A THESIS

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BY

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HISTORICAL.

The naturally occurring secondary alcohols, known as the sterols, are classified biologically as zoosterols, phytosterols, and mycosterols, according to the source, animal, vegetable, or fungoid, from which they are obtained. The discovery that irradiation with short wave-length ultra-violet light confers powerful anti-rachitic properties on ergosterol, the principal member of the mycosterol sub-division, has given a great impetus to the study of these compounds and of their derivatives. Mycosterols are mainly derived from the non-saponifiable matter in the fats of yeast and of ergot of rye, both of which contain complex mixtures of the individuals of this group. The separation of these mixtures has frequently been attempted, and the isolation of many new sterols has been claimed, but the views of the various investigators are in such conflict that an independent and complete examination has become necessary.

Tanret (1) was the first to obtain definite results from an investigation into the composition of the non-saponifiable matter in the fat of ergot of rye. By repeated alcohol and ether extractions of the ergot and by saponifying the extract, he obtained an oily mass containing small crystals. After many crystall-

isations from alcohol and ether these crystals were obtained pure, melting at 156° and having $[\alpha]_D$ -114°. Tanret proved this compound to be a mono-hydric alcohol and prepared the acetate, formate and butyrate. The free alcohol gave the typical cholesterol colour reactions with nitric acid and ferric chloride, but differed from cholesterol, however, in giving a modified colour reaction with concentrated sulphuric acid and chloroform. Tanret, while recognising the essential differences between this substance and cholesterol, concluded that it was a member of the same group and named it ergosterol.

Working on the non-saponifiable matter in different cultured fungi, Gerard (2) separated the sterols by a method similar to that of Tanret but repeatedly crystallised the benzoates as a final purification. He obtained two sterols, one from Penicillium glaucum, of melting point 135° and $[\alpha]_D$ -143.3°, and the other from Aethalium septicum of melting point 134.5° and $[\alpha]_D$ -28°. Later (3) the same author prepared a sterol in small yield from Mucor mucedo which appeared identical with Tanret's ergosterol, and also a sterol from the non-saponifiable matter in yeast fat of melting point 135-136° and $[\alpha]_D$ -105°. Hinsberg and Roos (4) repeated Gerard's

work on yeast fat and isolated, together with ergosterol, a second sterol having a melting point of 148-149°, but they carried out no further investigations. This latter observation was confirmed by Neville (5), who obtained the figure -75.54° for the specific rotation.

Using larger quantities of ergot Tanret (6) prepared crude ergosterol in bulk and subjected it to a fractional crystallisation from ether. He succeeded in obtaining two well defined fractions, the most insoluble being pure ergosterol melting at 165°, and having $[\alpha]_D$ -126°, and the more soluble being a sterol, fungisterol, of melting point 144° and $[\alpha]_D$ -22.4°, which yields an acetate of melting point 158.5°, having an $[\alpha]_D$ -15.9°. The three sterols isolated by Gerard and that isolated by Hinsberg and Roos appear to be mixtures of ergosterol and fungisterol.

The first definite and scientific investigation into the separation of the yeast sterol mixtures was that carried out by Smedley MacLean (7), who subjected large quantities of the non-saponifiable matter from brewer's yeast to an exhaustive fractionation from ether and acetone, and obtained, after the removal of the sparingly soluble ergosterol, a new sterol, zymosterol, having a melting point of 108-109° and possessing an

optical rotation of $+34.1^{\circ}$. Zymosterol acetate was prepared and has a melting point of 115°. This sterol, however, still contains ergosterol, estimated by the workers to be less than 5%. From the results of elementary analysis and the determination of the iodine number it was suggested that zymosterol is an isomeride of ergosterol possessing three ethenoid linkages, but this observation is of little value since the iodine numbers obtained for sterols are invariably high owing to the entrance of iodine into the molecule by substitution. The same investigator was unsuccessful in her attempts to obtain crystalline compounds on treating zymosterol with bromine in glacial acetic acid solution. Finally (8) it was definitely shown that irradiated zymosterol possessed no antirachitic action other than that due to the presence of minute traces of ergosterol.

In their search for an absolutely pure specimen of ergosterol for use in accurate biological and spectroscopical work, Bills and Honeywell (9) fractionated crude yeast ergosterol from a benzene-alcohol solvent, and obtained by this means two other sterols in addition to pure ergosterol. They stated that both cerevisterol, m.p. 240°, $[\alpha]_D$ -49°, and a sterol m.p. 80°, $[\alpha]_D$ $+7^{\circ}$, which

they suggested may be identical with zymosterol, were less soluble in the majority of common solvents than ergosterol; in the latter case the observation is entirely contrary to the results obtained by all other workers. An attempt was made to show that the melting point of ergosterol is of no value as an indication of purity, but that it varies from 166° to 183° according to the degree of hydration of the sample.

Häussler and Brauchli (10), endeavouring to determine whether or no the Tortelli-Jaffe reaction was specific for ergosterol, required a specimen of pure zymosterol. They used both chemical and physical methods in its final purification and found all specimens to possess a uniform specific rotation of +44°. The workers did not consider, however, that they had definitely proved zymosterol sensitive to that reaction, suggesting that the very weak colour produced may still have been due to the presence of ergosterol or its less reactive isomeride iso-ergosterol.

Until this time zymosterol had only been prepared from the yeast sterol mixtures, and Penau and Tanret (11) attempted to establish the presence of zymosterol among the sterols of ergot of rye. A crude sterol mixture from that source was fractionated from ether-alcohol

solvent, and by this means was separated into two fractions, ergosterol and zymosterol. The physical constants given by Penau and Tanret for their zymosterol are in remarkable agreement with those published by Smedley Maclean, and a determination of the number of ethenoid linkages by the iodine number method also confirms the latter author. The results of these workers, however, cannot be compared when it is realized that while Smedley Maclean obtained analytical figures corresponding to an empirical formula $C_{27}H_{42}O$, Penau and Tanret obtained figures indicative of a formula $C_{27}H_{42}O_2.H_2O$ containing two hydroxyl groups, a supposition which is supported by the results of the determination of the number of acetyl groups on the acetate.

In carrying out an extensive research on the principle sterols occurring in various different botanical fungi, Ikequchi (12) isolated a sterol common to all, to which he gave the name mycosterol. This sterol melted at 159-160° and possessed a specific rotation of -129°. These figures are in close agreement with those generally accepted as the characteristic constants of ergosterol, but it was observed that the sterol was not acted upon by bromine in glacial acetic acid solution and further that analysis of both the sterol and its acetate indicated an

empirical formula of $C_{30}H_{48}O_2$.

A more careful and detailed fractionation of the sterols of brewers' yeast was carried out by Smedley MacLean (13) in an endeavour to prepare a pure specimen of zymosterol. Yet another sterol was isolated in the process, of melting point 120-124° and $[\alpha]_{5461}$ +42 (Hg), yielding an acetate m.p. 135-137°. The zymosterol also obtained in this investigation was reported as possessing a melting point of 110-116° and $[\alpha]_{5461}$ +54, giving an acetate melting at 119-122°. It was stated that this specimen still contained a small proportion of ergosterol.

Hart and Heyl (14) repeated Tanret's work on the fractionation of the sterols of ergot of rye. They rejected the top fractions which contained the major portion of the ergosterol, and on recrystallising the residue from ethyl alcohol obtained a small amount of sterol m.p. 144-146°; a determination of the specific rotation of an impure specimen gave a figure -20°. The acetate melted at 156-157°, but the quantity isolated by these investigators was too small to permit of elementary analysis. A yet more soluble sterol was isolated from the filtrates; the acetate melted at 122-125° and on hydrolysis gave a sterol m.p. 120-125° having $[\alpha]_D$ -2°. This optical determination was also carried out on an

impure specimen.

In investigating the possibility of antirachitically activating mycosterols other than ergosterol, Rosenheim and Webster (15) endeavoured to isolate and purify Tanret's fungisterol. They used as a starting product the crude sterol mixture remaining after the removal of the larger portion of the ergosterol from ergot of rye. This mixture had a melting point of 107-110° and an $[\alpha]_{5461}$ -63°. The sterols were acetylated, and the acetates crystallised from acetic anhydride showed a melting point of 117-118° and an $[\alpha]_{5461}$ -50°. This product was fractionated from methyl alcohol; the fractions crystallising at 36° and 18° were rejected as still containing ergosterol. The solution was cooled to -5° and the crystals so obtained were again recrystallised from methyl alcohol. The final product possessed a melting point of 117-118° and an $[\alpha]_{5461}$ -43°. That this was not cholesteryl acetate was shown by the depression observed in a mixed melting point of the two. The authors considered this acetate to be fungisteryl acetate, an observation which has no apparent foundation since the fungisteryl acetate first isolated by Tanret, and later confirmed by Hart and Heyl, has a melting point nearly 40° higher. It was found from biological tests that this sterol possessed no active prop-

erties on irradiation other than those which could be attributed to traces of ergosterol as impurity.

A similar observation was reported by the same authors concerning an almost identical sterol isolated in the laboratories of Messrs. Burrough, Welcome & Co. Two further sterols isolated in the laboratories of this firm were also tested for antirachitic properties; one melting at 172° and having $[\alpha]_{5461}$ + 60° giving a benzoate melting at 173° and $[\alpha]_{5461}$ +72° was found to be completely inactive, and the second, of melting point 260° (approximately) and $[\alpha]_{5461}$ -90°, was observed to be only active to the extent of its ergosterol content. No details as to the separation of these latter sterols are given, neither are they named. The sterol melting at 260° approximates in its physical constants to the cerevisterol isolated by Bills and Honeywell from yeast sterol mixtures, but that melting at 172° corresponds to no other yet isolated sterol.

The isolation of a known hydrogenated derivative of ergosterol from ergot of rye was first recorded by Heyl and Swoap (16), who claimed to have obtained α-dihydroergosterol from the ether-petrol ether filtrates after the removal of ergosterol, fungisterol, and the new sterol previously reported by Hart and Heyl (loc.cit.)

The melting points and rotations (sterol m.p. 172.5-175°, $[\alpha]_D$ -20.6°, and acetate m.p. 176-177°) of the free sterol and its acetate agree well with those published (Windaus and Brunken, (17) but the melting point of the benzoate is 43° lower than that of the benzoate prepared from an authentic specimen of α-dihydroergosterol.

