XII. THE PURIFICATION OF ERGOSTEROL.

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Investigations proceeding in this laboratory on the formation of vitamin D made it necessary to obtain ergosterol in as pure a condition as possible. Great variations are recorded in the literature in the physical constants of ergosterol which has been the starting material for chemical and biochemical investigations, and it was considered essential to check the criteria of purity put forward by Tanret [1908] and subsequent workers. Although ergosterol of a high degree of purity may be obtained from certain specimens of crude yeast ergosterol by recrystallisation from a mixture of alcohol and benzene (2:1), as described by Bills and Honeywell [1928], the use of this solvent does not invariably yield products with equally high specific rotation [*cf.* Bills and Cox, 1929; Heilbron, Sexton and Spring, 1929]. According to the experience gained in this laboratory, recrystallisation from alcohol-benzene is extremely useful for the removal of zymosterol from ergosterol, but fails to free it from α-dihydroergosterol. α-Dihydroergosterol appears to be a normal constituent of the sterol mixture occurring in yeast [Callow, 1930, 1931], and its presence in varying proportions accounts almost entirely for the differences in the physical constants of ergosterol recorded in the literature. It is however removed by the method of fractional recrystallisation of the benzoates described here, and ergosterol from different sources, both British and Continental, has yielded purified specimens with no significant differences in their physical properties. No support is therefore given to the conclusion of Bills and Cox [1929] and Bills and McDonald [1930] that natural isomerides of ergosterol separable only with difficulty commonly occur in specimens of different specific rotation obtained from yeast grown under different conditions.

In the course of the preliminary investigations, ergosterol was distilled under reduced pressure. The conclusion of Reindel and Detzel [1929] that this is useless as a method of purification was confirmed.

Purification by means of the recrystallisation of an ester was then considered. The acetate [Windaus and Grosskopf, 1922] and the *iso*butyrate [Bills and Honeywell, 1928] have previously been used for this purpose. Preliminary experiments with ergosteryl ethyl carbonate, prepared from ergosterol by the action of excess of ethyl chloroformate in pyridine solution, indicated that this ester was not a suitable one. The benzoate, however, which has the advantage of being prepared under mild conditions of reaction, gave

an immediate separation into fractions of different specific rotation. Benzoylation of different specimens of yeast ergosterol was carried out with benzoyl chloride in pyridine under uniform conditions, as described in detail below, and the benzoates, which underwent an initial fractionation in the process of separation, were then further fractionally recrystallised from ethyl acetate. Purification was rapid when fractions were collected at 37°, and the head fractions attained a constant specific rotation after five or six recrystallisations at this temperature. The sterol was obtained from the purified benzoate by hydrolysis with alcoholic potassium hydroxide, and freed from traces of decomposition products by recrystallisation.

Three crystalline forms of ergosteryl benzoate were observed. It usually crystallises as a bulky mass of fine needles, a form which is actually stable only at high temperatures. The modification stable at ordinary temperatures forms rectangular plates. Rapidly cooled solutions deposit leaflets belonging to the monoclinic system, which soon pass into a mixture of the other two forms.

The specific rotations of the specimens of purified ergosteryl benzoate and ergosterol were concordant within the limits of the probable errors of determination. In Table I are given the values obtained, together with the melting-points and the figures recorded in the literature.

Table I.

	Ergosterol [α] in chloroform		Ergosteryl benzoate		
M.P.	Anhydrous	Hydrated	M.P.	[α] in chloroform	Observer
165°	$[\alpha]_D$ −132°	$[\alpha]_D$ −126°	—	—	Tanret [1908]
166–183°	$[\alpha]^{20}_{5461}$ −170·6°	—	164–168°	$[\alpha]^{25}_{D}$ −177°*	Bills and Honeywell [1928]
	$[\alpha]^{20}_{D}$ −132°				
	—	—	168–170°	$[\alpha]^{23}_{D}$ − 68°	Windaus and Rygh [1928]
160–161°	$[\alpha]^{20}_{D}$ −133·1°	—	168–170°	$[\alpha]^{23}_{D}$ − 71°	Wieland and Asano [1929]
160–163°	$[\alpha]^{20}_{5461}$ −174·2°	$[\alpha]^{20}_{5461}$ −167·2°	169–171·5°	$[\alpha]^{20}_{5461}$ − 88·3°	Callow
	$[\alpha]^{20}_{D}$ −135°	$[\alpha]^{20}_{D}$ −128·7°	—	—	—

* This exceptional value is not a misprint, as suggested by Windaus and Rygh [1928]; *cf.* Bills and McDonald [1930].

