Summary

The fact that N-1-N-3-disubstituted unsaturated hydantoins with different groups in union with the nitrogen show practically the same absorption spectra may be applied in determining the configuration of the molecule.

A strikingly close analogy has been observed between the relationship indicated by a comparison of absorption curves and the chemical properties of the substances studied.

Three closely related isomers have been discovered where only two might normally be predicted on the basis of geometrical isomerism.

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[A COMMUNICATION FROM THE LABORATORIES OF ORGANIC AND AGRICULTURAL CHEMISTRY OF THE UNIVERSITY OF WISCONSIN]

THE PREPARATION AND ANTIRACHITIC ACTIVATION OF SOME DERIVATIVES OF ERGOSTEROL AND CHOLESTEROL

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Inasmuch as biochemical tests revealed that extraction of yeast and plant materials with alcohol, or even with concentrated solutions of potassium hydroxide in alcohol, failed to free them of substances capable of antirachitic activation (unpublished work) it occurred to us that ergosterol was probably present therein in the form of derivatives. Speculation concerning the possible nature of such derivatives led us to attempt the synthesis of the *d*-glucoside of ergosterol and N-ergosterol glycine, as representatives of carbohydrate and protein substances, respectively. We were successful in our attempt to synthesize the carbohydrate compound, but failed with the latter. In order to avoid waste of ergosterol, preliminary experiments were always carried out with cholesterol.

The glucoside of cholesterol was first described by Salway[1] who obtained it by the condensation of the sterol with tetra-acetylbromoglucose by means of dry silver oxide, and subsequent saponification of the acetyl derivative. This method was found to be entirely satisfactory for the preparation of the glucoside of ergosterol, the only precaution necessary being the rigid exclusion of moisture.

A large number of experiments were made in an unsuccessful attempt to obtain an ergosteryl halide which, it was hoped, might be condensed with the amino acids to produce secondary amines. Darzens' method[2] for the preparation of halides by treatment of the appropriate alcohol with thionyl chloride in the presence of pyridine or other tertiary base when applied to

[1] Salway, *J. Chem. Soc.*, **103**, 1026 (1913).

[2] Darzens, *Compt. rend.*, **152**, 1314 (1911).

cholesterol yielded a white crystalline compound which analysis showed to be cholesteryl sulfite. After this work was completed a paper appeared by Daughenbaugh and Allison[3] in which they described the preparation of cholesteryl sulfite by Darzens' method. The properties given by them for this compound are in excellent agreement with those found by us. Various proportions of ergosterol with thionyl chloride and pyridine in dry ether gave small yields of what was apparently the sulfite or in other cases the chlorosulfite. In dry chloroform the reaction product was an oily tar. Phosphorus trichloride and phosphorus tribromide in dry benzene and dry 1,4-dioxane produced small yields of organic compounds containing phosphorus. Phosphorus tri-iodide in carbon disulfide also produced an organic phosphorus compound. None of these was further investigated.

Efforts were next directed toward the preparation of an ergosteryl ester of an amino acid. Abderhalden and Kautsch[4] prepared the cholesteryl ester of glycine by reacting cholesterol with glycyl chloride hydrochloride in dry chloroform and liberating the ester from its salt by means of alkali. Attempts to carry out this reaction with ergosterol proved unsuccessful due to the destructive action of the hydrogen chloride liberated in the reaction. The use of pyridine to avoid this proved ineffective because it reacted with the glycyl chloride hydrochloride liberating glycyl chloride which immediately underwent condensation. When the amino group was protected by benzoylation, good yields of cholesteryl and ergosteryl hippurates were obtained.

Schmidt, Knilling and Ascherl[5] have used acetobromo-amide as a reagent for the addition of hypobromous acid to the ethylene bond and it seemed that this method might serve to throw additional light on the nature of the unsaturation in ergosterol. The reaction was found to work very smoothly in the case of cholesterol, practically quantitative yields of the bromohydrin being produced, but with ergosterol decomposition was rapid and extensive.

Heilbron and Sexton[6] have described the results of some experiments on the catalytic hydrogenation of the free ergosterol with a palladium catalyst. When this paper appeared we were engaged in some similar experiments using the platinum catalyst of Voorhees and Adams and we likewise found that at a pressure just slightly greater than atmospheric two moles of hydrogen were absorbed with the formation of the α-ergostenol of Reindel and Walter.[7] The reductions were carried out in a semi-micro hydrogenation apparatus at room temperature, and the solvents used were ethyl acetate, ethyl alcohol and glacial acetic acid, all three solvents giving the same re-

[3] Daughenbaugh and Allison, THIS JOURNAL, **51**, 3666 (1929).