From the foregoing short resume of the confused and contradictory results obtained by different workers in this field, it is obvious that little or no definite progress can be achieved until the method of attack is radically modified. This was first realized by Wieland and Asano (18), who found that there was a greater difference in the solubilities of the various steryl benzoates than of the sterols themselves. They therefore benzoylated a large quantity of the crude yeast sterol mixture and subjected the mixed benzoates to an exhaustive fractionation. These investigators succeeded by this method in isolating four sterols in addition to ergosterol. The melting points and rotations of the sterols, acetates and benzoates are shown in the following table.

	Sterol		Acetate.		Benzoate	
	M.p.	$[\alpha]_D$	M.p.	$[\alpha]_D$	M.p.	$[\alpha]_D$
Ergosterol	160-1°	+133.1°	-	-	168-170°	-70.5°
Neosterol	164-5°	-105°	173-4°	-	172-4°	-50.6°
Zymosterol	108-110°	+47.3°	104-6°	+33.5°	126-8°	+36.4°
Faecosterol	161-163°	+42.1°	159-61°	-	144-6°	+35.4°
Ascosterol	141-2°	+45°	-	-	130-1°	+37°

The impure ergosteryl benzoate was purified by recrystallisation from ethyl acetate. It crystallises from this solvent in characteristic fibrous needles, and on evaporation of the filtrates it was observed that thin leaves of a presumably new steryl benzoate crystallised together with needles of ergosteryl benzoate. After many recrystallisations from ethyl acetate the plates were obtained uncontaminated by needles, the melting point rising and remaining constant at 173-175°. On hydrolysis a sterol was obtained which was indistinguishable from ergosterol in its colour reactions with the Rosenheim reagent; with the antimony trichloride reagent and with the Tortelli-Jaffe reagent, and it was also observed that this sterol and ergosterol possessed identical absorption spectra. There was no depression to a mixed melting

point with ergosterol, but on rebenzoylation the benzoate melted unchanged at 173-175°, and was again obtained free from needles. On this evidence, although analytical figures indicate the presence of only two ethenoid linkages, it is suggested that neosterol is a stereo-isomeride of ergosterol, or that it differs from ergosterol in the position of an ethenoid linkage.

Of the remaining three sterols, ascosterol, and faecosterol were isolated in small yield by fractionating the benzoates of medium solubility from acetone. The more soluble faecosteryl benzoate was finally purified by repeated recrystallisation from methyl ethyl ketone, ethyl acetate,and dioxan until constant melting point was reached. On hydrolysis pure faecosterol was obtained, which gave a depression of 10° to a mixed melting point with ergosterol. Analytical figures indicate the presence of only one ethenoid linking, and it was therefore suggested that faecosterol is an isomeride of cholesterol. Ascosteryl benzoate was obtained at constant melting point by repeated recrystallisation from acetone, and it was observed in this operation that ascosteryl benzoate is dimorphous. The benzoate gives a depression of 6° to a mixed melting point with faecosteryl benzoate. It is suggested, again from results

of analysis, that ascosterol is also an isomeride of cholesterol.

The most soluble benzoate was found to be principally zymosterol benzoate, which was readily purified by recrystallisation from acetone, from which solvent it can be obtained in dimorphous modifications. The zymosterol prepared by these investigators is not identical with that isolated by Smedley MacLean (loc.cit.), there being a slight difference in the melting points of the free sterols but a difference of 15° in the melting points of the acetates.

Later, working on different crude starting products, but using the same method of separation, Wieland and Gough (19) isolated four new sterols. They did not,however, obtain any trace of the sterols reported previously, ascosterol, faecosterol, and neosterol. A table of the physical constants of the new sterols, their acetates and benzoates, is appended below.

	Sterol		Acetate		Benzoate	
	M.p.	$[\alpha]_D$	M.p.	$[\alpha]_D$	M.p.	$[\alpha]_D$
Episterol	135-6°	+6.2°	-	-	161-3°	+11.8°
Anasterol	157-9°	-8.1°	-	-	180-2°	-19.3°
Hyposterol	100-2°	+12.5°	-	-	119-121°	+19.1°
(un-named)	144-6°	-33.8°	-	-	158-160°	-4.4°

The starting material from which episterol was isolated was a resinous mixture of the more soluble sterols of yeast remaining after the commercial removal of ergosterol. It was extracted with methyl alcohol and the residue benzoylated; the benzoates were then fractionated from acetone. The greater part of the ergosteryl benzoate was removed, and the new steryl benzoate crystallised together with small quantities of ergosteryl benzoate and zymosteryl benzoate. On recrystallisation from acetone and ethyl acetate, episteryl benzoate was obtained pure, and melted constantly at 161-163°. From analytical figures it is suggested that episterol is an isomeride of zymosterol, containing two ethenoid linkages.

From similar residues under the same treatment, anasteryl benzoate was isolated and obtained at constant melting point after repeated recrystallisation from acetone. Again, from analytical data anasterol was stated to be an isomeride of zymosterol. Anasterol does not give a positive colour reaction with Rosenheim's reagent, and only a faint green ring with the Tortelli-Jaffe reagent.

The third sterol, hyposterol, was obtained as the p-nitro benzoate after the saponification of dark greasy

residues from which it was not possible, without the preliminary treatment, to isolate crystalline esters. From this observation it is suggested that hyposterol does not exist in the free state, but as an ester of an unknown acid which, it is also stated, gives the characteristic sterol colour reactions. The residues, after saponification, were treated with p-nitro benzoyl chloride in pyridine solution, and the crude p-nitro benzoate crystallised from acetone until constant melting point was reached. On hydrolysis hyposterol was obtained, which the workers compare in its reactions and stability, to dehydroergosterol (cf. Windaus and Linsert (20). The number of ethenoid linkages in the molecule is not definitely stated, but the analytical figures and the great instability of the sterol are accepted by the investigators as indicating that hyposterol is an isomeride of ergosterol. The discrepancies in the literature as to the physical constants, colour reactions, and stability of zymosterol preparations when exposed to the atmosphere are explained as due to the incomplete removal of hyposterol, which gives both the Tortelli-Jaffe reaction and the antimony trichloride reaction, and which rapidly decomposes in the free state even when kept in sealed tubes. Wieland and Asano succeeded in hydrogenating hyposterol, using platinum

oxide as a catalyst, but they do not give any absorption figures from which the number of reactive ethenoid linkages could be estimated. The hydrogenated product is stable and can be dehydrated without decomposition.

From a further benzoate fractionation a small quantity of a steryl benzoate, m.p. 158-160° was isolated, which, on hydrolysis, yielded a sterol of m.p. 144-146°. Although absorption spectra measurements carried out on this sterol indicate the presence of 10% of ergosterol, the benzoate obtained by rebenzoylation melted unchanged at 158-160°.

A method of separation, based more on the chemical properties of the sterols, was introduced by Heilbron and Sexton (21), who treated a crude yeast sterol mixture, partially freed from ergosterol and dissolved in anhydrous ether, with a 10% solution of bromine in dry glacial acetic acid. A precipitate of insoluble bromides was obtained which could be separated by fractional crystallisation. The most insoluble fraction, zymosterol dibromide, m.p. 168°, yielded, on debromination by refluxing with zinc dust in industrial alcohol, zymosterol, m.p. 108-110°, giving an acetate melting at 106-107°. The specific rotation of the sterol, +38.6°, is low in comparison with the figures published by other authors. On catalytic

hydrogenation α-dihydrozymosterol was obtained, which, being unsaturated as evidenced by the positive Tortelli-Jaffe and Liebermann-Burchard colour reactions, contains the inert double bond.

Reindel and Weickmann (22) carried out a detailed investigation into the constitution and properties of zymosterol, and used the following method as a means of separation.

The crude sterol mixture, containing ergosterol, was subjected to a preliminary purification by successively and repeatedly recrystallising from ligroin and methyl alcohol. The impure zymosterol so obtained possessed a specific rotation of +30-35^{o} which it was impossible to increase by this method alone. Further purification was effected by refluxing the impure zymosterol in alcohol with animal charcoal, the ergosterol, being oxidised by the occluded oxygen, can then be removed by one recrystallisation. The optical rotation rose and remained constant at +49.5^{o}, which is the highest value so far recorded for zymosterol. These authors followed the course of the purification by means of the gradual disappearance of the green colour given by impure specimens with the Tortelli-Jaffe reagent, it being claimed that zymosterol, possessing a specific rotation greater than +46^{o}, does

not give the Tortelli-Jaffe reaction, and it is also stated that pure zymosterol gives a pink colouration with the antimony trichloride reagent, observations which have not been supported by any other authors.

By means of the per-benzoic acid titration zymosterol was shown to possess two ethenoid linkages, one of which could be easily saturated by shaking the sterol with platinum in an atmosphere of hydrogen.

Zymosterol acetyl dibromide, m.p. 156-157°, was prepared by bromination of zymosterol acetate in the presence of ammonium acetate. In a further publication (23) the same authors used the acetyl dibromide as an intermediate in a more rapid and complete purification of zymosterol. Pure zymosterol acetyl dibromide, m.p. 169°, $[\alpha]_{5463}$ -9.7°, was hydrolysed by shaking with cold alcoholic caustic potash to the dibromide of m.p. 158° and $[\alpha]_{5463}$ +7.1°, which was debromominated by shaking in cold glacial acetic acid with oxide-free zinc dust. The zymosterol so obtained differs only from that previously prepared in being of a slightly higher optical rotation, and in giving a negative reaction with the antimony trichloride reagent. The dihydrozymosterol, previously reported, was obtained in larger quantities and was isomerised by dry hydrochloric acid gas to the β-compound,

which was not identified with any known fully saturated sterol.

The investigators do not definitely attribute any molecular formula to zymosterol, merely pointing out that the analytical figures agree best with the formula $C_{30}H_{50}O$.