The values of the specific rotation now recorded are slightly higher than those found originally by Tanret [1908] for ergosterol from ergot. The molecule of water of crystallisation in ergosterol crystallised from aqueous alcohol is difficult to remove completely without decomposition. The specific rotation of the hydrated sterol which has been kept over calcium chloride for 3–4 days is the most reliable index of its purity, accompanied by a confirmatory determination of the loss of weight on heating. Prolonged storage of ergosterol over a drying agent causes dehydration which is followed by rapid oxidation in air. In sealed tubes it is unchanged for long periods.

Experimental.

Material.

The following data were obtained for the samples of yeast ergosterol used for this work: (A) M.P. 160–162°, $[\alpha]^{20}_{5461}$ – 156·9°; (B) M.P. 159–162°, $[\alpha]^{20}_{5461}$ – 157·7°; (C) M.P. 160–163°, $[\alpha]^{20}_{5461}$ – 156·6°; (D) M.P. 160–163°, $[\alpha]^{20}_{5461}$ – 158·4°; (E) M.P. 160–163°, $[\alpha]^{20}_{5461}$ – 161·2°; (F) M.P. 155–160·5°, $[\alpha]^{20}_{5461}$ – 137·2°. The specimens were from four different sources, and all except the last had been recrystallised from alcohol-benzene at least once. The melting-points were determined in sealed capillary tubes. All specific rotations recorded here were measured in chloroform containing alcohol (B.P.) in a concentration of about 1 % in a 4 dm. tube, the solutions being generally made up at 20° and measured at the same temperature with an instrument reading to 0·01°. Even purified ergosterol melts over a range of temperature in a capillary tube, and the melting-point is a less reliable and sensitive index of purity than the specific rotation.

One specimen of ergosterol (A), distilled from a flask in a metal-bath at 260–270°/0·1–1·0 mm., gave a pale yellow distillate, $[\alpha]^{20}_{5461}$ – 145·3°. A purified sample of ergosterol, $[\alpha]^{20}_{5461}$ – 164°, began to distil at 192°/0·001–0·01 mm., and the bulk passed over at 198° (bath at 250–260°). A sublimate began to come off at an appreciable rate at a bath temperature of 180°. The distillate was white, and had M.P. 158–161·5°, $[\alpha]^{20}_{5461}$ – 164·1°; the specific rotation had therefore fallen, since the distillate was anhydrous. Recrystallised from alcohol-benzene (2:1) it had $[\alpha]^{20}_{5461}$ – 161·2°.

Another specimen of ergosterol (F) was fractionally sublimed under the conditions used for distillation of vitamin D [Askew *et al.*, 1930]. Fractions were obtained from 0·5 g. at 135° (0·062 g., M.P. 160–164°, $[\alpha]^{20}_{5461}$ – 135°), at 145° (0·044 g., M.P. 156–163°), at 155° (0·317 g., M.P. 156·5–163°, $[\alpha]^{20}_{5461}$ – 148°), and a residue was left (0·06 g., M.P. 154·5–161·5°). These results did not justify further work.

The distilled materials were hygroscopic and very readily oxidised in air. Over calcium chloride in a desiccator, kept in a cupboard away from bright light, and opened at intervals, weighed samples turned yellow and gained in weight owing to absorption of oxygen. Reindel and Detzel [1929] made a similar observation, but, contrary to their statement, no break was observed in the curve of increase in weight at a composition corresponding to a "labile moloxide," $C_{27}H_{42}O_3$. The absorption of oxygen, after a perceptible initial acceleration, went on at a continuously decreasing rate until practical constancy of weight was attained after a year at an increase of 19·5–21 %, corresponding to the addition of five atoms of oxygen (calculated, 20·9 %). The measurements are illustrated by the curve in Fig. 1. It seems probable that this rapid oxidation is due to the combined effects of autocatalysis, fine state of division, and complete initial dehydration. Samples of recrystallised ergosterol kept under the same conditions show a slight initial loss of weight followed, after two or three months, by an increase in weight, the rate of

which at first increases and then decreases, accompanied by a deepening yellow colour. One sample, after a year, gained 18 % in weight, and was still gaining. It appeared likely to reach the same limit as the distilled products

