XIII. OCCURRENCE OF α -DIHYDROERGOSTEROL AS AN IMPURITY IN YEAST ERGOSTEROL.

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THE fractionation of the benzoates obtained from ordinary ergosterol [Callow, 1930, 1931] has led to the isolation of α -dihydroergosterol, the occurrence of which among the yeast sterols has not hitherto been reported. This compound is the only one found present in sufficient amount for separation and identification.

The attempt to isolate α -dihydroergosteryl benzoate by means of fractional crystallisation was a laborious process. The more soluble fractions of the benzoates from ordinary ergosterol crystallised from ethyl acetate in stable leaflets isomorphous with the unstable form of ergosteryl benzoate, and the properties of the material closely resembled those of neosteryl benzoate [Wieland and Asano, 1929]. On repeated recrystallisation from other solvents, however, the laevorotation diminished and the melting point rose, whilst the product still contained a large proportion of ergosteryl benzoate. It became evident that the unstable leaflet form of ergosteryl benzoate formed a stable mixed crystal phase with another benzoate crystallising in isomorphous leaflets. After the accumulation of larger quantities of material an attempt was made to remove the ergosterol by fractional crystallisation from ethylene dichloride, which was the best of the various solvents tried, but the results indicated that the isolation of any considerable quantity by this method would be impracticably slow. Recourse was had therefore to a chemical method of separation. It was found that partial bromination decomposed the ergosteryl benzoate preferentially, and ultimately the ergosterol content of the product of hydrolysis was reduced to 0.25 %. The last traces of ergosterol could be removed by ultra-violet irradiation.

The identity of the sterol thus isolated with α -dihydroergosterol, indicated by the correspondence of the properties with those recorded in the literature, was confirmed by comparison with an authentic specimen. The *p*-nitrobenzoate, which was prepared for the first time, is remarkable for the large range of temperature (46°) over which the anisotropic liquid phase is stable. This range is small in all derivatives of ergosterol hitherto examined, and the crystalline liquid is difficult to observe at all in some cases [cf. Gaubert, 1908].

No indication of the presence of sterols other than α -dihydroergosterol was obtained, although their presence in small amount in the most soluble

fractions is not impossible. The similarity of the neosteryl benzoate of Wieland and Asano [1929] to the products obtained in the initial stages of the separation of α -dihydroergosteryl benzoate strongly suggests that the former material was a similar isomorphous mixture. The data given, in particular the absorption spectrum, and the comments made by Wieland and Gough [1930] give some support to this suggestion. It was actually found that a mixture of ergosterol and α -dihydroergosterol yielded a benzoate, resembling neosteryl benzoate, which formed homogeneous crystals from ethyl acetate and showed little change in specific rotation after recrystallisation from that solvent. The existence of neosterol as a chemical entity cannot of course be denied without examining a sterol mixture similar to that used by Wieland and Asano as their starting product. The "cervisterol" of Bills and Honeywell [1928], which would be expected to yield a benzoate of high melting point and slight solubility, was not present, nor has any indication of its presence been obtained in numerous other specimens of ergosterol in this laboratory.

It is evident that in the specimens of ergosterol examined the variations in specific rotation could be accounted for almost entirely by the presence of α -dihydroergosterol in varying amounts, and that this is the only considerable impurity. The specific rotation of a mixture of 95 % of ergosterol (+ H_2O ; $[\alpha]_{5461}^{20} - 167.2^\circ$) with 5 % of α -dihydroergosterol (+ 0.5 $\text{C}_2\text{H}_5\text{OH}$; $[\alpha]_{5461}^{20} - 21.1^\circ$) would be $[\alpha]_{5461}^{20} - 159.9^\circ$, which is close to the average values shown by ordinary "pure" ergosterol.

α -Dihydroergosterol shows absorption only in the extreme ultra-violet, and it is unaffected by radiation of wave-lengths above $275\mu\mu$. Windaus, Gaede, Köser and Stein [1930], in a recent paper describing the products of over-irradiation of ergosterol through "Uviolglas," record that 4 % of the product was precipitated as a digitonide which proved to be that of α -dihydroergosterol. In the light of the results now described it is probable that the latter was present in their starting material.