A slight modification of Wieland's method of separation was used by Callow (24) in a fractionation of crude yeast ergosterol. Ergosteryl benzoate was fractionated from ethyl acetate, the temperature of the solution at crystallising point was maintained at 37^{o}. It was observed at this stage that ergosteryl benzoate is trimorphous, crystallising in fibrous needles, plates, or thin leaves, depending on the concentration and temperature of the solution. A high melting fraction was obtained which was repeatedly recrystallised from ethylene dichloride, but this process failed to remove all the ergosteryl benzoate, and an attempt was made to destroy the ergosterol by partial bromination; the crystals, however contained 0.25% ergosterol, which was finally removed by prolonged irradiation with short wave length ultra-violet light. The table appended shows the points of resemblance by which this investigator proves the high melting benzoate

to be α-dihydroergosteryl benzoate.

Author	Sterol		Benzoate		Acetate.	
	M.p.	[α]	M.p.	[α]	M.p.	[α]
Callow	172.5-4°	$[\alpha]^{5461}$ -23	192-5°	$[\alpha]^{5461}$ -13.6	180-1°	
Windaus and Brunken	173-4°	(D) -19.3°			180-1°	(D) -21
Heilbron and Webster (unpublished work)	177-8°	(D) -21.1	197-8°		182-3°	

Callow found that there was no depression in a mixed melting point of the sterol from yeast and an authentic specimen of α-dihydroergosterol, and a determination of the number of ethenoid linkages, by means of bromine in carbon tetrachloride, gave the figures 1,9: 1,8: 1,7.

No trace of neosterol as reported by Wieland (loc. cit) was found, it is suggested that neosterol is a eutectic mixture of ergosterol and α-dihydroergosterol, as mixtures of these two compounds can be prepared approximating very closely to the published properties of neosterol.

Physical Constants of Mycosterol and their Derivatives.

Sterol	Auther		Sterol		Acetate		Benzoate.	
			M.p.	[α]	M.p.	[α]	M.p.	[α]
Ergosterol $C_{26}H_{40}O_2.H_2O$	Tanret	E	154°	-114	169°	-80		
Ergosterol $C_{26}H_{44}O.H_2O$	Hinsberg	Y	159°					
Ergosterol $C_{27}H_{42}O.H_2O$	Tanret	E	165°	-126	180.5°	-91.8		
Ergosterol	Bills	Y	166-83°				168°	-177
"	Wieland	Y	160-1°	-133			168-70°	-70.5
"	Author	Y	163-4°	-130			167-70° 160-8	-68.4
Fungisterol $C_{25}H_{40}O.H_2O$	Tanret	E	144°	-22.4	158.5°	-15.9		
"	Hart	E	145-6°	-20	156-7°			
"	Rosenheim	E			117-8°	-43		
"	Burroughs Welcome	E			117-20°	-34		
Zymosterol $C_{27}H_{42}O$	Smedley-MacLean	Y	108-9°	+34.1	115°			
"	"	Y	110-6°	+54	119-			
" $C_{27}H_{42}O_2.H_2O$	Tanret	E	106-8°	+34	115	+20		

Sterol	Author		Sterol		Acetate		Benzoate	
			M.p.	[α]	M.p.	[α]	M.p.	[α]
Zymosterol	Haussler		105-7°	+44				
"	Wieland	Y	108-10°	+47.3	104-6°	+33.5	126.8°	+36.4
" $C_{27}H_{44}O$	Reindel	Y	107-10°	+49.2	102-4°	+49.2		
Zymosterol $C_{30}H_{50}O$	"	Y	"		"			
Zymosterol	Heilbron	Y	108-10°	+38.6	106-7°			
"	Bills	Y	80°	+7				
" $C_{87}H_{44}O$	Author	Y	110-11°	+48	123-4°	+35.7		
Cerevisterol	Bills	Y	240°	-49				
?	Burroughs Welcome	E	260°	-90				
Mycosterol $C_{30}H_{48}O_2$	Ikequchi	F	159-60°	-129				
α-Dihydro-ergosterol	Heyl	E	172.5°	-20.6	176-7°		153-5°	
"	Callow	Y	172-4°	-23	180-1°		192-5°	-13.6
"	Author	Y	177-8°	-27.5	184-5°		200-2°	-23.2
Neosterol	Wieland	Y	164-5°	-105	173-4°		172-4°	-50.6
"	Author	Y	167-9°	-62.5	178-80°		180-3°	-21.6
Faecosterol	Wieland	Y	161-3°	+42.1	159-61°		144-5°	+35.4

Sterol	Author		Sterol		Acetate		Benzoate	
			M.p.	[α]	M.p.	[α]	M.p.	[α]
Faecosterol	Author	Y	163-4°	+33.5	158-60°		144-5°	+28.2
Ascosterol	Wieland	Y	141-2°	+45			130-1°	+37
"	Author	Y	151°	+32.8	153-4°		134-5°	+29
Episterol	Wieland	Y	135-6°	+6.2			161-3°	+11.8
Anasterol	Wieland	Y	157-9°	-8.1			180-2°	-19.3
Hyposterol	Wieland	Y	100-2°	+12.5			119-21°	+19.1
?	Wieland	Y	144-6°	-33.8			158-60°	-4.4
?	Hinsberg	Y	148-9°					
?	Neville	Y	145-7°	-75.5				
?	Hart & Heyl	E	144-6°	-20	156-7°			
?	Smedley MacLean	Y	120-4°	+42	135-7			
?	Hart & Heyl	E	120-5°	-2	122-5°			
?	Burrough Welcome	E	172°	+60			173°	+72
?	Gerard	F	135°-	-1433				
?	"	F	134.5°	-28				
?	"	Y	135.6°	-105				

THEORETICAL

Part I.

This portion of the work on sterol mixtures was carried out on Messrs.Berk's "Sparingly soluble" sterols, a mixture of the more soluble sterols of yeast remaining after the removal of the greater part of the ergosterol. The crude sterol mixture gave a very pronounced colour reaction with the Rosenheim reagent, and its melting point was very undecided, lying between the limits 88-112°. The specific rotation, determined at 20° using the blue mercury line, was +15°. A spectroscopical determination of the ergosterol content indicated the presence of approximately 30%, but feeding tests were reported as being only "slightly positive".

An endeavour was made to isolate zymosterol by the precipitation of the insoluble bromine addition compound (Heilbron and Sexton, 21) without any preliminary purification. No precipitate was obtained, and the solid precipitated by pouring the acetic-ether solution into a large volume of water could not be obtained in crystalline form from any solvent. The failure of this method to yield crystalline products was undoubtedly due to the tarry substances produced by degradation of the ergosterol molecule, and perhaps of sterol molecules similar in stability to ergosterol, under the drastic bromine treatment. Probably the

presence of these tarry decomposition products and also the presence of hydrogen bromide, which was evolved in large quantities during the actual bromination, rendered the zymosterol dibromide itself unstable, since prolonged refluxing with charcoal failed to remove any colouring matter, nor did it enable crystalline compounds to be isolated.

With the failure of the bromine precipitation method, a fractionation of the steryl acetates was attempted. After an exhaustive fractionation,however, no definite individual acetates were isolated. It has been pointed out by Bills and Honeywell (9) that both sterols and their acetates readily form mixed crystals and eutectic mixtures, probably due to the large absorbing surfaces presented by such compounds crystallising in plates. The fractionation amply corroborates the observation of these authors, the melting points of the various crops ranging from 77-167°.

An endeavour was then made to repeat the method followed with success by Wieland and Asano (18) in their work on yeast sterol mixtures, but this also was proved useless. The purest positive-rotating fraction, the melting point of which remained constant on recrystallis-

ation from acetone and benzene-alcohol, was shown to contain on spectroscopic examination 33% of ergosterol.

The purely physical methods of separation were therefore abandoned, and a return was made to the bromine separation. Häussler and Brauchli (10) give four methods for the purification of zymosterol containing small quantities of ergosterol as follows :-

(1) A 1% solution of the mixture in alcohol is irradiated with short wave-length ultra-violet light, whereby the photo-labile ergosterol is destroyed and trans-formed to its isomeride iso-ergosterol, from which zymosterol can be purified by crystallisation.

(2) The ergosterol, being more unstable than zymosterol, is preferentially oxidised by refluxing with potassium permanganate in acetone solution.

(3) A chloroform solution of the mixture is allowed to stand several weeks; the ergosterol, which reacts with the chloroform, can be removed by one crystallisation.

(4) The mixture is repeatedly recrystallised from ether.

The method, refluxing with potassium permanganate in acetone solution was tried with success. The resulting sterol still gave the antimony trichloride reaction but to a greatly reduced degree. The products of bromination were much less contaminated with tarry matter and consid-

erably less hydrobromic acid was produced in the reaction. Since mineral acids are known to isomerise sterols, the addition of ammonium acetate (Reindel and Weickmann,(22) to the solution of bromine in glacial acetic acid, to act as an acid absorber, was the final modification of the original method.

The bromides were fractionated from acetone and chloroform-methyl alcohol, and three constant-melting fractions were obtained, m.p. 168°, 147°, and 138° respectively. Of these three, the least soluble fraction (m.p. 168°) is identical with the sterol dibromide reported by Heilbron and Sexton (21) and gave analytical figures in good agreement with the molecular formula $C_{27}H_{44}OBr_2$. Debromination was effected by refluxing the bromide in industrial alcohol with oxide-free zinc dust. The sterol appears to be slightly contaminated by the products of side reactions in this operation, and, by repeated recrystallisation of the acetate from ethyl acetate-methyl alcohol, was finally obtained in a pure state, m.p. 123-124°. The pure sterol obtained by hydrolysis of the acetate, melted at 110-111°, and gave negative reactions with the Rosenheim and antimony trichloride reagents, but a brilliant green ring with the modified Tortelli-Jaffe reagent, indicating the presence

of the inert double bond in the molecule.

On rebromination of this sterol, a bromide m.p. 168° was obtained. In the latter portion of this work a separation was effected by means of a fractionation of the crude steryl-p-nitrobenzoates without the previous intervention of the bromine treatment. From this fractionation a slightly impure sterol was obtained, m.p. 105-107°, acetate m.p. 119-120°, which on bromination yielded as main product an acetyl dibromide of m.p. 182° which on deacetylation yields a sterol di-bromide of m.p. 168°. From these two results it is apparent that the sterol generated by this method of debromination is identical in all respects with the naturally occurring sterol. The method of preparation and purification of the zymosterol in this work precludes any possibility of ergosterol being present as impurity, and therefore the physical constants given by the present author (sterol m.p. 110-111°, acetate m.p. 123-124°) are more accurate than those of Smedley MacLean (13) (sterol m.p. 110-116°, acetate m.p. 119-122°) given for a specimen of zymosterol which is stated to contain a small percentage of ergosterol.