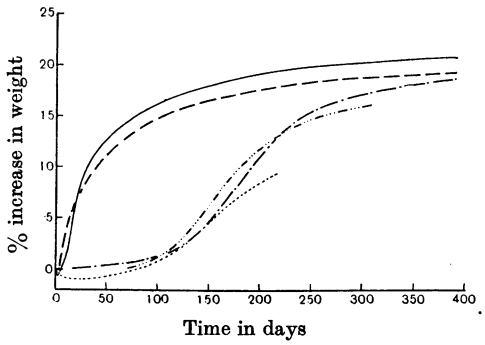

Fig. 1. Increase in weight of ergosterol kept over $CaCl_2$ in air.

——— Distilled, purified ergosterol. — — — Distilled ergosterol (A).
–·–·–·– Ergosterol (E).
·—·—· Ergosterol (A), recrystallised from alcohol-benzene.
- - - - - - - Ergosterol purified *via* the benzoate.

after a further six months. The nature of the products formed has not yet been investigated.

Ergosteryl ethyl carbonate. Ergosterol (3·4 g.) was dissolved in dry pyridine (60 cc.) and ethyl chloroformate (14 cc.) was added slowly with constant shaking and cooling. After standing for 30 minutes the mixture was poured into water and the precipitated solid was collected and washed. Much frothing occurred in both stages of the preparation. Recrystallised from absolute alcohol and from ethyl acetate, *ergosteryl ethyl carbonate* was obtained as thin plates, M.P. 150–153·5°, $[\alpha]_{5461}^{20}$ – 111·1°. The yield was 2 g. (Found (micro): C, 79·3; H, 10·4 %. $C_{30}H_{46}O_3$ requires C, 79·3; H, 10·1 %.) The ultra-violet absorption spectrum in alcoholic solution closely resembled that of ergosterol. When the carbonate was irradiated in solution under the same conditions as ergosterol, the product showed equal antirachitic activity. The ester, in this respect, thus resembles the acetate.

A small fraction of the crude product was insoluble in alcohol, and crystallised from ethyl acetate in needles, M.P. 206–212°. Fractionation of the alcoholic mother-liquors showed the presence of unchanged ergosterol. Hydrolysis of the ester with alcoholic potassium hydroxide yielded ergosterol, which, recrystallised from 95 % alcohol, had M.P. 159–162°, $[\alpha]_{5461}^{20}$ – 164·2°. No further work was done with the ergosteryl ethyl carbonate, since the conditions of its preparation were less satisfactory than those of the benzoate, and it had no advantage with respect to purification.

Preparation of ergosteryl benzoate. Ergosterol (1 part) in dry pyridine (20 volumes) was treated with redistilled benzoyl chloride (2·5–3 volumes) in small portions while the flask was shaken and cooled under the tap. The

development of a permanent pink colour was a reliable indication of the presence of a slight excess of benzoyl chloride. It was necessary to use much more than the calculated quantity of benzoyl chloride in order to obtain complete esterification, and the excess required varied considerably with different specimens, probably according to the amount of solvent of crystallisation or the degree of dryness of the pyridine. Under these conditions part of the benzoate crystallised out directly from the reaction mixture, together with pyridine hydrochloride. The necessity of pouring the whole into water was obviated, and a fractionation was obtained. After cooling to room temperature, the solid which separated (fraction I) was collected and washed with a very little pyridine, and the filtrate was cooled to − 4°. Fraction I was, in the meantime, washed thoroughly with water and finally with alcohol. Fraction II, obtained at − 4°, was treated similarly, and the aqueous washings from the two crops (in all 40 volumes) were added to the filtrate. The precipitate thus produced (fraction III) was collected and washed. Ethyl benzoate, in some cases in large amount, separated with the precipitate. In the light of subsequent work, it is evident that this was derived from the alcohol of crystallisation of the α-dihydroergosterol originally present. Pyridine was recovered from the filtrate.

