LXI. THE ISOLATION OF CAROTENE AND STEROLS FROM THE UNSAPONIFIABLE MATTER OF COCKSFOOT.

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FIVE years ago a comprehensive analysis was made of the ether extract of cocksfoot (*Dactylis glomerata*) [Pollard *et al.*, 1931; Smith and Chibnall, 1932]. During the work, β-carotene, xanthophyll and sterols were isolated in good yield and their properties were studied. The results were not considered to be of sufficient importance for publication, since about that time Kuhn and Lederer [1931, 1] showed that "grass" contained only β-carotene, and the spectrographic examination of the sterols had been undertaken by Prof. Heilbron.

A recent investigation of the cocksfoot carotene by Gillam [1935] has however emphasised the purity of the product, and as dried grass is now available commercially in large quantities and as the pure carotene is readily obtained in good yield, it seems worth while to describe the method of isolation employed. Some further investigations on the sterols and a brief description of the xanthophyll are also given.

The principal constituent of most leaf carotenes has been shown to be the β-isomeride by Kuhn *et al.* [1931] and more recently by Miller [1935], Mackinney [1935] and Strain [1935], who have shown that in many grasses it is the only form that can be detected. Among thirteen members of the Gramineae examined by Miller, the bamboo was the only one in which α-carotene could be found. A measurement of the optical rotation of the carotene isolated by the author from cocksfoot was made at the National Institute for Medical Research through the courtesy of the late Dr Dudley. Within the limits of experimental error, the carotene had no rotation, $[\alpha]_{Cd} = -10°$. Since α-carotene has $[\alpha]_{Cd} = +380°$ and β-carotene has a rotation of zero, the specimen examined appeared to consist only of β-carotene.

The amount of carotene in the grass may be increased very considerably by suitable nitrogen fertilisation, and specimens with a carotene content much higher than in the present case have been examined. The comparative ease with which the carotene has been isolated from the cocksfoot was chiefly due to the previous removal of the wax material. Unless these compounds are separated at the beginning of the preparation, they have a considerable influence on the solubility of the carotene and on its adsorption in chromatographic analysis. This point has been discussed by Strain [1935], who encountered some difficulty in the later stages of the purification.

When these experiments were undertaken, the presence of ergosterol in forage grasses had not been established, and the possibility that this substance might play some part in the production of the antirachitic vitamin of butter fat was considered to be a point of some interest. The chief constituent of the cocksfoot sterol appeared to be sitosterol and no evidence could be found for the

presence of stigmasterol on brominating the mixture by the method of Windaus [1906; 1907]. Spectrographic examination of the sterol by Prof. Heilbron showed that the spectrum of ergosterol was detectable and corresponded to an ergosterol content of approximately 1 % of the mixture [Gillam *et al.*, 1933].

Recently, using the method of Windaus and Borgeaud [1928], it has been possible to isolate from the mixture of sterols a substance which has been identified by melting-points and analysis as the pinacol of ergosterol. The amount obtained corresponded to 0·3 % of the mixture, but as ergosterol itself gives the pinacol only in 70 % yield, the amount isolated from the grass sterol would indicate a minimum content of 0·4 % of ergosterol.

Evidence has been brought forward by Waddell [1934] that the antirachitic vitamin from animal sources is not identical with the irradiation product of ergosterol, and that the pro-vitamin present in specimens of cholesterol gives on irradiation a product of greater antirachitic power than that derived from ergosterol. Furthermore, Windaus [1935] has shown conclusively that some sterols with the same system of conjugated double bonds as ergosterol, but differing in the constitution of the side chain, have properties very similar to those of ergosterol and give rise to irradiation products possessing antirachitic activity.

The identities of compounds of this nature cannot be determined with certainty by a comparison of melting-points alone, and the melting-points of mixtures of such compounds do not necessarily show a depression. Again, the absorption spectrum of 22:23-dihydroergosterol has been found by Windaus [1935] to be identical with that of ergosterol itself.

The possibility still exists therefore that the substance in the cocksfoot sterol may be not ergosterol itself, but some very closely related sterol, differing perhaps only in the constitution of the side-chain. Since however the sterol from cocksfoot gives a pinacol and its acetate which both correspond exactly in melting-point with those derived from ergosterol, and since the absorption spectra are the same, the conclusion that the substance is ergosterol appears to be justified.

Experimental.

