessary to discard the records of all rats which (a) lose weight during the 7 day treatment, and which (b) eat less than 2 gm. of food in any 1 day or less than 3 gm. per day for the 7 day period.

The protocols of the rats are combined in Table I. Experimental findings were obtained by the Shipley "line test" procedure, in which a longitudinal section through the proximal end of the tibia was illuminated in silver nitrate until the calcified structures blackened. The principal criteria of healing are the development of the "line" at the zone of provisional calcification and the reappearance of bony trabeculae in the metaphysial osteoid. For convenience in tabulation the degree of healing is expressed on a

scale of four plus signs, which the reader should realize bear no numerical relation to each other.

EXPERIMENTAL.

In my original investigation of the nitrous fumes reaction (4) I employed the oxides of nitrogen generated by the interaction of sodium nitrite and acetic acid. In the present work I found that the reaction can be made more precise by using *n*-butyl nitrite, which is freely miscible with oils and can be accurately measured. The cod liver oil used in this experiment was found to effect advanced healing when administered at 4 per cent, this dose being, however, more than sixteen times the minimum required to initiate healing in 7 days.

Action of n-Butyl Nitrite on Cod Liver Oil.—8 gm. of cod liver oil were mixed with 1 ml. of *n*-butyl nitrite, and kept for 1 hour in a small test-tube at room temperature (29°). A calculated quantity of the mixture was then triturated with Diet 3143 so as to give a product containing 4 per cent of oil. This preparation was exposed to air for 2 hours to dissipate the odor. When fed to rats it induced advanced healing, which shows that the antiricketic vitamin in cod liver oil is not rapidly destroyed by *n*-butyl nitrite at room temperature.

A second tube of cod liver oil and *n*-butyl nitrite was prepared the same as the first, except that this was allowed to stand for 95 hours. In this time the oil became dark reddish brown, and when administered at 4 per cent it induced no healing. It is evident that *n*-butyl nitrite slowly destroys the antiricketic vitamin in cod liver oil at room temperature.

A third tube of oil and nitrite was prepared the same as the first, except that this tube was immediately plugged with cotton and immersed in boiling water for 1 hour. Bubbles of gas developed, and the oil quickly darkened. The tube was promptly cooled, and the product made up to 4 per cent as before. The tests with rats indicated that the antiricketic vitamin in cod liver oil is destroyed by heating the oil with *n*-butyl nitrite for 1 hour. A control tube of oil heated without nitrite gave advanced healing at 4 per cent. This observation is the basis for the subsequent examination of activated cholesterol.

TABLE I.

For convenience in tabulation, the quality of healing observed by the line test is here expressed on a scale of four degrees. One plus sign (+) indicates a just perceptible healing; two plus signs (++) , a distinct healing; three plus signs (+++) , an advanced healing; and four plus signs (++++) , a practically complete healing. The reader should realize that these values bear no numerical relation to each other.

Rat No.	Sex.	Preparation administered.	Grade of test.	Average daily consumption.	
				gm.	gm.
1013	M.	Diet 3143 control.	—	64-64	3.9
1014	"	" " "	—	52-57	5.0
1015	F.	" " "	—	59-61	3.9
1016	M.	" " "	—	58-61	4.4
1069	F.	" " "	—	51-59	5.9
1045	F.	Cod liver oil 4 per cent.	++++	53-54	3.9
1046	"	" " "	++	52-54	4.0
1073	"	" " "	++++	50-50	3.6
1074	M.	" " "	+++	52-53	3.7
1075	F.	" " "	++++	52-53	4.9
1029	M.	Cod liver oil $\frac{1}{4}$ per cent.	+++	60-61	3.7
1030	"	" " "	+++	53-61	4.7
1031	F.	" " "	+++	56-58	3.9
1032	"	" " "	+++	56-61	4.4
1033	M.	" " "	+++	51-53	3.3
1098	M.	Cod liver oil 4 per cent with <i>n</i> -butyl nitrite 1 hr. cold.	++++	64-65	5.3
1114	"	" " "	+++	68-68	5.6
1140	"	" " "	++++	66-67	4.6
1141	F.	" " "	+++	52-53	4.4
1142	M.	" " "	++++	55-58	5.6
1057	F.	Cod liver oil 4 per cent with <i>n</i> -butyl nitrite 95 hr. cold.	—	56-56	3.3
1058	M.	" " "	—	52-53	3.4
1105	"	" " "	—	58-58	5.0
1157	F.	" " "	—	70-75	5.9
1170	M.	" " "	—	53-59	4.4

TABLE I—Concluded.

