147. *The Ether-soluble Constituents of Sarsaparilla Root. Part I.*

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The nature of the constituents of sarsaparilla root has become, on biogenetic grounds, a matter of considerable interest in view of the recent advances that have been made in the chemistry of its two principal sapogenins, sarsasapogenin and smilagenin (van der Haar, *Rec. trav. chim.*, 1929, **48**, 726; Jacobs and Simpson, *J. Biol. Chem.*, 1934, **105**, 501; *J. Amer. Chem. Soc.*, 1934, **56**, 1424; Simpson and Jacobs, *J. Biol. Chem.*, 1935, **109**, 573; **110**, 565; Askew, Farmer, and Kon, J., 1936, 1399). The literature is seemingly devoid of information on this subject, with the notable exception of a paper by Power and Salway (J., 1914, **105**, 201), who carried out a qualitative investigation of the root and claimed to have isolated as the principal lipoids sitosterol, stigmasterol, cetyl alcohol, and a mixture of acids. The identification of these products was however based on somewhat meagre evidence; their characterisation of the sterols, in particular, must now be regarded as inadequate in the light of recent developments in phytosterol chemistry, for no comparison with authentic derivatives appears to have been made. Thus the evidence for the presence of stigmasterol (isolation of a tetrabromoacetate) does not exclude the possible presence of brassicasterol, which forms a tetrabromoacetate of similar melting point (Windaus and Welsch, *Ber.*, 1909, **42**, 612). The presence of sitosterol (also incompletely characterised) has likewise been observed by van der Haar (*loc. cit.*).

In the hope of placing these earlier findings on an unequivocal basis we have commenced a systematic examination of the ether-soluble (*i.e.*, non-saponaceous) constituents of the root. The results, now described, of a preliminary semi-quantitative survey of certain

fractions of the material show in some respects noteworthy differences from those of Power and Salway (*loc. cit.*).

The ether-soluble material was first separated into several main fractions by means of the scheme shown in the experimental part. The bulk of the sterols was found in fraction D, from which they were readily isolated by saponification and benzoylation of the non-saponifiable material, crystallisation of the mixed benzoates yielding a product of constant m. p. 144—145°. The materials obtained by hydrolysis and subsequent acetylation of this product had physical constants approximating to those of the sitosterol and sitosteryl acetate of Power and Salway, but consisted of a complex mixture of sterols, of which a partial separation was effected as follows.

Bromination of the mixed acetates by the method of Windaus and Hauth (*Ber.*, 1906, **39**, 4378) furnished a considerable proportion of a sparingly soluble bromoacetate, m. p. 210°; this was debrominated, hydrolysed, and converted into the corresponding *anisate*, m. p. 174°, and p-*nitrobenzoate*, m. p. 203°. Through the kindness of Professor Windaus, to whom we are greatly indebted for the supply of a sample of stigmasterol, we have been able to prepare authentic derivatives of the latter for comparison with those of the sarsaparilla sterol. The identity of this sterol with stigmasterol has by this means been conclusively established.

The filtrate from the stigmasteryl tetrabromoacetate was debrominated and saponified, and the physical constants of the product were found to resemble those of an ordinary sitosterol mixture. In view of the difficulty of separating such a mixture (or the acetates) into chemical individuals by fractional crystallisation from alcohol (Anderson and co-workers, *J. Amer. Chem. Soc.*, 1926, **48**, 2976, 2987; Bonstedt, *Z. physiol. Chem.*, 1928, **176**, 269) systematic fractional crystallisation from *cyclo*hexane of the 3 : 5-dinitrobenzoates of the mixed sterols was adopted and two apparently pure compounds were thus isolated. The less soluble *ester*, m. p. 215—217°, gave analytical figures indicating the formula $C_{35}H_{56}O_6N_2$, corresponding to the formula $C_{29}H_{50}O$ for the free sterol, which had m. p. 143—144° (acetate, m. p. 127—128°). Since none of the isomeric sitosterols or their acetates has physical constants in agreement with those of this sterol or its acetate, we propose for it the name ε-sitosterol. It appears to be present only in minute amount.