Preparation of the light petroleum extract of cocksfoot. Fresh cocksfoot was dried in the experimental grass drying plant of the Imperial Chemical Industries at Jealott's Hill. Under these conditions, the grass was dried in about 8 min. without scorching, and no breakdown of the carotene occurred. 32·7 kg. of the dried grass were extracted with light petroleum by British Drug Houses and gave a dark yellow-green extract containing 797 g. of solids, as found by the evaporation of an aliquot portion. This was equivalent to 2·44 % of the dry weight of the grass.

Separation of wax and phosphatide. The extract was concentrated to a thick syrup and poured into 7 l. of boiling acetone to precipitate the wax and phosphatide. After cooling to 7° the solution was filtered at the pump and the precipitate taken up in 1 l. of warm ether and again precipitated with 2 vols. of acetone. The acetone solutions containing the glycerides, unsaponifiable matter and pigments were combined and evaporated under reduced pressure and the residue was dissolved in light petroleum.

Colorimetric estimation of the pigments in the solution showed the presence of 10·7 g. of carotene equivalent to 0·033 % of the dry grass and 7 g. of xanthophyll equivalent to 0·021 % of the dry grass, the comparatively small amount of xanthophyll being due to its low solubility in the light petroleum used for the extraction.

Separation of carotene. The light petroleum solution was diluted with the same solvent to a total volume of 7 l. and the solution divided into three equal parts. Each portion was shaken with successive quantities of 92 % methanol which had previously been saturated with light petroleum, using in all 12 l. of methanol. A carotene solution in petroleum and a xanthophyll solution in methanol were thus obtained, and at the same time the carotene solution had been freed from a considerable part of the chlorophyll and of the general unsaponifiable material, which had passed over into the methanol. The greenish brown petroleum solution was divided into three equal parts. The first was shaken mechanically for half an hour with 200 ml. of a saturated solution of potassium hydroxide in methanol, the light petroleum solution decanted and shaken with another 200 ml. of alkali. The second alkaline solution was used for the first extraction of the next portion of the light petroleum solution and the process continued, so that eventually each portion of the light petroleum had been shaken with three successive amounts of alkali. The petroleum solutions were then combined and concentrated under reduced pressure to a thick syrup which was taken up in ether. The ethereal solution was then washed with water and dried over sodium sulphate.

From this point onwards all manipulations and crystallisations were carried out as far as possible in an atmosphere of nitrogen or carbon dioxide, and the solutions were kept in the dark. The deep red ethereal solution was concentrated to a syrup under reduced pressure and diluted with absolute alcohol to a total volume of 300 ml. In a short time carotene began to separate in small glistening crystals. These were collected at the pump and further crops were obtained by the addition of absolute alcohol to the mother-liquors. The last fractions of carotene were contaminated by small quantities of wax and sterol, which were removed by washing with warm alcohol and ether. The mother-liquors were concentrated and again treated with alcohol, when further small quantities of carotene were obtained. The crude carotene, which still contained a small amount of colourless impurities, amounted to 7 g. and colorimetric estimation showed that the mother-liquors still contained 2·7 g. of carotene in solution.

The carotene was dissolved in 1400 ml. of warm benzene and a few ml. of methanol added. On cooling, 4 g. carotene crystallised out in the form of large red crystals with a green lustre, which were collected by filtration and kept in an atmosphere of nitrogen in the dark.

The carotene had M.P. 182° (corr.), and I have to thank Mr Gillam for examining the material spectrographically. He found that the intensity of its absorption was about 10 % higher than that of the best specimen which he had examined previously and that it gave a value of $E^{1\%}_{1\,\mathrm{cm.}}$ (463 $m\mu$) = 2200 in chloroform solution. In light petroleum (B.P. 60–80°) the corresponding value was 2500 [Gillam, 1935]. This intensity of absorption is similar to or slightly less than that of Kuhn's β-carotene [Smakula, 1934; Kuhn and Lederer, 1931, 2].

Recently Ferguson [1935] has used a specimen of the same carotene to construct curves for use in the colorimetric estimation of carotene. The colour was compared against the yellow units of the Lovibond tintometer (B.D.H. Pattern) and against the usual potassium dichromate solution.

All the mother-liquors from the carotene solutions were combined, evaporated and found to contain 66 g. of a red gummy material. When this was stirred with a small quantity of 95 % alcohol and kept in the refrigerator, small quantities of sterol separated. After removal of the sterol, the solution was again concentrated and taken up in a small quantity of light petroleum. Further amounts of

crystalline sterol were obtained, giving in all 7 g. of crude material. The liquid residue still contained 5·8 g. of sterol as estimated by digitonin.