Rat No.	Sex.	Preparation administered.	Grade of test.	Weight.	Average daily consumption.
				gm.	gm.
1119	M.	Cod liver oil 4 per cent with <i>n</i> -butyl nitrite 1 hr. hot.	—	59-60	5.3
1120	"	" " "	—	58-58	6.0
1160	"	" " "	—	70-73	5.1
1161	F.	" " "	—	63-65	4.4
1162	M.	" " "	—	56-61	4.7
1052	F.	Cod liver oil 4 per cent heated 1 hr. without <i>n</i> -butyl nitrite.	+++	55-55	3.4
1053	M.	" " "	+++	54-54	3.7
1056	F.	" " "	++++	55-56	3.7
1076	"	" " "	+++	52-52	6.0
1077	"	" " "	++++	57-59	3.9
1048	M.	Seal oil 4 per cent.	—	55-58	3.4
1049	F.	" " "	—	55-60	4.6
1050	"	" " "	—	56-58	4.0
1051	M.	" " "	—	64-65	4.4
1068	"	" " "	-(?)	60-64	4.9
1017	F.	Seal oil 4 per cent plus cholesterol $\frac{1}{10}$ per cent.	—	53-54	3.4
1018	M.	" " "	—	57-58	3.9
1019	"	" " "	—	60-64	3.9
1020	"	" " "	—	58-60	4.4
1110	"	" " "	-(?)	55-57	6.1
1024	M.	Seal oil 4 per cent plus irradiated cholesterol $\frac{1}{10}$ per cent.	++	64-68	3.9
1025	"	" " "	+	53-55	4.3
1026	"	" " "	++	54-54	3.4
1027	"	" " "	++	59-60	4.1
1028	"	" " "	+	61-65	4.1
1034	M.	Cod liver oil 4 per cent plus irradiated cholesterol $\frac{1}{10}$ per cent with <i>n</i> -butyl nitrite 1 hr. hot.	—	64-64	4.0
1035	"	" " "	—	63-66	4.1
1036	"	" " "	—	61-61	3.7
1038	"	" " "	—	67-70	4.3
1039	"	" " "	—	62-66	4.0

Preparation of Cholesterol.—The cholesterol was originally obtained from cod liver oil by Wilkins and Bills in the course of some unpublished investigations on the antiricketic vitamin. The crude product was purified by repeated crystallization from hot alcohol and treatment with decolorizing charcoal. The final product consisted of large, colorless, thin laminae. The melting point of the anhydrous material, determined by the conventional capillary tube method, was 147° corrected. One must be especially careful in determining this constant for cholesterol, because the pulverized crystals include so much air that sintering may obscure the true melting point.

Activation of Cholesterol.—The cholesterol was activated in the dry, powdered state by exposing a very shallow layer for 15 minutes to the radiations from a Cooper Hewitt horizontal "Uviarc" operated at 150 volts. The burner was 36 cm. from the cholesterol. After irradiation, the cholesterol powder was thoroughly stirred to insure uniformity throughout the mass.

Properties of Seal Oil.—To determine the biological effect of the cholesterol before and after irradiation, and to secure results comparable with those obtained with 4 per cent of cod liver oil, it was decided to dissolve the cholesterol in a similar quantity of an inactive oil. Mineral or vegetable oil might suffice, but a marine oil similar to cod liver oil seemed preferable.

It is noteworthy that seal oil, which chemically and physically resembles cod liver oil, and is sometimes used to adulterate the latter, is not appreciably antiricketic. Through the kindness of Messrs. Job Brothers and Company, Ltd., of Saint John's, Newfoundland, a supply of pure "pale" seal oil was obtained. This oil was said to have been rendered the 1st week in April, 1925, by briefly steaming the fresh pelt fat of the young harp seal (*Phoca granlandica*). After settling, the oil was bleached in tanks by exposure to sunlight for a few days. As received in this laboratory, it was almost colorless and odorless. Administered to rats at 4 per cent, its antiricketic value was nil.

Action of n-Butyl Nitrite on Activated Cholesterol.—Purified cholesterol was dissolved in seal oil and incorporated in Diet 3143 in such proportions that the modified diet contained 4 per cent of seal oil and 0.1 per cent of cholesterol. This preparation induced no definite healing, which shows that the purified cholesterol was

inactive. A second modified diet was prepared the same as the first, except that the irradiated cholesterol was employed. This gave a moderate healing in all five rats, which shows that the irradiated cholesterol was antiricketic. Contemporaneously a third modified diet was prepared as follows: 200 mg. of the same irradiated cholesterol were dissolved in 8 gm. of cod liver oil in a small test-tube. 1 ml. of *n*-butyl nitrite was added, and the tube plugged with cotton and immersed in boiling water for 1 hour. The cooled product was triturated with Diet 3143 in such proportions that the modified diet contained 4 per cent of cod liver oil and 0.1 per cent of irradiated cholesterol. The line tests were negative, which shows that the antiricketic potency of irradiated cholesterol, like that of cod liver oil, is destroyed by *n*-butyl nitrite.

SUMMARY.

1. The antiricketic vitamin in cod liver oil was destroyed by *n*-butyl nitrite, slowly at room temperature, and rapidly on heating.

2. Some seal oil was found to be inactive, but irradiated cholesterol dissolved in the seal oil was moderately antiricketic. When the irradiated cholesterol was dissolved in cod liver oil and heated with *n*-butyl nitrite the antiricketic activity was destroyed.

3. The above reactions support, but do not prove, the hypothesis that the antiricketic vitamin is identical with the antiricketic derivative of cholesterol produced by irradiation.

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