The second apparently pure 3 : 5-dinitrobenzoate had m. p. 207—209°; the corresponding sterol (which, allowing for the necessarily incomplete separation, probably constitutes about 50% of the total sitosterol mixture) melted at 135° and yielded an acetate, m. p. 126—127°, and benzoate, m. p. 146°. The close agreement between the melting points and specific rotations of these derivatives (see experimental) and those of the β-sitosterol of wheat-germ oil (Ichiba, *Inst. Phys. Chem. Res., Tokyo*, 1935, **28**, 112) indicates that the two substances are identical.

Fraction A (see scheme) consisted largely of fats and waxes, which liquefied at room temperature, together with a mixture of paraffins. The latter were isolated by fractional crystallisation and appeared, from the results of analysis and molecular-weight determinations, to have the mean formula $C_{18}H_{38} \pm CH_2$. In view of the illuminating work of Chibnall, Piper, Pollard, Smith, and Williams (*Biochem. J.*, 1931, **25**, 2095) on the composition of apple-wax, the possibility of resolution into individual substances by fractional crystallisation of the sarsaparilla paraffins would appear remote. The latter substances appear to be of lower molecular weight than the apple paraffins, and it is by no means certain that they are straight-chain compounds, since they melt at appreciably higher temperatures than normal paraffins of similar molecular weight (compare Piper, Chibnall, Hopkins, Pollard, Smith, and Williams, *ibid.*, p. 2072).

After the paraffins had been removed as completely as possible from fraction A, the filtrates were saponified. The non-saponifiable material (fraction B) was a complex mixture, of which little separation has as yet been effected. By means of phthalic anhydride there were isolated (i) a paraffin fraction, m. p. 55—57°; (ii) a small quantity of sterols, apparently a mixture similar to that already described; and (iii) after benzoylation of the original material, an unidentified compound which we are provisionally designating as *substance X*. This compound gave analytical data suggestive of the formula $C_{25}H_{24}O_7$. It is apparently the dibenzoate of a dihydric phenol $C_{11}H_{14}O_3(OH)_2$, this partial formula being supported

by the results of quantitative saponification. Attempts to isolate the product of hydrolysis —other than benzoic acid—proved unsuccessful. Small amounts of this substance, which has m. p. 124—125°, were also isolated from fractions C and F. Filixic acid, which occurs in male ferns, forms a benzoate of similar melting point (Daccomo, *Ber.*, 1888, **21**, 2962), but in view of the highly controversial literature dealing with this acid (see, for instance, Boehm, *Annalen*, 1901, **318**, 253) the identity of the two substances can only be regarded as speculative.