Xanthophyll. The methanol solutions containing the xanthophyll were combined and concentrated *in vacuo* to about 8 l. This solution was saponified in portions of 2 l. by boiling under a reflux condenser with 100 g. KOH for 1·5 hours. The methanol was distilled off *in vacuo* and the combined residues (1500 ml.) diluted with water and extracted with ether. The ethereal solution (6 l.) was washed with water, dried over sodium sulphate and concentrated to 300 ml. On pouring this solution into 2 l. of light petroleum, 2 g. of xanthophyll separated and were collected at the pump. The mother-liquor was concentrated to a syrup (44 g.) and on treatment with light petroleum (100 ml.) and absolute alcohol (300 ml.) gave 10 g. of crystalline sterol. The residual solution was partitioned between light petroleum and 90 % methanol, and from the methanol solution, which contained the greater part of the original red colour, small quantities of xanthophyll were obtained on concentration and treatment with light petroleum as before. The mother-liquors were estimated to contain 2 g. of xanthophyll and 2·8 g. of sterol.

The xanthophyll (2·5 g.) crystallised from benzene in dark red crystals, M.P. 174°. On further recrystallisation from chloroform-methanol it melted at 182°. The material appears to be the usual mixture of xanthophylls and to consist largely of lutein. Although the crystalline fractions gave no colour in ethereal solution with 25 % HCl, the mother-liquors gave the characteristic blue colour given by violaxanthin, which has been shown to be present in the great majority of leaf xanthophylls by Kuhn *et al.* [1931]. Analysis of the crystals, M.P. 182° (uncorr.), corresponded with that of dihydroxyxanthophyll. (Found: C, 83·9; H, 10·0 %. $C_{40}H_{56}O_2$ requires C, 84·4; H, 9·93 %. $C_{40}H_{56}O_4$ requires C, 82·08; H, 9·40 %.)

Sterols. The several fractions of crude sterol were combined (19·5 g.), dissolved in 95 % alcohol and filtered from a small quantity of carotene. The sterol was recovered from the solution and recrystallised from acetone, methanol and light petroleum. Finally 12·85 g. of sterol were obtained, M.P. 138–139° (uncorr.) and $[\alpha]_D = -36°$.

Isolation of ergopinacol. The sterol (10 g.) was dissolved in a mixture of absolute alcohol (350 ml.) and benzene (50 ml.) and eosin (1 g.) added. The solution was boiled for 5 min. to expel dissolved oxygen, stoppered and after cooling was exposed overnight to the light from a 150-watt electric bulb. On cooling to room temperature the ergopinacol separated in characteristic films of small needle-shaped crystals, which were collected and recrystallised from pyridine-alcohol. The ergopinacol was obtained as fine needles and weighed 30 mg. When melted side by side with an authentic specimen, both substances melted at 201°. The acetate, first obtained by H. H. Inhoffen in Göttingen (unpublished), was prepared by heating the pinacol with a few ml. of pyridine and acetic anhydride for half an hour on a boiling water-bath. On adding a trace of water and allowing the solution to cool, the acetate crystallised and was collected. After recrystallisation from a mixture of chloroform and alcohol the substance formed colourless needles, M.P. 204°. A specimen prepared from the authentic ergopinacol melted at 205°, while a mixture of the two melted at 204°. (Found: C, 82·3; H, 10·4 %. $C_{60}H_{90}O_4$ requires C, 82·0; H, 10·2 %.) As the melting-points of the pinacols and derivatives are dependent on the rate of heating, all specimens for comparison were melted side by side; the recorded values are consequently uncorrected.

SUMMARY.

The light petroleum extract of a common forage grass, cocksfoot, has been investigated.

From the unsaponifiable fraction carotene, xanthophyll and sterols have been isolated.

The carotene appeared to consist entirely of the β-isomeride and has been isolated in a state of purity. The xanthophyll, which was a mixture, has not been further investigated.

From the sterol fraction, which consisted chiefly of sitosterol, ergosterol has been isolated as the pinacol in an amount corresponding to 0·4 % of the total sterol. The presence of approximately 1 % of ergosterol had previously been determined spectrographically.

The problem of the identification of such substances is discussed.

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