[4] Abderhalden and Kautsch, *Z. physiol. Chem.*, **65**, 69–77 (1910).

[5] Schmidt, Knilling and Ascherl, *Ber.*, **59B**, 1279–1282 (1926).

[6] Heilbron and Sexton, *J. Chem. Soc.*, 921–926 (1929).

[7] Reindel and Walter, *Ann.*, **460**, 212 (1928).

sults. In a typical experiment, 0.1093 g. of ergosterol dissolved in 40 cc. of glacial acetic acid was shaken with hydrogen and 0.100 g. of catalyst previously reduced and saturated with hydrogen. At the end of twenty-five minutes the contraction amounted to 16.9 cc. (calcd. for two moles, 17.5 cc.) and after an additional forty-five minutes it was 17.2 cc. There was no break in the curve to indicate the formation of the dihydroergosterol

Experimental

Tetra-acetylbromoglucose.—This was prepared by the method of J. K. Dale.[8] It was found that a mixture of dry ether and dry petroleum ether was a much better solvent for recrystallization than ether alone. From the mixed solvent the product was obtained in beautiful white needle-like crystals which melted at 88–89°.

Ergosterol-*d*-glucoside.—Ergosterol, obtained from The Fleischmann Company was purified by recrystallization from a mixture of alcohol and benzene as described by Bills and Honeywell.[9] Two crystallizations gave a product which had $[\alpha]_D^{20}$ −131.5° and melted at 164°. This ergosterol was dried at room temperature and 2-mm. pressure for one hour and then at 80° and the same pressure for fifteen minutes. Then 2.00 g. of this was dissolved in 100 cc. of thoroughly dried ether and 3.00 g. of pure freshly recrystallized tetra-acetylbromoglucose and 3.00 g. of dry freshly precipitated silver oxide added. This was shaken for about eight hours, after which the container was centrifuged to collect the silver bromide and silver oxide at the bottom. The clear ethereal solution was poured through a filter and the ether distilled off on the water-bath. The crude tetra-acetyl-ergosterol-*d*-glucoside thus obtained was crystallized once from 95% alcohol. The partially purified product was dissolved in boiling 95% alcohol and an excess of hot 10% alcoholic potash was added. The glucoside precipitated immediately and after warming for a few minutes on the water-bath the product was filtered from the hot solution and washed thoroughly with boiling 95% alcohol. For complete purification it was boiled with redistilled pyridine and filtered from a small amount of insoluble material. The filtrate was diluted somewhat with distilled water and allowed to cool, whereupon the glucoside separated in pure white crystals which were filtered off and washed with cold dilute pyridine and then with 95% alcohol. The yield was 0.94 g. Another recrystallization from dilute pyridine yielded 0.84 g. of product. The glucoside is insoluble in water, alcohol, ether, chloroform, acetone, benzene and ethyl acetate. It dissolves with difficulty in hot amyl alcohol and glacial acetic acid, and with ease in hot pyridine. It melts at 308° (corr.) with decomposition; $[\alpha]_D^{25}$ −98.5 when $C = 1$ in pyridine.

Anal. Calcd. for $C_{33}H_{52}O_6$: C, 72.75; H, 9.63. Found: C, 72.64; H, 10.07.

Upon hydrolysis with hydrochloric acid in a mixture of amyl alcohol and ethyl alcohol, glucose and isoergosterol are formed.

Tetra-acetyl-ergosterol-*d*-glucoside.—One gram of pure ergosterol-*d*-glucoside was boiled for thirty minutes with 10 cc. of pure acetic anhydride, the excess of the latter then being removed by distillation under diminished pressure on the water-bath. The residue was dissolved in 25 cc. of hot absolute alcohol and filtered through a hot funnel. Upon cooling, the filtrate deposited the tetra-acetyl-ergosterol-*d*-glucoside in fine white needle-like crystals. This product was filtered off, washed with ice-cold absolute alcohol and the crystallization repeated. The yield was 0.80 g. of product which melted at 167° corrected. It dissolves readily in ether, acetone, ethyl acetate, chloroform,

[8] Dale, THIS JOURNAL, **38**, 2187 (1916).