In a typical preparation sample D of ergosterol (45 g.) gave the following fractions: (I) 35·8 g., M.P. 164–171°, $[\alpha]^{20}_{5461}$ − 86·8°; (II) 7·1 g., M.P. 163–168·5°, $[\alpha]^{20}_{5461}$ − 82·9°; (III) 7·0 g., M.P. 135–150°, $[\alpha]^{20}_{5461}$ − 36°; total yield, 91 %.

Purification of ergosteryl benzoate. In general, fractions I and II were worked up in the same scheme of fractional crystallisation, whilst fraction III was treated separately for the isolation of admixed sterols whose presence was suspected. The system ultimately adopted was to recrystallise fraction I repeatedly until no further alteration in specific rotation took place, and to utilise the mother-liquors for the recrystallisation of subsequent batches of crude benzoate. In this way there accumulated mother-liquors in which impurities of the original ergosterol collected. The ethyl acetate used as solvent was freed from acetic acid and higher esters and dried. The crude benzoate was dissolved, in a current of nitrogen, in 25–35 volumes of boiling ethyl acetate to give a nearly saturated solution, which was then allowed to cool in a closed flask to 37° in the hot room. It was found in the first stages of the work that crystallisation at 37° rather than at room temperature gave a much more efficient purification, and much reduced the number of recrystallisations necessary. Oxidation took place to an undesirable extent in the mother-liquors if hot solutions were exposed freely to the air. A mixture of butyl alcohol and benzene (2:1), and 80 % aqueous pyridine were also tried as solvents, but had the disadvantage of being difficult to remove and of hindering the recovery of material from mother-liquors. Pyridine seemed particularly to favour decomposition and oxidation. Ethylene dichloride was unsuitable because of the difficulty of keeping it free from traces of hydrogen chloride,

to which ergosterol is very sensitive. None of these solvents, moreover, gave evidence of further purification after exhaustive crystallisation from ethyl acetate.

The course of the purification of the benzoate from sample F may be taken as an example. Fraction I, twice recrystallised from ethyl acetate at 37° yielded material (1), $[\alpha]^{20}_{5461}$ − 83·7°. Crystallisation of this (33 g.) from ethyl acetate (1100 cc.) yielded (2), $[\alpha]^{20}_{5461}$ − 86·2° (20 g.), and so, in succession, (3), $[\alpha]^{20}_{5461}$ − 86·7° (10·8 g.); (4), $[\alpha]^{20}_{5461}$ − 87·2° (6·4 g.); (5), $[\alpha]^{20}_{5461}$ − 88·1° (4 g.); (6), $[\alpha]^{20}_{5461}$ − 88·0° (2·8 g.). In another case (sample D above), the successive fractions were: (1) M.P. 167–172°, $[\alpha]^{20}_{5461}$ − 87·6°; (2) (from butyl alcohol-benzene) M.P. 167–171·5°, $[\alpha]^{20}_{5461}$ − 88·0°; (3) M.P. 166–171·5°, $[\alpha]^{20}_{5461}$ − 88·5°; (4) (from butyl alcohol-benzene) M.P. 169–171·5°, $[\alpha]^{20}_{5461}$ − 88·7°. A third specimen of benzoate (from samples B and C), after purification, had $[\alpha]^{20}_{5461}$ − 88·0°, and then, in two successive recrystallisations from ethyl acetate, gave material having $[\alpha]^{20}_{5461}$ − 88·15°, and $[\alpha]^{20}_{5461}$ − 88·2°. Comparable figures were obtained in all cases for the benzoate recovered from the mother-liquors of the head fractions, separated in later stages of the fractionation. Thus, after five or six recrystallisations from ethyl acetate, a pure, homogeneous benzoate, having a mean specific rotation $[\alpha]^{20}_{5461}$ − 88·3°, without significant variation, was obtained from all the samples of ergosterol.