The recognition of α -dihydroergosterol as a companion of ergosterol in yeast indicates that the simultaneous occurrence of sterols in different stages of saturation is a general phenomenon connected with the synthetic cellular activity of plants and animals. Evidence for such a view has been accumulating during the course of this work. The occurrence of dihydroergosterol along with fungisterol and ergosterol in ergot [Heyl and Swoap, 1930] is analogous to its association with zymosterol and ergosterol in yeast. Dihydrositosterol has been found to be a constant companion of sitosterol in cereals [Anderson, Nabenhauer, and Shriner, 1927] and in the soya bean [Bonstedt, 1928]. The latter also contains a doubly unsaturated sterol, stigmasterol, which is found associated with sitosterol in the Calabar bean and cacao. Finally, it has recently been found [Schönheimer, Behring, Hummel, and Schindel, 1930], that the main sterol constituent of animal cells, cholesterol, contains dihydrocholesterol as a constant admixture. The significance of the occurrence of these saturated sterols is uncertain. The last named authors consider that dihydro-

cholesterol is formed by hydrogenation of cholesterol, but there is as yet little evidence to indicate whether the formation of the more saturated sterols by reduction is general. It is possible that in some cases they are intermediates in the formation of the unsaturated compounds.

EXPERIMENTAL.

Material. The specimens of ergosterol which were the starting material for this work were the same as those used in the purification of ergosterol previously described [Callow, 1931]. After benzoylation of the crude ergosterol with benzoyl chloride in pyridine and separation of the material which crystallised out directly, a final fraction (10–15 % of the total yield) was obtained by the addition of water to the pyridine solution. The presence of the benzoate of another sterol was immediately evident when this fraction was recrystallised from ethyl acetate, for a new stable crystalline form appeared—homogeneous leaflets belonging to the monoclinic system in the case of the least pure specimen of ergosterol (F; $[\alpha]_{5461}^{20} - 137.2^\circ$), and mixtures of leaflets with needles of ergosteryl benzoate in the case of other specimens ($[\alpha]_{5461}^{20} - 156.9$ to -161.2°). In addition, the higher fractions of the benzoates from specimen F yielded mixtures of needles and leaflets, and a small quantity of leaflets was obtained from the mother-liquors of the higher fractions of the benzoates from other specimens. Mixtures of leaflets and needles could be separated by dissolving in boiling ethyl acetate and collecting fractions at 37° and -4° . In this way batches of leaflets were collected with M.P. ranging from 159 – 165° to 165 – 170° , and values of $[\alpha]_{5461}^{20}$ ranging from -59° to -65° . In a typical case 50 g. of specimen A of ergosterol gave a total of 6.8 g. of leaflets.

Some difficulty was encountered in the initial stages of the work owing to the change which took place in ethyl acetate mother-liquors kept exposed to the air. This change, the nature of which is still uncertain, was indicated by an increase in solubility, yellowing of the solution on concentration, and the subsequent deposition of poorly crystallised products. The lower crystalline fractions from ethyl acetate also showed signs of oxidation when stored in contact with air. Simultaneously, the material developed an intense colour reaction with a 25 % solution of mercuric acetate in nitric acid ("mercury reagent") [Rosenheim and Callow, 1931]. When a chloroform solution of the material was mixed with the reagent and shaken the chloroform layer turned bluish-red, changing to blue, and then, after an interval, to green, the acid layer remaining colourless. In the ultimate systematic fractionation the composition of all fractions obtained was checked by the use of this reagent.

After hydrolysis with 3 % alcoholic potassium hydroxide these fractions of altered material yielded a brown solution from which a colourless sterol mixture separated, and the latter, when re-benzoylated, gave a mixture of benzoates which showed only the usual ergosterol reaction with the "mercury reagent" (chloroform layer pink $\rightarrow$ orange $\rightarrow$ yellow). The difficulty of working up sterol mixtures from the mother-liquors of technical ergosterol may be due

to the presence of similar transformation products of ergosterol, and the re-saponification process which Wieland and Gough [1930] applied to such material may owe its usefulness to the destruction of these products by alkali.