The analytical figures obtained on micro combustion of the second constant melting fraction (m.p. 147°), indicated

that it was an addition compound of hydrogen bromide and a steryl acetate, and therefore an investigation was made into the products of the reaction between zymosterol and hydrogen bromide. The zymosterol was dissolved in dry ether and a solution of dry hydrogen bromide in acetic acid added at 0^{o}. The compound isolated contained no bromine but melted constant after two recrystallisations from methyl alcohol at 75^{o}, and yielded an acetate m.p. 73^{o}. By analogy with the known action of mineral acids on other sterols containing the inert double bond it can be assumed that this product is iso-zymosterol.

A similar attempt was made to isolate the hydrogen bromide addition compounds of zymosteryl acetate, and also that of the sterol and its acetate obtained from the second fraction of bromides. These experiments were unsuccessful and the negative results, while not conclusive, strongly indicate that zymosterol and the other sterol, if pure, do not form stable addition compounds with hydrobromic acid, and therefore the two more soluble bromine-containing fractions are not pure compounds but eutectic mixtures of zymosterol di-bromide and free sterols. This view receives experimental support from the fact that when the sterol obtained by

debromination of the 147° bromide is rebrominated, small quantities of the pure 168° dibromide and of the 138° bromine-containing body are isolated.

The conclusions which can be drawn from this portion of the work are :-

(1) Zymosterol forms a stable dibromide, m.p. 168°, which on debromination yields the free sterol, m.p. 110-111°, whose acetate melts at 123-124°.

(2) That the original sterol mixture contains unsaturated sterols inert to the additive and disruptive action of bromine.

(3) Zymosterol is isomerised by the action of mineral acids to iso- zymosterol of melting point 75°, and acetate of melting point 73°.

E X P E R I M E N T A L

Part I.

Separation by means of Bromine.

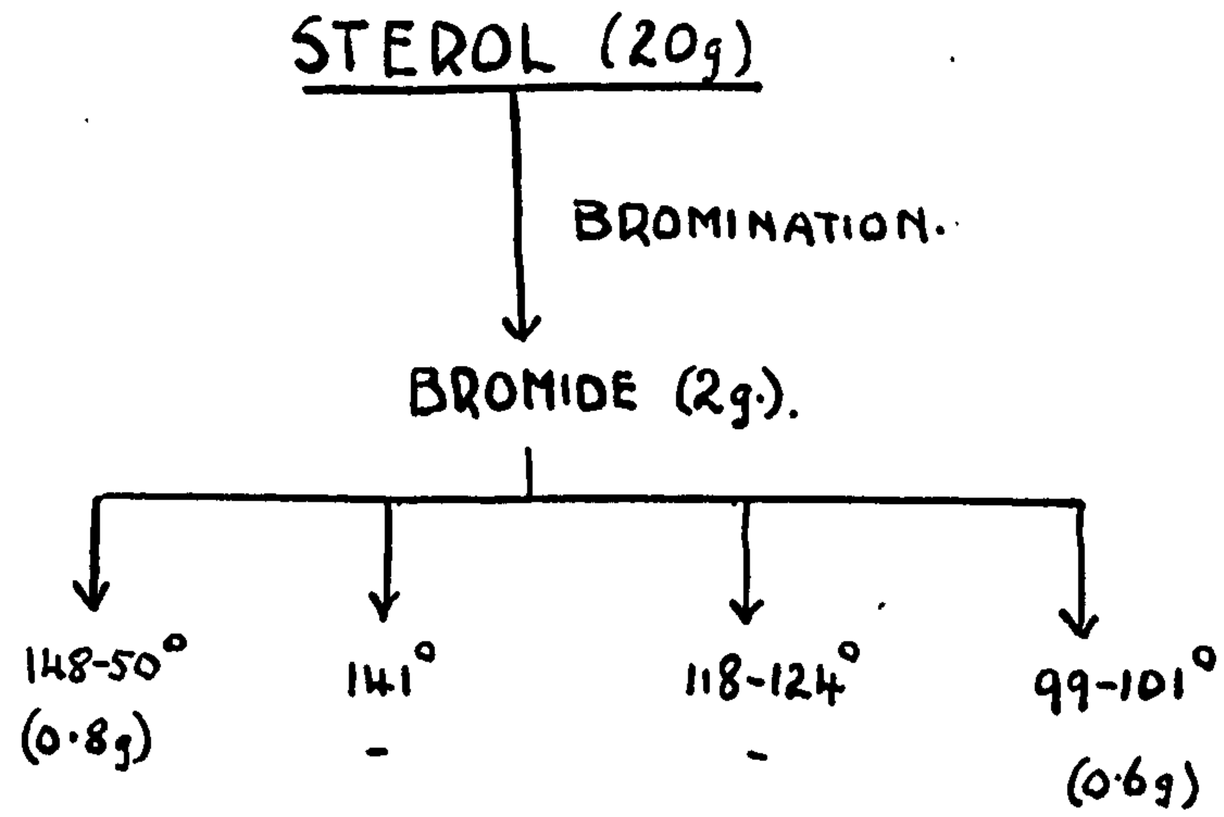
Bromination as a method of purifying zymosterol was first applied by Heilbron and Sexton (21), and was found to work successfully with their specimens of crude sterol. The method was strictly followed, but the presence of large quantities of sterols unstable to bromine was responsible for its failure.

Sterol (20 g.) was dissolved in sodium-dried ether (300 c.c.) and the solution cooled in ice. Bromine (12 g.) dissolved in dry glacial acetic acid (120 c.c.) was added. The solution became dark brown in colour, and large volumes of hydrogen bromide were evolved. There was no sign of a precipitate and the solution was poured into a large volume of water which precipitated quantities of a dark brown solid. This solid was dissolved successively in ether, ethyl alcohol, ethyl acetate, glacial acetic acid, ligroin and acetone, but could not be obtained in crystalline form from any of these solvents. Refluxing with charcoal in industrial alcohol had no effect on the colour.

The physical conditions were modified; the ether solution was cooled to -10^{o}, and the solid precipitated with methyl alcohol instead of water; the volume of ether was reduced as was also the volume of bromine

solution added. Partial success was achieved using the following method :-

Crude sterol (20 g.) was suspended in anhydrous ether (100 c.c.) and 10% bromine solution (80 c.c.) was added. A precipitate was obtained (2 g.) which, however, on crystallisation from chloroform-methyl alcohol, gave crops whose melting points ranged from 99-150°. The yield of high melting bromide was too small to be of practical value, and consequently this method had to be abandoned for one less destructive.

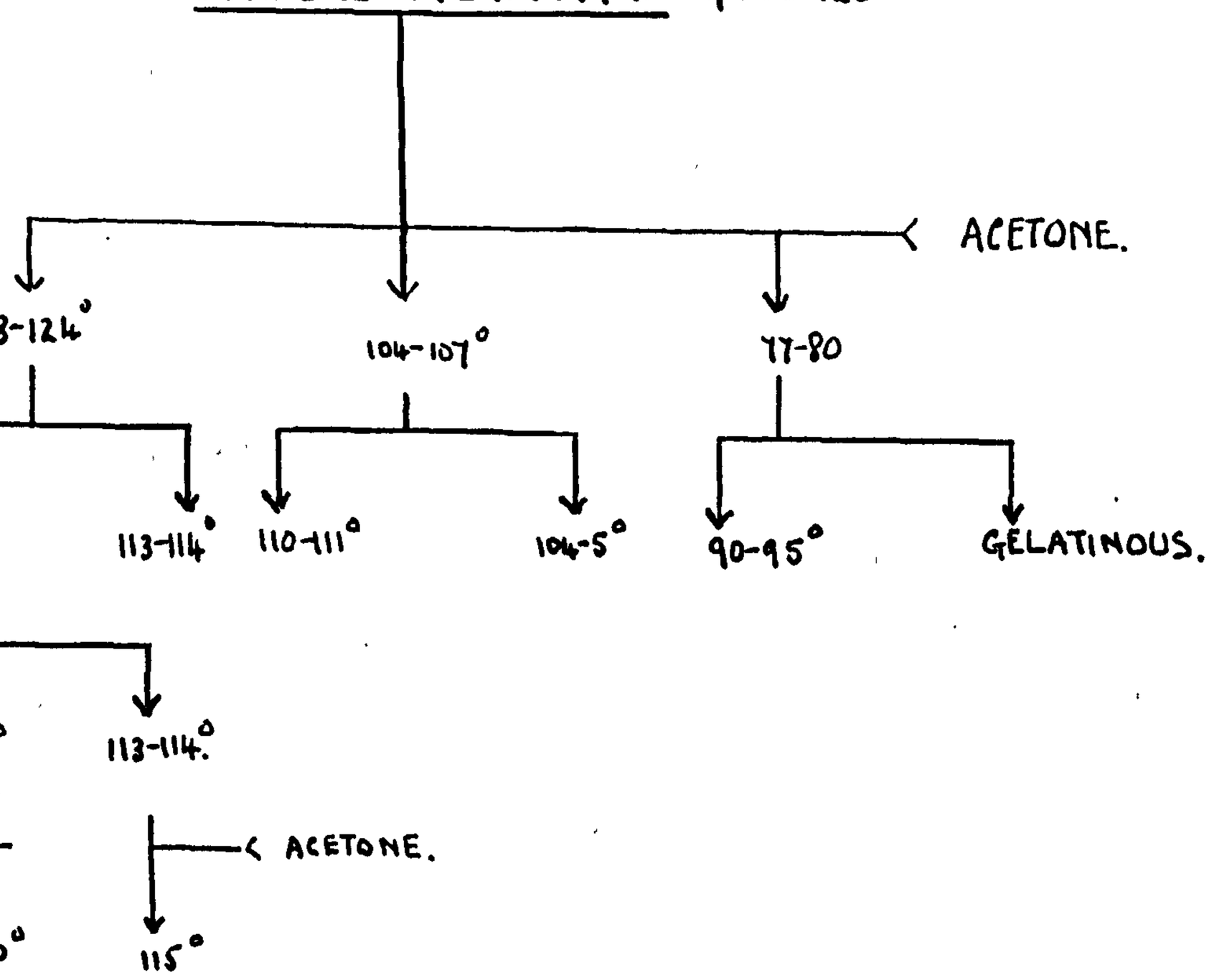


Fractionation of the Steryl Acetates.