[9] Bills and Honeywell, *J. Biol. Chem.*, **80**, 12 (1928).

benzene and glacial acetic acid. It is fairly soluble in hot alcohol but crystallizes out almost completely on cooling; $[\alpha]_D^{25}$ −43.0° when $C = 1$ in chloroform.

Anal. Calcd. for $C_{41}H_{60}O_{10}$: C, 69.06; H, 8.49. Found: C, 69.43; H, 8.60.

Cholesteryl Hippurate.—One gram of dry cholesterol was dissolved in 5 cc. of dry chloroform and heated on the water-bath for half an hour with 0.60 g. of hippuryl chloride. The solution was then evaporated to about 3 cc. and a large excess of hot methyl alcohol added. On cooling the cholesteryl hippurate separated in small spherical clusters of needles. The yield was 1.35 g. It was recrystallized three times from methyl alcohol containing a little chloroform and then melted at 153–154° corrected; $[\alpha]_D^{25}$ −30.5° when $C = 1$ in chloroform. It dissolves in about twenty-five parts of methyl alcohol and separates on cooling in small roset-like clusters of needles. From 80% ethyl alcohol it separates in long hair-like needles. It dissolves easily in chloroform, acetone, benzene, ethyl acetate and glacial acetic acid. It is insoluble in water. On saponification with dilute alcoholic potassium hydroxide it yields cholesterol and hippuric acid.

Anal. Calcd. for $C_{36}H_{53}O_3N$: N, 2.56. Found: N, 2.60.

Ergosteryl Hippurate.—One-half gram of pure ergosterol ($[\alpha]_D^{25}$ −131.5°) and 0.30 g. of hippuryl chloride were dissolved in 3 cc. of dry chloroform containing 0.5 cc. of pure dry pyridine and heated on the water-bath for half an hour. Then 25 cc. of dry methyl alcohol was added and the brown solution chilled in ice and salt. The crystalline ergosteryl hippurate was filtered off and washed with methyl alcohol. The yield of crude dry product was 0.63 g. It was recrystallized once from 95% ethyl alcohol containing a little chloroform and then three times from pure acetone. This gave 0.24 g. of product which was dried at 80° and 2 mm. and then melted at 166–167° corrected. Its solubility in ethyl alcohol roughly corresponds to that of cholesteryl hippurate in methyl alcohol and it crystallizes in the same manner. It dissolves readily in benzene and chloroform. It is moderately soluble in acetone, ethyl acetate and hot ethyl alcohol. It is only sparingly soluble in cold ethyl alcohol and almost insoluble in methyl alcohol. It does not dissolve in water. $[\alpha]_D^{25}$ −67.5°, when $C = 1$ in chloroform. On saponification with alcoholic potassium hydroxide it yields ergosterol and hippuric acid.

Anal. Calcd. for $C_{36}H_{49}O_3N$: N, 2.58. Found: N, 2.30.

Cholesterol Bromohydrin.—Three grams of cholesterol was dissolved in 8 cc. of pure chloroform and 8 cc. of distilled water was added. Then 1.10 g. of acetobromoamide was added and the mixture shaken at room temperature until the water layer no longer affected starch–iodide paper. This required about six hours. The chloroform layer was washed twice with distilled water and dried over anhydrous sodium sulfate, after which it was filtered into a 100-cc. distilling flask and the solvent removed by distillation in a vacuum at room temperature. The flask was finally heated for one and one-half hours on the water-bath to remove the last traces of chloroform. The product swelled up to a light fluffy solid mass which was easily broken up and removed from the flask. The yield of almost pure white product was 3.12 g. It melted, but not sharply, at about 85° and underwent rapid decomposition at a slightly higher temperature. It is very soluble in benzene, chloroform, ethyl acetate, ethyl alcohol, methyl alcohol, petroleum ether, ethyl ether, acetone and glacial acetic acid. It is insoluble in water. No suitable solvent was found for recrystallization.

Anal. Calcd. for $C_{27}H_{47}O_2Br$: Br, 16.53. Found: Br, 16.62.

Biological Tests

The biological tests were carried out with rats. They were young animals raised under standard conditions to a weight of about 60 g. in our

stock colony. After having attained this weight they were transferred to a rickets-producing diet for three to four weeks, by which time they had always developed a very pronounced degree of rickets. They were then taken for test. The diet used was our Ration 2965[10] consisting of yellow corn 76, wheat gluten 20, calcium carbonate 3 and sodium chloride 1.