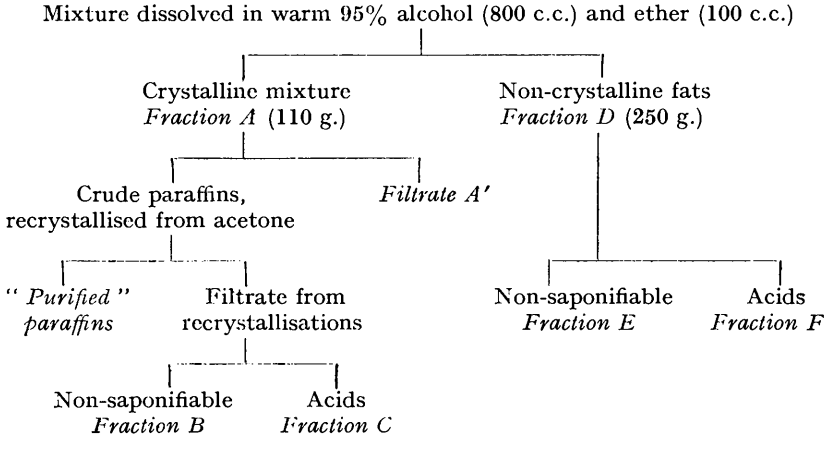
It is somewhat remarkable that the paraffins already mentioned have apparently not been encountered by previous workers, particularly as they appear to be highly characteristic components of the root, a fact which we have demonstrated by working with a different sample of root (commercial Mexican). The ether-soluble material was treated in the same way as that obtained from the Honduras root, the hydrocarbons being isolated as before from fraction A. The melting point (70°) was, however, considerably higher than that of the Honduras paraffins, and the analysis indicated contamination with non-paraffinoid substances. For the removal of the latter we employed the method of selective adsorption on aluminium oxide (Winterstein and Stein, *Z. physiol. Chem.*, 1933, **220**, 247, by this method quantitatively resolved a mixture of 99% of hentriacontane and 1% of pentadecyl ketone) and thus readily resolved the mixture of m. p. 70° into three distinct fractions. The main fraction (90% of the whole) was completely non-adsorbable and consisted of a paraffin or mixture of paraffins of m. p. 61—62° and mean formula $C_{23}H_{48}$. The second fraction (9%) was moderately strongly adsorbed; after purification by re-adsorption it had m. p. 82°. Analysis indicated the probable formula $C_{20}H_{42}O$, and its inertness towards acylating reagents and semicarbazide suggests that it is a tertiary alcohol. Owing to the small amount available it has been impossible further to characterise this substance. The third fraction, which was tenaciously held by the alumina, was a substance of probable formula $C_{29}H_{58}O_3$ and melting point 102—104°. It was present only in minute quantity (1% of the original mixture) and has not so far been characterised.

The investigation is being continued.

Experimental.

(Melting points uncorrected; specific rotations in chloroform.)

Honduras sarsaparilla root was exhausted by percolation with 95% alcohol, and the resultant solution concentrated under reduced pressure to a thick syrup. This was digested with a large volume of ether, and the ethereal solution decanted from the insoluble saponaceous gum, which was redigested with fresh ether. The united ethereal solutions were freed from solvent, and 1 kg. of the residue (corresponding to 43·5 kg. of root) was dissolved in ether (2 l.). After standing for 2—3 days at 10°, the solution was filtered from a little insoluble material and shaken first with 2*N*-sodium carbonate (5—6 times) and then with 6% aqueous sodium hydroxide until this was practically colourless. The neutral solution was then thoroughly washed with water, dried, and evaporated; the residual fatty material (360 g.) was resolved into fractions according to the scheme:



Fraction A.—This was dissolved in ethyl acetate to remove adhering oily material and three successive crops of crystalline material were removed (leaving the filtrate A′) which on fractionation from acetone by the triangle scheme yielded several fractions consisting of a mixture of paraffins (17 g.), m. p. 57—59° (Found for the main fraction : C, 85·2, 85·3; H, 15·3, 15·2; *M*, 211, 260, 236. $C_{18}H_{38}$ requires C, 84·9; H, 15·1%; *M*, 254).

The acetone filtrates from the most soluble fractions were united and evaporated, yielding an oil (44 g.), which was found to contain sterols. A portion was benzoylated in pyridine, and the product dissolved in acetone, in which the sterol benzoates (see below, fraction D) were sparingly soluble. No crystalline product could be obtained, indicating that the sterols were mostly, if not entirely, present in the form of waxes. The remainder of the oil (40 g.) was therefore refluxed for 2½ hours with alcoholic potassium hydroxide (500 c.c. of 6%), most of the solvent was then removed, and the residue largely diluted with water. Extraction with ether, followed by washing, drying, and evaporation of the extract, furnished a dark resinous material (fraction B, 17·5 g.).