An exhaustive fractionation of the steryl acetates was first attempted in the search for a method of separation in which the losses by decomposition and formation of tarry products, unavoidable in the bromine separation, would be reduced to a minimum. This method, as shown by the following table, was of little practical value, since considerable waste in such a large fractionation is unavoidable, and also the rate of separation was far too slow.

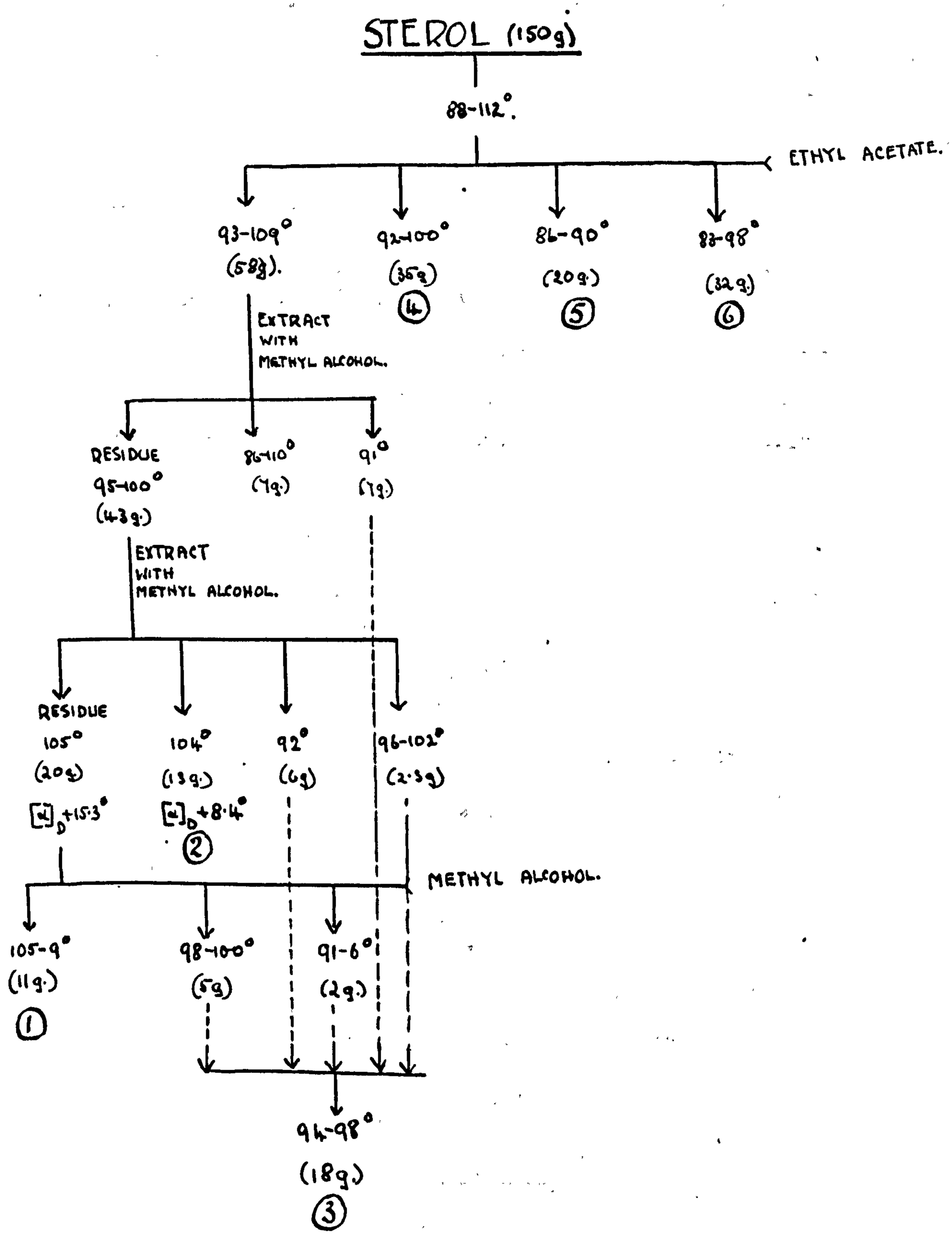
Acetylation.

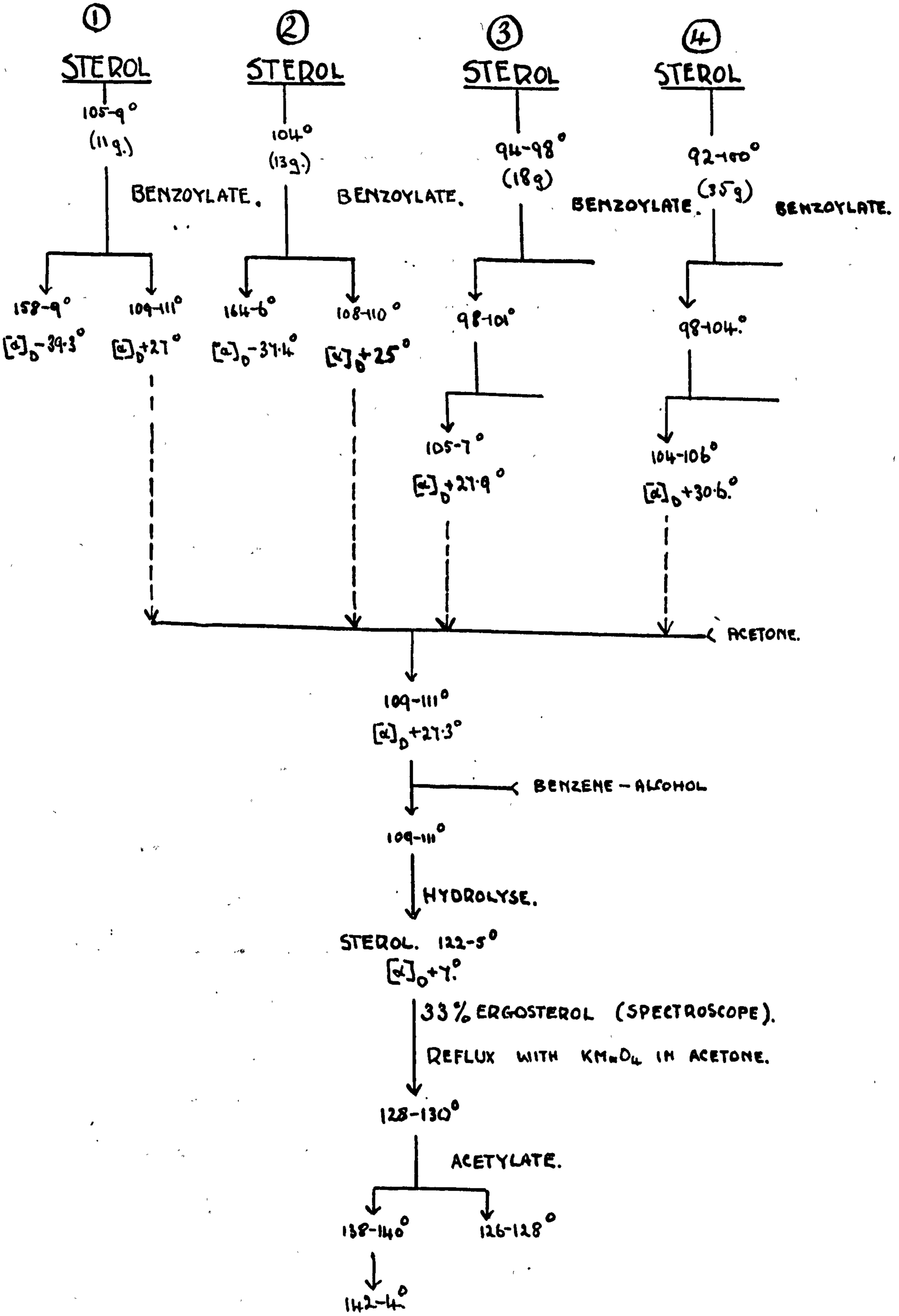
The sterol (100 g.) was refluxed with acetic anhydride (500 c.c.) containing a trace of d-camphor sulphonic acid catalyst, for 30 minutes. The unchanged acetic anhydride was converted to methyl acetate by refluxing with methyl alcohol (500 c.c.) and on slow cooling the acetate crystallised in large plates, m.p. 110-120°. The acetate was fractionated from acetone, fresh solvent being used for each crystallisation. The full course of the fractionation is shown in the following table.


CRUDE ACETATE m.p. 110-120°
ACETONE.
118-124°
104-107°
77-80
126-147°
113-114°
110-111°
104-5°
90-95°
GELATINOUS.
150-4°
125-6°
113-114°
ACETONE.
162-170°
126-30°
115°

Fractionation of the Steryl Benzoates.

The crude sterol was subjected to a preliminary fractionation from ethyl acetate and then a fractional extraction with methyl alcohol. The fractions obtained were benzoylated and further fractionated, the benzoates of high regative rotations being rejected as containing a large proportion of ergosteryl benzoate. The following tables are a summary of the work and results.