Rectangular plates accompanied the usual needles of ergosteryl benzoate, as recorded by Wieland and Asano [1929], most frequently when the mother-liquors of fractions collected at 37° were allowed to stand at room temperature. It was ultimately discovered that from butyl alcohol-benzene solutions, the separation of either plates or needles could be obtained by seeding with the required form, although rapid cooling favoured the separation of plates, and slow cooling the separation of needles. Both forms were obtained from the purified benzoate with the same specific rotation, $[\alpha]^{20}_{5461}$ − 88·4°. No attempt was made to determine the transition point of the two forms, but the slow replacement of needles by plates in the presence of various solvents shows that the plate form is the stable one at room temperatures. A specimen of plates began to melt at 165° to give a cloudy mixture, in which a mass of fine needles could be discerned with the aid of a lens, and became clear at 170°, so that the needle form is evidently the stable one at high temperatures. A third crystalline form was observed when solutions of the benzoate were cooled rapidly without agitation. Leaflets separated which were replaced by a felted mass of needles and a few plates when the mixture was stirred. Isolation of the leaflets in bulk was impossible, but in portions of the solution in butyl alcohol-benzene transferred to a microscope slide it was possible to observe hexagonal leaflets, belonging to the monoclinic system, which dissolved and were replaced by rectangular plates and a few needles, the latter afterwards gradually dissolving.

Ultra-violet irradiation of ergosteryl benzoate gave a product with about 1/8 the antirachitic activity of ergosterol similarly treated, but the activity

rose to that of the irradiated free sterol when the product was hydrolysed [*cf.* Windaus and Rygh, 1928].

Hydrolysis of ergosteryl benzoate. This was done by heating the benzoate with 25 parts of 3 % alcoholic potassium hydroxide and boiling for 5 minutes after solution was complete. As a precaution against oxidation, the reaction was carried out in an atmosphere of nitrogen. The ergosterol which separated on cooling was collected, washed with alcohol and water, recrystallised from 40–50 parts of 95 % alcohol, and dried for several days over calcium chloride. The yield was about 67 %, and a further 15 %, generally of slightly lower rotation, could be obtained by working up the mother-liquors.

Properties of purified ergosterol. The characteristics of the pure ergosterol thus obtained from different samples were as follows:

(A) M.P. 160–162°, $[\alpha]^{15}_{5461}$ − 165°, loss at 100°/12 mm., 4·5 %. (calculated for $C_{27}H_{42}O$, H_2O: 4·5 %);

(B + C) M.P. 160–163°, $[\alpha]^{20}_{5461}$ − 166·8°, − 167·7°, − 167·2° (different batches; mean, − 167·2°), $[\alpha]^{20}_{D}$ − 128·7°, anhydrous substance, $[\alpha]^{20}_{5461}$ − 174·2°, $[\alpha]^{20}_{D}$ − 135°, loss at 120°/0·1 mm., 4·5 %;

(D) M.P. 160–163°, $[\alpha]^{20}_{5461}$ − 166·5°, loss at 120°/10 mm., 4·7 %;

(F) M.P. 160–163°, $[\alpha]^{20}_{5461}$ − 165·9°.

Dehydration of ergosterol by heating under reduced pressure in an Abderhalden "pistol" with steam or xylene vapour was accompanied by slight decomposition, and the specific rotation was a little below the calculated value. The material was generally slightly sintered and had acquired a yellow tinge. Less decomposition occurred when the ergosterol was quickly heated to just above its melting-point in a current of pure, dry nitrogen. The anhydrous material gained in weight rapidly, owing to oxidation, when exposed to the air. Purified ergosterol gained weight in a similar way to unpurified ergosterol when kept over calcium chloride (see Fig. 1). No change in specific rotation or melting-point was found after six months in a specimen kept in the dark in a sealed, evacuated tube.

Summary.

1. Distillation of ergosterol and recrystallisation of ergosteryl ethyl carbonate are unsatisfactory methods for purifying ergosterol, and recrystallisation from alcohol-benzene (2:1) does not invariably yield a pure product.

2. A high degree of purity is attained by benzoylation of ergosterol, recrystallisation of the benzoate from ethyl acetate at 37°, and hydrolysis.

3. Ergosteryl benzoate is trimorphic.

4. No variation has been found in the properties of purified yeast ergosterol from different sources, and no evidence of the existence of natural isomerides has been obtained.

5. When ergosterol is kept over a dehydrating agent in air it takes up five

atoms of oxygen. This oxidation is rapid in the case of distilled material, but also takes place with recrystallised hydrated material after an induction period.

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