Fraction B.—Separation of this mixture was attempted by the method of Chibnall *et al.* (*loc. cit.*); it was accordingly heated for 19 hours with phthalic anhydride (20 g.) at 120 ± 5°. The product was shaken with hot water, and the insoluble residue dissolved in ether. Only a negligible amount of material was extracted by shaking with warm 4% sodium carbonate solution, indicating the absence of primary aliphatic alcohols. The ethereal solution was then shaken with water, which removed the sparingly soluble sodium salts of the acid phthalates of a mixture of sterols. The latter were liberated by saponification and converted into the benzoates; the product appeared to be identical (m. p., mixed m. p., solubility) with the mixture of sterol benzoates, m. p. 143—144°, described under fraction D.

The residue (9·75 g.) from the dried and evaporated neutral ethereal solution was easily soluble in alcohol and was assumed from analogy with the experiments of Chibnall *et al.* (*loc. cit.*) to consist mainly of the sodium salts of the acid phthalates of secondary alcohols. It was therefore saponified under reflux for 18½ hours with 20% alcoholic potash (100 c.c.); after working up by the usual method a brown semi-crystalline product was obtained, which was esterified with benzoyl chloride in pyridine. The benzoylated material was isolated by the usual method (10·7 g.) and crystallised from acetone. The less soluble fractions consisted of a mixture of paraffins, m. p. 55—57° (Found : C, 85·4; H, 14·7; *M*, 314. $C_{23}H_{48}$ requires C, 85·1; H, 14·9%; *M*, 324). (In previous experiments direct crystallisation of fraction B, the phthalic anhydride treatment being omitted, yielded a paraffin mixture of m. p. 49—50°. Found : C, 85·2, 85·4; H, 15·2, 15·3%; *M*, 200, 263.)

The filtrate from the paraffins was evaporated, and the residue (6·9 g.) digested with warm ligroin; the crystalline material which separated was repeatedly crystallised from acetone–ligroin, ether–ligroin, and finally benzene, from which *substance X* separated in needles, m. p. 124·5—125° (Found : C, 69·0; H, 5·6. $C_{25}H_{24}O_7$ requires C, 68·8; H, 5·5%). The substance was recovered unchanged after refluxing with *N*/10-potassium hydroxide, but was saponified completely by refluxing for 8 hours with 5% alcoholic potassium hydroxide; with methyl-red as indicator, 69·7 mg. required 34·2 mg. of potassium hydroxide [$C_{11}H_{14}O_3(O{\cdot}CO{\cdot}C_6H_5)_2$ requires 35·8 mg. for 4 equivs.]. The saponified solution after titration was again made alkaline, distilled in steam, extracted with ether, and acidified. The benzoic acid was extracted with ether and purified by sublimation [yield, 36·5 mg. Calc. for $C_{11}H_{14}O_3(O{\cdot}CO{\cdot}C_6H_5)_2$, 39 mg.]. It was identified by m. p. and mixed m. p.; a mixture with the original substance melted at 80°.

The first ligroin filtrate from substance X yielded a small amount of the mixture of sterol benzoates, m. p. 143°.

Fraction C.—After acidification of the alkaline solution the acids were removed by threefold extraction with ether. The aqueous solution which remained was then re-extracted with ether for 3 days in a continuous-extraction apparatus. Evaporation of the dried extract left an oily residue, which gave positive colour reactions for glycerol; it was treated with benzoyl chloride in pyridine, but the only product isolated was a further quantity of substance X, m. p. 122—123° (from ether–ligroin) (Found : C, 68·5; H, 5·5%).

Filtrate A′.—This (43 g.) was saponified, and the non-saponifiable fraction benzoylated in the usual manner; a small quantity of the mixture of sterol benzoates was obtained as the sole crystalline product.

Fraction D.—After removal of solvent this was refluxed for 5½ hours with potassium hydroxide in 80% alcohol (2 l. of 12%). The non-saponifiable material (fraction E, 90 g.) was isolated by the usual method and benzoylated in pyridine. The product was treated with acetone (100 c.c.) and the crude crystalline material which separated was recrystallised from acetone

(which removed considerable amounts of easily soluble, crystalline substances) until it had m. p. 143—144° (4·8 g.).