Separation of mixtures rich in α -dihydroergosteryl benzoate by fractional crystallisation. After preliminary investigations which indicated that the fractions of the benzoate crystallising in leaflets contained a large proportion of ergosteryl benzoate, and after trials with various solvents, a systematic fractionation was begun with 180 g. of material by crystallisation from ethylene dichloride. The benzoate was dissolved in eight parts of boiling ethylene dichloride in an atmosphere of nitrogen, cooled, and fractions were collected at 37° and at -4°. The mother-liquors were either used for the recrystallisation of lower fractions or were evaporated in a current of nitrogen. In this way at one end of the system a series of less soluble fractions was obtained with decreasing laevorotation and rising m.p., whilst ergosteryl benzoate became concentrated in the more soluble fractions and ultimately separated in needles. The progress of the separation is illustrated by the following figures for successive head fractions: (1) m.p. 176–181°, $[\alpha]_{5461}^{20} - 55.8^\circ$; (2) m.p. 179–183.5°, $[\alpha]_{5461}^{20} - 45.5^\circ$; (3) m.p. 180.5–187°, $[\alpha]_{5461}^{20} - 38.7^\circ$; (4) m.p. 185–190°, $[\alpha]_{5461}^{20} - 32.2^\circ$, 12.1 g.; (5) m.p. 187.5–192°, $[\alpha]_{5461}^{20} - 26.6^\circ$, 6.3 g. When the m.p. and specific rotation were plotted on curves against number of recrystallisations it appeared that at least six further recrystallisations would be necessary before practical constancy could be attained.

Removal of ergosterol by bromination. Bromination of the mixed benzoates was initially tried in the hope that the sterol, then unidentified, accompanying ergosterol would form an easily separable bromide. Preliminary experiments, however, in which bromination was carried out in ether, chloroform or carbon tetrachloride, yielded no product sparingly soluble in alcohol when excess of bromine was used. When smaller proportions of bromine, preferably in carbon tetrachloride solution, were used, the addition of alcohol precipitated products free from bromine, and these were found to have the same properties as the products obtainable from the same starting material by fractional crystallisation.

The following method was found suitable for removing ergosterol. The mixture of benzoates (30.7 g.; containing about 30 % of ergosterol) was dissolved in carbon tetrachloride (400 cc.), and a solution of bromine in carbon tetrachloride (31 cc. of a 5 % solution by volume; about 2.5 mols./mol. of ergosterol) was added slowly with shaking. After allowing to stand for a few minutes, 95 % alcohol (1500 cc.) was added. The precipitate (18.8 g.) was collected, washed with alcohol, and recrystallised from ethylene dichloride. The slightly discoloured product had m.p. 189–193°, $[\alpha]_{5461}^{20} - 12^\circ$, and contained 3 % of ergosterol, determined colorimetrically. The ergosterol content was not reduced below 1 % by repeating the bromination. A specimen obtained in this way contained 1 % of ergosterol, and had m.p. 194–196°, $[\alpha]_{5461}^{20} - 13.0^\circ$. (Found (micro): C, 83.4; H, 10.0 %; $C_{34}H_{48}O_2$ requires C, 83.5;

H, 9.9 %.) It was found best, when dealing with fractions of high ergosterol content, to remove the ergosterol by two successive partial brominations.

α -Dihydroergosterol. Hydrolysis of the benzoate (14 g.; 3 % ergosteryl benzoate) was carried out by boiling for 45 minutes with 3 % alcoholic potassium hydroxide solution (800 cc.). Water was added to the hot solution until it was slightly cloudy, and the crystals which separated on cooling were collected, washed, dried, and recrystallised from alcohol-benzene (2:1). α -Dihydroergosterol (8.6 g.), containing 0.25 % of ergosterol, separated in leaflets, m.p. 172–174°, containing 0.5 mol. of alcohol of crystallisation. The solvent-free substance had $[\alpha]_{5461}^{20} - 23.8^\circ$. (Found: loss at 130°/10 mm. over P_2O_5 , 5.7, 5.7 %, and, in solvent-free substance (micro), C, 83.6; H, 11.6 %. $C_{27}H_{44}O$, 0.5 C_2H_5OH requires loss, 5.65 %. $C_{27}H_{44}O$ requires C, 84.3; H, 11.5%.) The digitonide was precipitated by the addition of 1 vol. of a 1 % solution of digitonin in 90 % alcohol to 2 vols. of a 0.1 % solution of the sterol in 90 % alcohol. (Found: digitonide: sterol = 3.9:1.)