STEROL (150g)
88-112°.
ETHYL ACETATE.
93-109°
(58g).
92-100°
(35g)
4
86-90°
(20g.)
5
88-98°
(32g.)
6
EXTRACT WITH METHYL ALCOHOL.
RESIDUE
95-100°
(43g.)
86-110°
(7g.)
91°
(1g.)
EXTRACT WITH METHYL ALCOHOL.
RESIDUE
105°
(20g)
[α]D +15.3°
104°
(13g.)
[α]D +8.4°
2
92°
(6g)
96-102°
(2.5g)
METHYL ALCOHOL.
105-9°
(11g.)
1
98-100°
(5g)
91-6°
(2g.)
94-98°
(18g)
3


①
STEROL
105-9°
(11g.)
BENZOYLATE.
158-9°
[α]D -39·3°
109-111°
[α]D +27°
②
STEROL
104°
(13g.)
BENZOYLATE.
164-6°
[α]D -34·4°
108-110°
[α]D +25°
③
STEROL
94-98°
(18g)
BENZOYLATE.
98-101°
105-7°
[α]D +27·9°
④
STEROL
92-100°
(35g)
BENZOYLATE.
98-104°
104-106°
[α]D +30·6°
ACETONE.
109-111°
[α]D +27·3°
BENZENE - ALCOHOL
109-111°
HYDROLYSE.
STEROL. 122-5°
[α]D +7°
33% ERGOSTEROL (SPECTROSCOPE).
REFLUX WITH KMnO4 IN ACETONE.
128-130°
ACETYLATE.
138-140°
126-128°
142-4°

Although the melting point of the benzoate was obtained constant, the melting point of the corresponding acetate was still rising, and the spectroscopic determination of the ergosterol content also showed the futility of this method. At this stage it was apparent that the foregoing physical methods, depending on the difference in solubility of sterols, acetates, and benzoates, are of no practical value in the separation of such complex sterol mixtures. A modification of the bromine precipitation of Heilbron and Sexton was therefore investigated.

Modified Bromine Precipitation Method.

(1) Preliminary purification.

Crude sterol (525 g.) was refluxed with potassium permanganate (52.5 g.) in acetone solution. The oxidation was carried out in seven stages, and the resulting products were combined and brominated according to the following method.

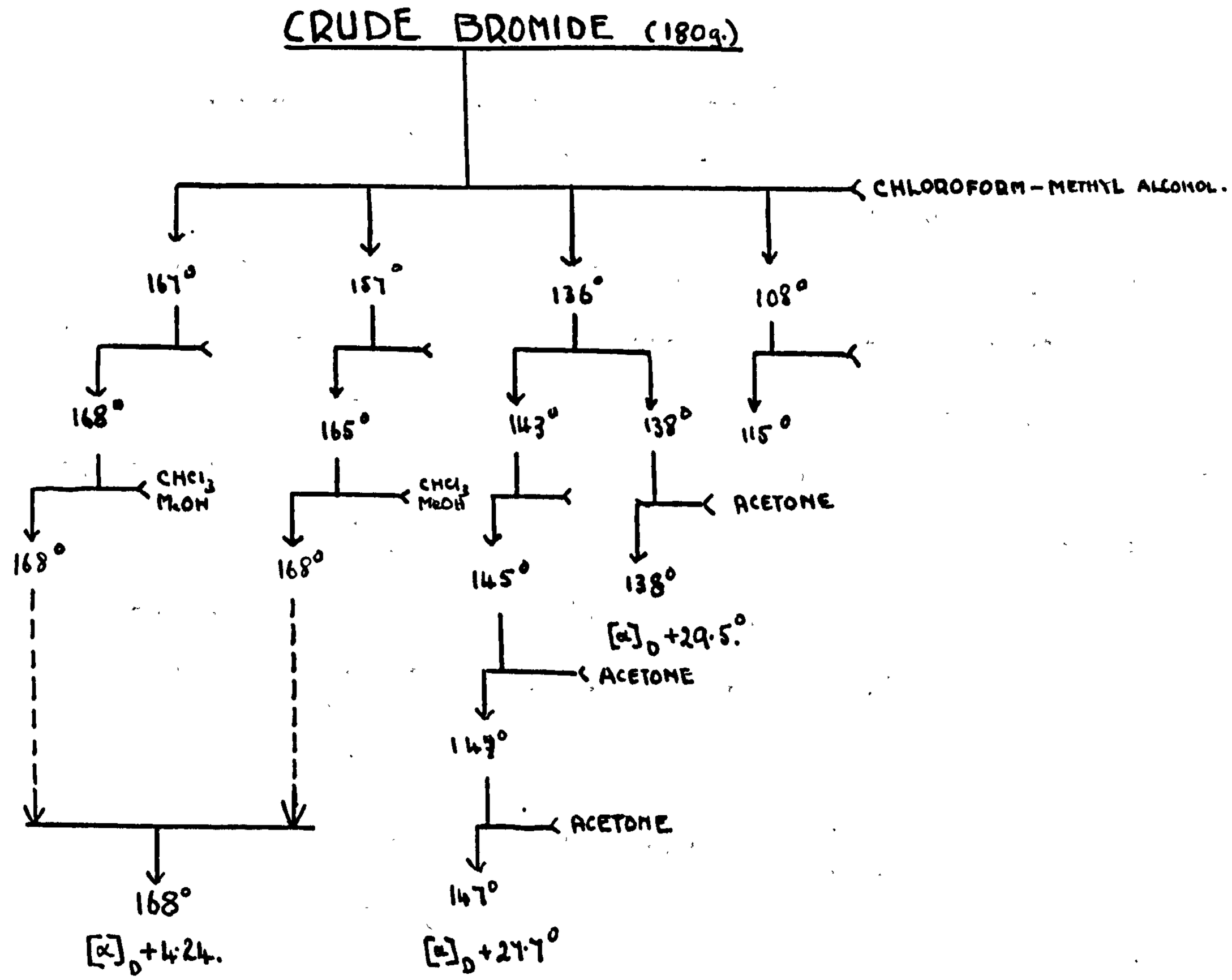
(a) Bromination.

Sterol (10 g.) was dissolved in a mixture of anhydrous ether (250 c.c.) and dry glacial acetic acid (100 c.c.). A 10% solution of bromine in glacial acetic acid (50 c.c.), containing anhydrous ammonium acetate (2 g.) was added during 15 minutes, the solution being

efficiently stirred and maintained at 0°. There was no sign of a precipitate, and after standing 10 minutes, the mixture was poured into industrial alcohol (250 c.c.). The solution still remained clear, and on dilution with much water a semi-colloidal precipitate was thrown down. It was filtered through cloth and washed well to remove all traces of acetic acid and hydrobromic acid. The product was slightly yellow but entirely free from tarry matter.

The total yield of bromide was 180 g.

The crude bromide was crystallised from a mixed solvent of chloroform (500 c.c.) and methyl alcohol (500 c.c.). The solution was nearly black but three crystalline crops were obtained, the fourth crop being tarry. The fractionation is shown fully in the following table.


CRUDE BROMIDE (180g.)
CHLOROFORM – METHYL ALCOHOL.
167°
168°
CHCl3 MeOH
168°
168°
157°
165°
CHCl3 MeOH
168°
136°
143°
145°
[α]D +29·5°
ACETONE
147°
ACETONE
147°
[α]D +27·7°
138°
ACETONE
138°
108°
115°
168°
[α]D +4·24.

Zymosterol dibromide, m.p. 168°.

The top fraction, m.p. 168°, is identical with the dibromide isolated by Heilbron and Sexton (21). The bromide crystallises in small plates and is sparingly soluble in all solvents with the exception of chloroform.

Analysis :-

(Found (micro): C,59.8, 59.7; H,7.9, 8.1; Br, 27.2,27.8.

F_1 $C_{27}H_{44}OBr_2$ requires C,59.5; H, 8.1; Br,29.5%

F_0 $C_{27}H_{46}OBr_2$ requires C,59.3; H,8.4; Br,29.3%

$[\alpha]_D^{20}$ +4.24 (C_{CHCl_3} 3.04).

Preparation of Zymosterol from Zymosterol dibromide.

Zymosterol dibromide (5 g.), m.p. 168°, was refluxed in industrial alcohol (100 c.c.) with oxide-free zinc dust (10 g.) for one hour. The solution was filtered hot to remove the excess zinc, and water was added until the sterol was just precipitated. On allowing to cool slowly the sterol deposited large crystals, m.p. 102-103°, $[\alpha]_D$ +47.9 (C_{CHCl_3} 3.77). On repeated recrystallisation from alcohol and ethyl acetate-methyl alcohol, zymosterol was obtained melting constantly at 107-108° (Yield 1.9 g.).

Zymosterol acetate.

Pure zymosterol (1.5 g.) was refluxed with acetic anhydride (10 c.c.) for 15 minutes; the excess acetic anhydride was destroyed by refluxing with methyl alcohol (10 c.c.). The crude product melted at 119-120°. After two recrystallisations from ethyl acetate-methyl alcohol the acetate melted constant at 123-124°, $[\alpha]_D$ +35.7° (C_{CHCl_3} 1.82). (Yield 1 g.).

Analysis :-

Found (micro): C,81.2; 81.3; H,10.6, 10.9.

$(C_{29}H_{46}O_2)_2$ requires C,81.6; H,10.9%.

A small quantity of pure zymosterol acetate was hydrolysed by refluxing with alcoholic caustic potash for two hours. The crude product, precipitated by water, melted at 109-111°. After two recrystallisations from ether-methyl alcohol the sterol showed a constant m.p. 110-111°. This specimen of zymosterol gave the following colour reactions :-

Rosenheim.	Negative, even in concentrated solution.
Antimony trichloride.	Negative.
Tortelli-Jaffe	Brilliant green ring which persists on shaking.

Rebromination of Zymosterol, m.p. 107-108°.

Zymosterol, m.p. 107-108° (small quantity) was

dissolved in sodium-dried ether, and a solution of 10% bromine in glacial acetic acid was added until the mixture just retained a faint yellow colour. On standing for a short time a white precipitate was obtained which melted at 165-167°. After one re-crystallisation this compound melted constant at 168°.