When the animals were taken for the curative tests the afore-mentioned ration had incorporated in it the sterol preparation mixed in the desired quantity with 50 g. of the ration. This was fed *ad libitum*, daily consumption records being taken. When the 50 g. was completely consumed the unsupplemented ration was fed until ten days had elapsed from the beginning of the experimental period. The animals were then killed and the distal ends of the radii and ulnae examined for the deposition of calcium phosphate in the rachitic lesions.[10]

TABLE I

CALCIFICATION WITH IRRADIATED STEROL PREPARATIONS

Rat	Mg.	Dietary addition	Initial wt., g.	Final wt., g.	Av. consumption, g.	Line test
9509	0.01	Cholesterol glucoside Mc 185	87	92	10.0	−
9510	.10	Cholesterol glucoside Mc 185	73	78	7.5	−
9511	2.00	Cholesterol glucoside Mc 185	77	82	9.5	−
9515	0.01	Ergosterol glucoside Mc 193	88	95	10.0	−
9516	.10	Ergosterol glucoside Mc 193	100	104	9.2	++
9517	2.00	Ergosterol glucoside Mc 193	84	80	9.2	++
9520	2.00	Acetyl ergosterol glucoside Mc 194	85	82	7.5	+++
9523	2.00	Acetyl ergosterol glucoside Mc 195	88	89	8.1	+++
9784	0.01	Ergosterol *d*-glucoside Mc 200	80	73	6.9	++
9785	0.10	Ergosterol *d*-glucoside Mc 200	72	75	6.9	++ or +++
9786	2.00	Ergosterol *d*-glucoside Mc 200	84	86	6.4	++++
9792	0.01	Isoergosteryl acetate Mc 203	91	107	9.3	−
9794	2.00	Isoergosteryl acetate Mc 203	74	77	8.7	−
9839	0.10	Isoergosterol Mc 204	87	97	6.3	−
9842	2.00	Isoergosterol Mc 204	90	98	6.6	−
9969	0.01	α-ergostenyl acetate Mc 209	79	80	5.1	++++
9970	.10	α-ergostenyl acetate Mc 209	84	90	6.1	++++
9972	.01	α-ergostenol Mc 210	82	78	5.7	++++
9973	.10	α-ergostenol Mc 210	84	70	4.0	++++
217	.10	Ergosteryl hippurate Mc 221	94	90	7.4	+++
220	.10	Cholesteryl hippurate Mc 219	80	87	7.0	−
9975	.01	Ergosterol *d*-glucoside Mc 212	85	90	9.0	+++
9976	.10	Ergosterol *d*-glucoside Mc 212	85	95	7.5	++++
9977	2.00	Ergosterol *d*-glucoside Mc 212	87	98	5.7	++++

Key to table: − = wide rachitic metaphyses; + = narrow line; ++ = medium metaphyses or medium line; +++ = wide line or narrow metaphyses; ++++ = almost complete healing.

[10] Steenbock and Black, *J. Biol. Chem.*, **64**, 263 (1925).

For their activation the sterol derivatives were exposed to the radiation of a Hanovia Alpine Sun Lamp at a distance of 25 cm. from the burner. In order to secure thorough exposure and to facilitate subsequent mixing with the ration, 10 mg. of the preparation was ground in a mortar with 990 mg. of sucrose. The mixture was then spread out on a glass dish measuring 22.2 by 16.5 cm. and irradiated with mixing at intervals of fifteen minutes for a period of one hour. The mixing of the preparations with sucrose is permissible because sucrose is not antirachitically activatable. The results of the tests are listed in Table I. Because the various preparations differ as to purification, treatment, etc., a brief description of each is necessary.

Cholesterol-*d*-glucoside, Mc 185.—This was prepared by Salway's method,[1] the cholesterol used having a melting point of 147–148°. The glucoside was crystallized four times from dilute pyridine and melted at 265–270° with decomposition.

Ergosterol-*d*-glucoside, Mc 193.—This preparation was made from an incompletely purified specimen of ergosterol having a melting point of 157–159° (short thermometer). The crude glucoside was recrystallized once from pure dilute pyridine and was then extracted for one hour with pure dry ether in a Soxhlet extractor. The crystallization and extraction were repeated after which the dry product had a melting point of 308° (corrected) with decomposition.