Isolation of stigmasterol. The mixture of sterol benzoates was combined with the product obtained by similar treatment of a further kg. of starting material, and the whole (10·7 g.) was refluxed with alcoholic potash (5%). The free sterols (8·1 g., m. p. 135° from alcohol) were refluxed with acetic anhydride and the mixture of acetates (m. p. 128°) in ether (125 c.c.) was brominated by the method of Windaus and Hauth (*loc. cit.*). The product (2·25 g.) which separated on standing (m. p. 210—211°; crystallised from chloroform–alcohol) was refluxed for 1 hour with zinc dust (3 g.) and glacial acetic acid (55 c.c.). The product obtained by precipitation with water was recrystallised from alcohol and separated in glistening laminæ, m. p. 140—141° either alone or on admixture with authentic stigmasteryl acetate.

Saponification of the above acetate with alcoholic potash gave the free sterol, which formed plates from alcohol, m. p. 170°, $[\alpha]_D^{17°}$ − 45·8° (c = 1·26, l = 1), and gave no depression in m. p. when mixed with authentic stigmasterol of m. p. 171° and $[\alpha]_D^{17°}$ − 46·2° (c = 1·36, l = 1).

Stigmasteryl p-*nitrobenzoate.* This was prepared by heating the sterol (0·1 g.) with *p*-nitrobenzoyl chloride (0·2 g.) in pyridine (3 c.c.) on the water-bath for 3 hours; it separated from ligroin in silky needles which melted at 203° to a liquid of characteristic bluish-green fluorescence, and had $[\alpha]_D^{17°}$ − 13·4° (c = 1·835, l = 1). The ester gave no depression in m. p. when mixed with a sample prepared from authentic stigmasterol, m. p. 203°, $[\alpha]_D^{17°}$ − 13·3° (c = 0·58, l = 1) (Found: C, 76·9; H, 8·8; N, 2·7. $C_{36}H_{51}O_4N$ requires C, 77·1; H, 9·15; N, 2·5%).

Stigmasteryl anisate. This was prepared in a similar manner to the *p*-nitrobenzoate and separated from chloroform–methyl alcohol in plates, m. p. 173·5—174·5° to a fluorescent liquid; $[\alpha]_D^{17°}$ − 14·3° (c = 1·212, l = 1). Authentic stigmasteryl anisate had the same m. p. and $[\alpha]_D^{17°}$ − 15·1° (c = 1·017, l = 1), and a mixture of the two samples gave no depression in m. p. (Found: C, 81·7; H, 9·8. $C_{37}H_{54}O_3$ requires C, 81·3; H, 10·0%).

Fractionation of sitosterol mixture. The filtrate from the stigmasteryl tetrabromoacetate was debrominated by means of sodium amalgam and finally with zinc dust and acetic acid. Saponification of the halogen-free product was effected in the usual manner, 3·84 g. of a crystalline mixture of sterols being obtained; this was thoroughly dried and esterified with 3 : 5-dinitrobenzoyl chloride in pyridine (35 c.c.), the product being decomposed with ice and sulphuric acid and extracted with ether. The solution was washed with 1% aqueous sodium hydroxide and water, dried, and evaporated. A solution of the residue in benzene–alcohol yielded 4·4 g. of crystalline material, m. p. approximately 196°, which was fractionated from *cyclo*hexane by the triangle method, specific rotations being determined after every five crystallisations. The rotation of the least soluble fraction attained the constant value $[\alpha]_D^{17°}$ − 10° (c = 1·025, l = 1) after fifteen crystallisations (m. p. 210—211°), but the m. p. still rose slowly, becoming constant at 215—217° after twenty-four crystallisations (Found: C, 71·2; H, 8·5. $C_{36}H_{52}O_6N_2$ requires C, 71·0; H, 8·6%). Hydrolysis of this *ester* yielded the free ε-sitosterol, m. p. 143—144°, $[\alpha]_D^{17°}$ − 38·7° (c = 0·35, l = 1); the corresponding acetate separated from alcohol in plates, m. p. 127—128°, $[\alpha]_D^{17°}$ − 44·7° (c = 0·645, l = 1).