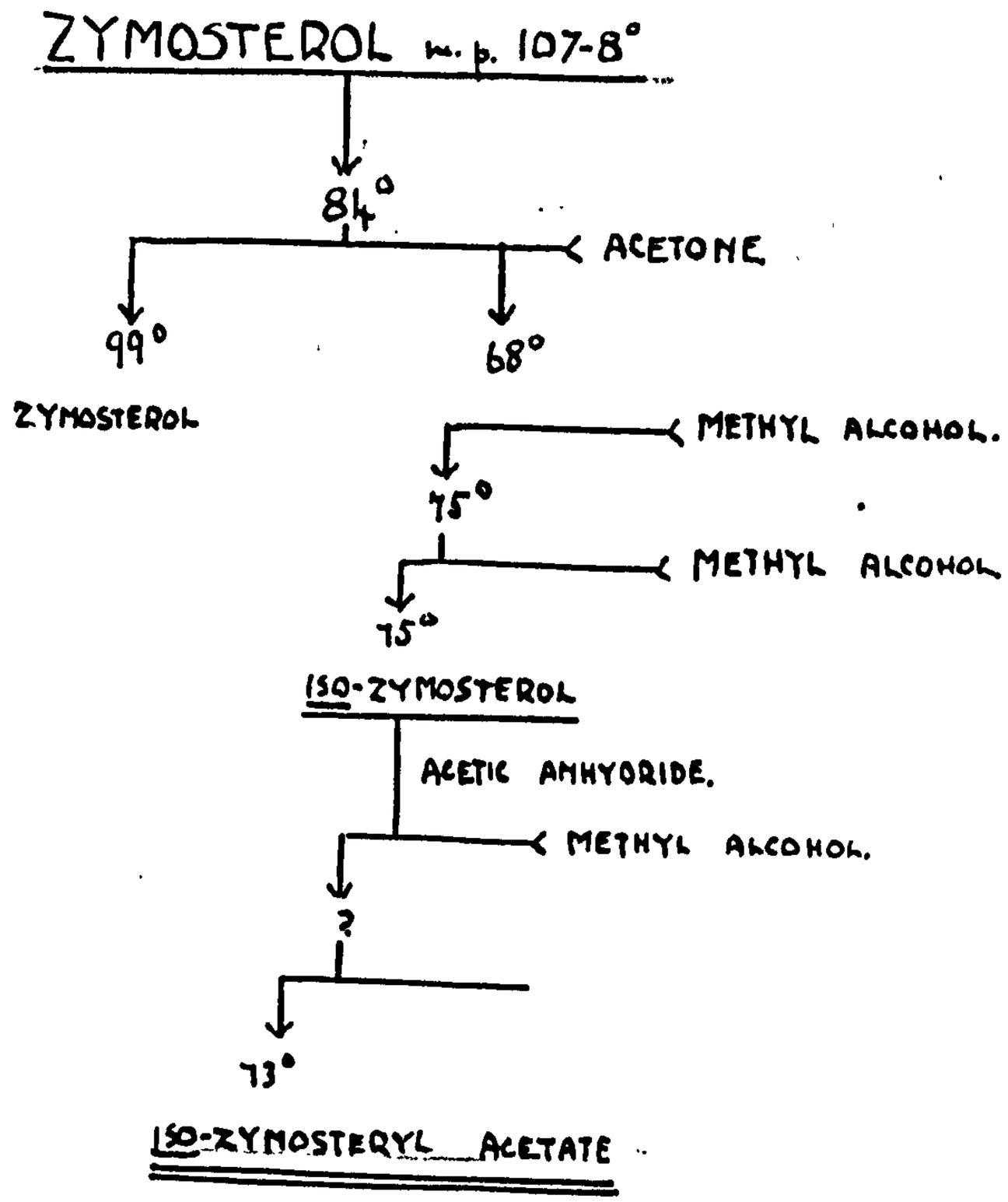
Preparation of iso-zymosterol, m.p. 107-108°.

Zymosterol, m.p. 107-108° (2 g.) was dissolved in dry ether (75 c.c.) and the solution cooled to 0°. Acetic acid (20 c.c.) saturated with dry hydrogen bromide was slowly added with efficient stirring. There was no visible reaction and the mixture was allowed to stand in ice water for 10 minutes. The acetic acid and excess hydrobromic acid were removed by aqueous extraction, the final washing containing a small quantity of sodium bicarbonate. The ether extract was dried over anhydrous sodium sulphate and evaporated to small bulk (approximately 5 c.c.) and then a few c.c. of 50% methyl alcohol were added. There was an immediate separation of a flocculent white precipitate of m.p. 84°. The precipitate was re-crystallised from acetone, the small quantity of high melting (99°) crystals obtained being assumed to be unchanged zymosterol. The second crop, m.p. 68°,

was twice recrystallised from methyl alcohol, when its melting point remained constant at 75°. There was insufficient sterol for any further work other than the preparation of the acetate.

Preparation of iso-zymosteryl acetate.

Iso-zymosterol, m.p. 75° (small quantity) was refluxed with acetic anhydride for 15 minutes and the excess acetic anhydride was destroyed by refluxing with methyl alcohol. Iso-zymosteryl acetate, on recrystallisation from methyl alcohol melted at 73° (mixed m.p. with sterol 59-63°).



The two remaining fractions in the bromide separation, melting constantly at 147° and 138° respectively, were shown on analysis to contain 16.2% and 13% of bromine. If the fractions were single pure compounds these figures could only correspond to addition compounds of steryl acetates and hydrogen bromide ($C_{29}H_{47}O_2Br$ requires Br, 15.8%).

Attempts were made to isolate the hydrogen bromide addition compound of zymosteryl acetate, and also of the addition compounds with the steryl acetates obtained by debromination of these two fractions, but in no case was a bromine-containing compound isolated. The only conclusion in agreement with these facts is that the fractions are eutectic mixtures of zymosterol dibromide and of sterols inert to the action of bromine.

T H E O R E T I C A L

Part 2.

The crude sterol mixture employed in this portion of the work was kindly supplied by Messrs. Boots, and was that remaining after the commercial removal of ergosterol. Neither a melting point nor a specific rotation could be attributed to it owing to its heterogeneous and resinous nature. On account of the presence of ergosterol, and also the fear of the destruction of sterols similar to ergosterol in stability, the drastic separation by means of the bromine addition compounds was postponed, and a fractional crystallisation, first of the free sterols and then of the steryl benzoates, was followed.

As a preliminary purification the crude sterol was extracted with ether, the ether extract being fractionally crystallised. The residue was found to be soluble in ethyl acetate, from which solvent it was obtained in good crystalline form. Four main crops were obtained from the ether extract, which were further subdivided by recrystallisation from different solvents, and fractions possessing similar melting points, specific rotations and absorption spectra were combined. These crops were then benzoylated (Wieland and Asano (18) and fractionated. Consistency of melting point and

optical rotation after crystallising from different solvents, and quantitative spectroscopical measurements, were the criteria of purity.

Great difficulty was encountered in the benzoate fractionation of the top fraction until it was proved beyond doubt that ergosteryl benzoate can exist in dimorphic crystalline forms (compare Callow (24). The crystals differed both in structure and melting point, but were readily interchangeable and were identical in those properties, optical rotation and absorption spectra, not dependent on the physical state of the crystals. For benzoates of medium solubility this method proved of great value, but in the easily soluble fractions no advance was made, and recourse was had to the fractionation of the sterol acetyl dibromides as given by Reindel and Weickmann (23). For the separation of the still more soluble fractions, which contained unstable sterols, the p-nitro benzoates (Wieland and Gough (19) were prepared and fractionated.

From the sparingly soluble benzoate fraction two steryl benzoates, together with pure ergosteryl benzoate, were isolated. The more insoluble of the two melted at 200-202° (corr.), and had an $[\alpha]_D$ -23.2°. On hyd-

rolysis a sterol was obtained of m.p. 177-178° having $[\alpha]_D$ -27.5°. There was no depression observed in a mixed melt of this benzoate and α-dihydroergosteryl benzoate, nor of this sterol and α-dihydroergosterol. Spectroscopical examination showed the presence of less than 2.5% of ergosterol, and this explains the faint colour obtained with the antimony trichloride reagent. Analysis is in good agreement with that required for a sterol containing two double bonds, and on comparison of the physical constants of this sterol and its derivatives with those of pure α-dihydroergosterol, as prepared by Heilbron and Webster (unpublished work), there is little doubt that the isolation of this sterol confirms Callow (24) in the observation that α-dihydroergosterol occurs naturally in the non-saponifiable matter of yeast fat.

	Sterol		Benxoate		Acetate	
	M.p.	$[\alpha]_D$	M.p.	$[\alpha]_D$	M.p.	$[\alpha]_D$
Sterol obtained in this work	177-8°	-27.5°	200-2°	-23.2	184-5°	-
α-Dihydroergo-sterol (Heilbron and Webster)	177-8°	-21.1°	197-8°	-	182.3°	-

The more soluble benzoate, m.p. 180-183° was obtained pure by repeated recrystallisation from benzene-alcohol solvent and gave, on hydrolysis, a sterol of melting point 167-169°. The absorption spectra on investigation showed bands identical with those of ergosterol at approximately 50% intensity. From this observation it was first thought that the new sterol was a mixture of 50% ergosterol and α-dihydro-ergosterol, but on refluxing in permanganate stable acetone with potassium permanganate, and finally refluxing with charcoal in alcohol, no alteration in the melting point was obtained; also from consideration of the specific rotation values it is apparent that for this sterol to be a mixture containing 50% ergosterol, the second sterol must have a zero rotation while its benzoate must have a specific rotation of +20°. These facts entirely rule out any possibility of the new sterol being a mixture of ergosterol and α-dihydroergosterol. Analytical figures indicate the presence of two ethenoid linkages which, since the antimony trichloride, the Rosenheim and the modified Tortelli-Jaffe reactions are positive, occupy positions identical with two of the three double bonds present in the ergosterol molecule. It appears from the

spectroscopical result that it is the combination of these two ethenoid linkages which is responsible for the typical absorption bands in the ultra-violet, while the added double bond in ergosterol merely enhances their intensity without causing any shift.

It was in this fraction that neosterol was expected to occur, and it appears probable that this sterol is pure neosterol and that that prepared by Wieland and Asano (loc.cit.) is impure, containing appreciable quantities of ergosterol, thus accounting for the higher values obtained by those authors for the specific rotations, and for the extinction coefficients of the absorption bands in the ultra-violet. The table appended below compares the published physical constants of neosterol and its derivatives with those of the sterol isolated in this investigation.

	Sterol		Benzoate		Acetate	
	M.p.	$[\alpha]_D$	M.p.	$[\alpha]_D$	M.p.	$[\alpha]_D$
Sterol obtained in this work.	167-9°	-62.5°	180-3°	-21.6°	178-180°	-
Neosterol	164-5°	-105°	173-4°		173-4°	-

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From the fractionation of the benzoates of medium solubility two further benzoates were isolated, in the case the final traces of ergosterol were removed by refluxing with potassium permanganate in acetone solution. The more insoluble of the sterols corresponds very closely to the faecosterol isolated by Wieland and Asano (loc.cit.), while the second appears to be a pure form of ascosterol reported by the same authors.