A portion of this specimen of α -dihydroergosterol was irradiated in 0.5 % solution in benzene by a quartz mercury vapour lamp for a period sufficient to destroy the ergosterol present. After evaporation and crystallisation of the residue twice from 95 % alcohol (charcoal), the product, freed from solvent at 130°/10 mm. (loss 5.9 %), had m.p. 172.5–174° (unchanged by mixture with an authentic specimen of α -dihydroergosterol of the same m.p.), and $[\alpha]_{5461}^{20} - 23.0^\circ$. (Windaus and Brunken [1928] give m.p. 173–174°, $[\alpha]_D^{17} - 20.4^\circ$; Heilbron and Sexton [1929] give m.p. 173°, $[\alpha]_{5461}^{20} - 21.7^\circ$.) The colour reactions were as follows: Liebermann-Burchard: momentary purple $\rightarrow$ dark-blue $\rightarrow$ bottle-green $\rightarrow$ emerald. Bromine (Tortelli-Jaffé): negative in dilute solution; in concentrated solution, yellow $\rightarrow$ emerald $\rightarrow$ blue-green. Iodine [Rosenheim and Callow, 1931]: negative. Salkowski: chloroform colourless, acid deep yellow with green fluorescence. Antimony trichloride: negative, red after standing. Trichloroacetic acid: negative, dull green with light green fluorescence after 6 hours. Mercuric acetate in nitric acid [Rosenheim and Callow, 1931]: negative. The slow, secondary reactions with antimony trichloride and trichloroacetic acid, which have not been described before, were also given by an authentic specimen of α -dihydroergosterol. The digitonide was precipitated in 90 % alcohol. (Found: digitonide: sterol = 4:1.)

Spectrographic examination of purified α -dihydroergosterol in 0.1 % alcoholic solution showed that absorption was negligibly small at wave-lengths exceeding $250\mu\mu$, but then increased rapidly down to $230\mu\mu$, the limit of measurement [cf. De Boe, 1930]. Investigation of the effect of irradiation in alcoholic solution by a quartz mercury vapour lamp with and without filters [cf. Askew *et al.*, 1930] indicated that a small amount was destroyed by the total radiation or by wave-lengths between $210\mu\mu$ and $280\mu\mu$, and a slight diminution of absorption occurred. The product of irradiation by the unscreened lamp, in which about 3 % was destroyed, showed no antirachitic activity in rats in doses of 8 γ daily. Irradiation with light of wave-lengths

exceeding $275\mu\mu$, under conditions in which ergosterol is destroyed to the extent of 50 %, caused no significant alteration of the absorption or of the amount precipitated by digitonin. A trace of a substance of high melting point appeared to be formed.

Derivatives of α -dihydroergosterol. These were prepared from material containing 0.25 % of ergosterol.

The *benzoate*, prepared with excess of benzoyl chloride in pyridine, and recrystallised, had m.p. $192-195^\circ$, $[\alpha]_{5461}^{20} - 13.6^\circ$. The m.p. of the benzoate prepared from 10 mg. of an authentic specimen of α -dihydroergosterol was $188-194^\circ$, and a mixture melted at $190-195^\circ$. Heyl and Swoap [1930] record m.p. $153-155^\circ$, but from their description it is evident that, owing to the use of insufficient benzoyl chloride, their material must have been a mixture of free sterol and benzoate.

The *acetate*, prepared by boiling with acetic anhydride for 20 minutes, and recrystallised from ethyl acetate, had m.p. $178-180^\circ$, $[\alpha]_{5461}^{20} - 24.2^\circ$. (Windaus and Brunken [1928] give m.p. $180-181^\circ$. Heilbron, Johnstone, and Spring [1929] give m.p. $179-180^\circ$, $[\alpha]_{5461}^{20} - 25.3^\circ$.) (Found (micro): C, 81.9; H, 10.9 %. $C_{29}H_{46}O_3$ requires C, 81.6; H, 10.9 %.)

The *p-nitrobenzoate* was prepared by adding a solution of *p*-nitrobenzoyl chloride (0.3 g.) in chloroform (2 cc.) to α -dihydroergosterol (9.1 g.) in pyridine (2 cc.). The reaction mixture was poured into water, chloroform was removed by evaporation, and the solid was collected, washed with hot alcohol, and recrystallised twice from 80 % aqueous pyridine. It separated in lanceolate plates, melting at 202° to a cloudy, anisotropic liquid, clearing point 248° . It separated from other solvents in gelatinous form. (Found (micro): C, 76.9; H, 8.8 %. $C_{34}H_{47}O_4N$ requires C, 76.5; H, 8.9 %.)

The number of ethylenic linkings in the acetate and benzoate was determined by titration with bromine in carbon tetrachloride according to the modification of Kaufmann's method described by Reindel and Niederländer [1929]. (Found: acetate, 1.9, 1.8; benzoate, 1.7. Control analyses: ergosteryl benzoate, 2.4, 2.4; cholesteryl acetate, 1.1.)