Tetra-acetyl-ergosterol-*d*-glucoside, Mc 194.—A portion of the glucoside Mc 193 was not extracted the second time with ether but was acetylated by boiling with acetic anhydride. The product was recrystallized twice from absolute alcohol and melted at 163° (uncorr.).

Tetra-acetyl-ergosterol-*d*-glucoside Mc 195.—The preparation of this was identical with that of Mc 194 except that the ergosterol used was of a fairly high degree of purity having a specific rotation of −128° and a melting point of 161.5–162.5° (short thermometer).

Ergosterol-*d*-glucoside Mc 200.—The ergosterol used for this preparation was rather crude, having a melting point of 152–156° (short thermometer). Purification of the glucoside was the same as that of Mc 193.

Isoergosteryl Acetate Mc 203.—This was prepared by treatment of a chloroform solution of ergosteryl acetate with dry hydrogen chloride according to the directions of Reindel, Walter and Rausch.[11] The product was recrystallized three times from a mixture of methyl alcohol and ethyl acetate (4:1) and then twice from glacial acetic acid. The crystals began to sinter at 128° and melted at 131–132° (short thermometer).

Isoergosterol Mc 204.—A sample of ergosteryl acetate which had a melting point of 174° uncorrected was treated with hydrogen chloride in chloroform solution and the isoergosteryl acetate recrystallized twice from methyl alcohol and ethyl acetate (4:1) and once from glacial acetic acid. The ester was saponified with alcoholic potash and the isoergosterol recrystallized twice from a mixture of methyl alcohol and ethyl acetate (4:1). The crystals were found to have a melting point of 145–146° whereas Reindel, Walter and Rausch[11] give the melting point of their compound as 135–136°. The reason for this discrepancy became clear with the appearance of a paper by Heilbron and Spring[12] in which these authors have shown that the isoergosterol of Reindel, Walter and Rausch is a mixture of two isomers, α-isoergosterol melting at 143–144°, and β-

[11] Reindel, Walter and Rausch, *Ann.*, **452**, 34 (1927).

[12] Heilbron and Spring, *J. Chem. Soc.*, 2807 (1929).

isoergosterol melting at 135°. Clearly our compound was the α-isomer which we had separated by the repeated crystallization.

α-Ergostenyl Acetate Mc 209.—The ergosterol used was the same as that used for preparation Mc 195. It was acetylated and the acetate reduced catalytically in glacial acetic acid. The α-ergostenyl acetate was purified by recrystallization first from methyl alcohol and ethyl acetate (4:1) and then from methyl alcohol and ether (4:1). It melted at 110–111° (short thermometer).

α-Ergostenol Mc 210.—The ergosterol was the same as for Mc 209. It was hydrogenated in glacial acetic acid and then precipitated with water. The product was warmed with alcoholic potash, diluted and chilled. It was then recrystallized twice from methyl alcohol and then melted at 130–131° (short thermometer).

Ergosteryl Hippurate Mc 221 and Cholesteryl Hippurate Mc 219.—These were the preparations previously described in detail.

Ergosterol *d*-Glucoside Mc 212.—This was prepared from highly purified ergosterol. Its preparation has already been described in detail.

These data show that isoergosterol, isoergosteryl acetate, cholesterol-*d*-glucoside and cholesteryl hippurate are entirely unactivatable. The glucoside, the tetra-acetylglucoside and the hippurate of ergosterol are shown to be antirachitically activatable, although to a somewhat less degree than the free sterol. The tests show α-ergostenol and α-ergostenyl acetate to be activatable but we do not wish these results to be accepted as final. The feeding tests of these compounds were unsuccessful in several cases as the consumption of the ration was incomplete. However, this apparently was not due to any properties peculiar to the ergostenol, for at the same time the consumption of a ration containing ergosterol glucoside was also incomplete.

Summary

1. Five new compounds, ergosterol-*d*-glucoside, tetra-acetyl-ergosterol-*d*-glucoside, ergosteryl hippurate, cholesteryl hippurate and cholesterol bromohydrin have been prepared and their properties described.

2. Ergosterol-*d*-glucoside, tetra-acetyl-ergosterol-*d*-glucoside and ergosteryl hippurate and perhaps α-ergostenol have been shown to be antirachitically activatable by ultraviolet light, while cholesterol-*d*-glucoside, cholesteryl hippurate, isoergosterol and isoergosteryl acetate have been shown to be unactivatable.

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