From the more soluble dinitrobenzoate fractions a product was obtained having m. p. 207—209° and $[\alpha]_D^{17°}$ − 21·7° (c = 1·20, l = 1), these values being unchanged by further crystallisation. The sterol, obtained by hydrolysis of this ester with 6% alcoholic potash, separated from alcohol in plates, m. p. 135—135·5°, $[\alpha]_D^{17°}$ − 34·2° (c = 1·96, l = 1). The acetate, prepared in the usual manner, melted at 126—127° (124—125° when mixed with ε-sitosteryl acetate) and had $[\alpha]_D^{17°}$ − 34·7° (c = 1·105, l = 1); the corresponding benzoate (plates from acetone) had m. p. 145—146° and $[\alpha]_D^{17°}$ − 14·2° (c = 0·94, l = 1). The values recorded by Ichiba (*loc. cit.*) for derivatives of β-sitosterol are m. p. 136—137° and $[\alpha]_D$ − 31·5° for the free sterol, 122—123° and − 36·7° for the acetate, and 146—147° and − 12·3° for the benzoate.

Fraction F.—This was treated as described for fraction C, a small amount of substance X being isolated from the water-soluble material after benzoylation (m. p. 123°; crystallised from ether–ligroin).

Paraffin Fraction from Mexican Sarsaparilla Root.—The root was extracted and the saponins and free acids were removed as already described. The neutral fatty material (400 g.) was dissolved in alcohol–ether (8 : 1, 900 c.c.) and the crystalline product (47 g.) which separated was refluxed for 3 hours with 10% alcoholic potash (500 c.c.). The non-saponifiable fraction (24 g.), isolated by the usual method, was dissolved in warm acetone; on cooling, 2·25 g. of crystalline material separated, m. p. 70—70·5° after recrystallisation (Found: C, 85·8; H, 14·0%; M, 347). A solution of this mixture (1·55 g.) in chloroform (100 c.c.), light petroleum

(100 c.c.), and benzene (50 c.c.) was filtered through a column of alumina (Merck's "standardisiert nach Brockmann"). The filtrate contained a paraffin fraction, m. p. 61—62° after crystallisation from acetone (Found: C, 85·4; H, 14·7; *M*, 318. $C_{23}H_{48}$ requires C, 85·1; H, 14·9%; *M*, 324).

The chromatogram was developed with chloroform–benzene (75 c.c., 1 : 1); the material in the filtrate had m. p. 76—79° and was clearly a mixture. The column was then divided into three sections of equal length, each of which was eluted with light petroleum–methyl alcohol. The middle and bottom zones gave products of identical m. p. 80—81°, which were combined and recrystallised from acetone without change in the m. p. (Found: C, 82·6; H, 14·1%; *M*, 386). This substance was readsorbed on alumina; the filtrate both before and after development contained only traces of material, the bulk of the *compound* being recovered by elutriation of the middle section of the column. It crystallised from acetone in small plates, m. p. 82—82·5°. It was recovered unchanged after refluxing both with semicarbazide acetate in alcohol and with acetic anhydride, and after heating with 3 : 5-dinitrobenzoyl chloride in pyridine (Found: C, 80·2; H, 13·7. $C_{30}H_{42}O$ requires C, 80·4; H, 14·2%).

The elutriate from the top section of the first adsorption-column crystallised from acetone in small nodules, m. p. 102—104° (Found: C, 76·3; H, 12·6; *M*, 514. $C_{29}H_{58}O_3$ requires C, 76·6; H, 12·9%; *M*, 454). The *compound* gives no coloration with tetranitromethane.

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