Mixtures resembling neosterol. Neosterol, m.p. $164-165^\circ$, $[\alpha]_D^{24} - 105^\circ$, was isolated by Wieland and Asano [1929] from material derived from the mother-liquors of yeast ergosterol, and the benzoate was described as leaflets having m.p. $173-175^\circ$, $[\alpha]_D^{24} - 50.6^\circ$. The sterol gave the same colour reactions as ergosterol, and its absorption in the ultra-violet [Wieland and Gough (with Page), 1930] was almost identical. The fractions of benzoate crystallising in leaflets encountered in the first stages of the separation strongly resembled the description of neosteryl benzoate. One fraction, for example, had m.p. $170-177^\circ$, $[\alpha]_{5461}^{20} - 60.0^\circ$, and another had m.p. $172-176^\circ$, $[\alpha]_{5461}^{20} - 63.8^\circ$. However, recrystallisation of the first from ethyl acetate and twice from acetone gave a product of m.p. $173-185^\circ$, $[\alpha]_{5461}^{20} - 47.4^\circ$, and two recrystallisations of the second from ethylene dichloride at 37° gave a product of m.p. $180.5-185^\circ$, $[\alpha]_{5461}^{20} - 46.7^\circ$. The hydrolysis of these products yielded sterols which, re-

crystallised from alcohol, had, respectively, M.P. 165.5–170°, $[\alpha]_{5461}^{20} - 86.9^\circ$ and M.P. 168–172°, $[\alpha]_{5461}^{20} - 91.5^\circ$. (Found (micro), in material dried at 130°/10 mm. over P_2O_5 : C, 83.3; H, 9.8 %. $C_{34}H_{48}O_2$ requires C, 83.6; H, 9.8 %.) The anti-rachitic potency after irradiation was in each case half that of ergosterol, and the extinction coefficients of the alcoholic solution at 281.7, 271.5, and 247.3 $\mu\mu$ were 55–57 % of those of ergosterol. In addition, colorimetric measurement of the second specimen (described below) indicated the presence of about 55 % of ergosterol.

It was thus evident that the material resembling neosteryl benzoate was, in fact, an isomorphous mixture, and this was confirmed, after the recognition of α -dihydroergosterol as one of the constituents, by the preparation of a homogeneous mixed benzoate of ergosterol and α -dihydroergosterol. Ergosterol (0.7 g.) and α -dihydroergosterol (0.3 g.) were dissolved in pyridine (10 cc.) and treated with benzoyl chloride (2.5 cc.). The crude product, separated by pouring into water and washing with alcohol, had M.P. 167–174°, $[\alpha]_{5461}^{20} - 65.4^\circ$. It crystallised from ethyl acetate in leaflets, M.P. 170–175°, $[\alpha]_{5461}^{20} - 62.5^\circ$. This entirely confirms the conclusions as to the composition of the early fractions of benzoates, and lends additional support to the suggestion that neosteryl benzoate is also such a mixture.

Colorimetric determination of ergosterol. The blue colour obtained when a chloroform solution of ergosterol or ergosteryl benzoate is mixed with a 90 % aqueous solution of trichloroacetic acid [Rosenheim, 1929] attains a maximum intensity after 5 minutes, and is then readily matched by a combination of blue and yellow Lovibond tints in the Rosenheim-Schuster colorimeter [Rosenheim and Schuster, 1927]. The intensity of the blue is maximal when the reagent and the chloroform solution are mixed in the proportion 2:1, and is then approximately proportional to the concentration of ergosterol, thus providing a rapid method for the determination of ergosterol or the benzoate. Example: a 0.2 % solution of a sterol mixture gave 8.1 blue units (+ 2.7 yellow) in a 10 mm. cell, whilst a 0.1 % solution of ergosterol gave 7.5 blue units (+ 3.2 yellow); the mixture therefore contained $0.1/0.2 \times 8.1/7.5 \times 100 = 55$ % of ergosterol, a result which was in agreement with that of spectroscopic analysis.

SUMMARY.

1. Ordinary yeast ergosterol commonly contains considerable amounts of α -dihydroergosterol.

2. α -Dihydroergosteryl benzoate has been isolated by fractionation of the benzoates from yeast ergosterol, followed by a process of partial bromination which destroys the ergosterol preferentially. α -Dihydroergosterol has been identified and characterised by the preparation of derivatives.

3. The neosterol of Wieland and Asano [1929] has not been found, but the benzoates of ergosterol and α -dihydroergosterol, as well as the free sterols, form mixed crystals whose properties agree with those of neosteryl benzoate and neosterol, respectively.

4. The coloration given by ergosterol with trichloroacetic acid has been applied to its determination.

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