An examination of the absorption spectrum of a concentrated solution of faecosterol shows that the sterol possesses no powers of selective absorption, and further, indicates that it is free from ergosterol. In agreement with Wieland and Asano, the analytical figures leave no doubt but that faecosterol is an isomeride of cholesterol, possessing one ethenoid linkage, which, since the sterol gives an intense green ring with the modified Tortelli-Jaffe reaction, must be the inert ethenoid linkage, a supposition which is supported by the fact that it is impossible to prepare a bromine addition compound. A table comparing the faecosterol isolated in this investigation with that isolated by Wieland and Asano is appended below.

	Sterol		Benzoate		Acetate	
	M.p.	$[\alpha]_D$	M.p.	$[\alpha]_D$	M.p.	$[\alpha]_D$
Faecosterol (author)	163-4°	+33.5°	144-5°	+28.2°	158-160°	-
Wieland	161-3°	+42.1°	144-6°	+35.4°	159-161°	-

From the filtrates of the faecosterol separation a benzoate was obtained which it was impossible to purify further by recrystallisation; it was therefore hydrolysed and the sterol fractionated. During this operation peculiar crops were obtained consisting of large flat plates covered with opaque spots which, on microscopical examination, were shown to be clusters of minute crystals. After repeated recrystallisation from ethyl acetate-acetone solvent the sterol was obtained homogeneous, the melting point, however, was still rising slowly and final purification was achieved by re-benzoylation and fraction-ation of the benzoate.

This sterol was obtained from fractions exactly similar to those from which Wieland and Asano isolated ascosterol. The following table shows that there is a general resemblance to ascosterol, and it is considered that this sterol is pure ascosterol.

	Sterol		Benzoate		Acetate	
	M.p.	$[\alpha]_D$	M.p.	$[\alpha]_D$	M.p.	$[\alpha]_D$
Ascoaterol. (author)	151°	+32.8°	134-5°	+29°	153-4°	
Wieland	141-2°	+45°	130-1°	+37°		

Analytical figures indicate that ascosterol is a stereo-isomeride of faecosterol, possessing one double bond, which, since the modified Tortelli-Jaffe reaction is positive, must be the inert ethenoid linkage.

The benzoate fractionation completely failed when applied to the separation of the more soluble sterol fractions, and the benzoates were therefore hydrolysed. The melting point of the sterol being approximately constant in the neighbourhood of 108° gave the impression that this fraction was an impure specimen of zymosterol, and Reindel's modification of the bromine precipitation method of Heilbron for its final purification was attempted. On acetylation, however, the melting point of the acetate, 115°, ruled out any possibility of its being the zymosterol of Reindel and Weickmann, and Wieland and Asano, but, as reported in the previous section of this investigation, it is possible to prepare a stable dibromide of a sterol giving an acetate of m.p. 123°, and the products of the action of

bromine on the acetate were therefore studied. No decomposition was observed, and on fractionation of the resulting bromides an insoluble fraction of melting point 182° was readily obtained pure.

Debromination was effected in two ways, refluxing with zinc dust in alcohol,and shaking with zinc dust in cold 95% acetic acid. Two acetates were obtained, that from the former method melting at 123°, and that from the latter at 104°. Proof that refluxing the bromide with zinc dust in alcohol regenerates the naturally occurring sterol is afforded by the isolation of a slightly impure acetate, m.p. 119°, by means of the sparingly soluble p-nitro benzoate, the acetate yielding on bromination the steryl acetyl dibromide, m.p. 182°.

This work confirms Smedley MacLean in the observation that zymosterol and zymosteryl acetate have the melting points 110-6° and 119° respectively.

No trace of the sterols, Episterol, Anasterol and Hyposterol, isolated by Wieland, nor of the zymosteryl acetyl dibromide, m.p. 168°, characterised by Reindel has been observed.

PRELIMINARY PURIFICATION OF STEROL.

Crude sterol (2 Kg.) was extracted with ether (15 l.) and on evaporation of the extract the following fractions were obtained :-

(1) FRACTION B. (75 g.)

This fraction was microscopically crystalline of melting point 132-142°. No rotation figures were available since its intense yellow colour prevented the estimation. Fraction B gave very strongly all the colour reactions typical of ergosterol.

(2) FRACTION C. (265 g.)

This fraction crystallised in large flat plates, m.p. 95-101°.

(3) FRACTION D. (75 g.)

This fraction was not crystalline and was highly coloured, m.p. 76-80°. It was obtained by concentration of the ether filtrate and the addition of industrial alcohol.

(4) FRACTION E. (35 g.)

On further concentration, under reduced pressure, of the filtrate from fraction D and the addition of methyl alcohol the final solid fraction was deposited on standing several months.

(5) FRACTION F

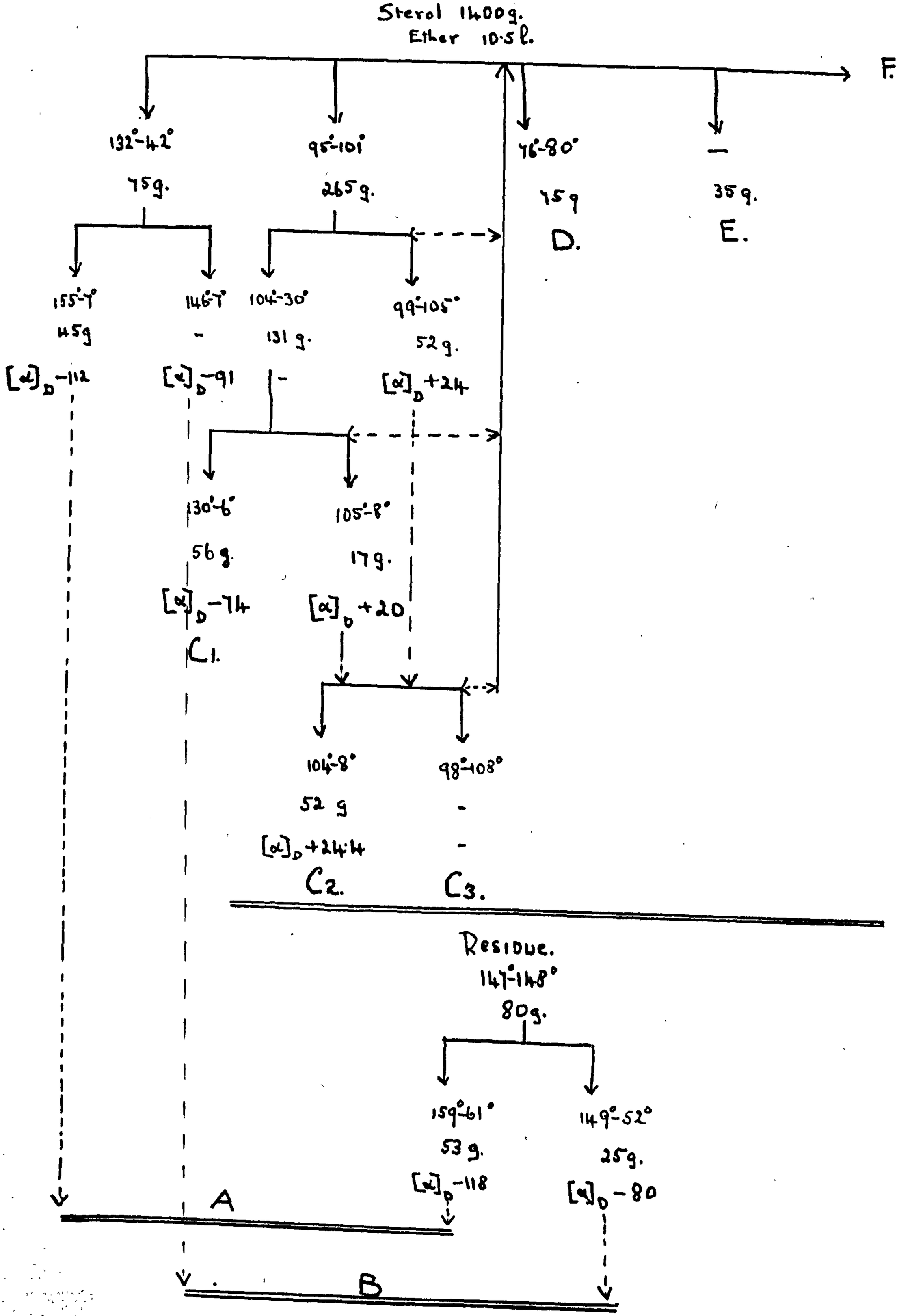
A thick oil was obtained on removal of the greater

part of the solvent.

(6) FRACTION A.

The residue remaining after the ether extraction was dissolved in ethyl acetate. On cooling, the solution deposited a crystalline solid (80 g.), which formed well defined hard needles melting at 147-148°.

TABLE I.


Sterol 1400g.
Ether 10·5l.
F.
132°-42°
75g.
95°-101°
265g.
76°-80°
15g
D.
—
35g.
E.
155°-7°
45g
[α]D -112
C1.
146°-7°
-
104°-30°
131g.
[α]D -91
99°-105°
52g.
[α]D +24
130°-6°
56g.
[α]D -74
105°-8°
17g.
[α]D +20
104°-8°
52 g
[α]D +24·4
C2.
98°-108°
-
-
C3.
Residue.
147°-148°
80g.
159°-61°
53g.
[α]D -118
149°-52°
25g.
[α]D -80
A
B

PURIFICATION OF FRACTION A.

The residues, after crystallisation from ethyl acetate, which remained after the ether extraction of 2 Kilos of sterol mixture were again crystallised from ethyl acetate, and gave two fractions :-

(1) Melting point 159-161°, $[\alpha]_D$ -118°.

(2) Melting point 149-152°, $[\alpha]_D$ -80°.

Fraction (1) approximates in its physical constants to ergosterol (m.p. 160-161°, $[\alpha]_D$ -133°).

A spectroscopic examination of Fraction (2) showed the presence in it of 73% of ergosterol.

PURIFICATION OF FRACTION B.

Fraction B (75 g.) was recrystallised from ethyl acetate (500 c.c.) and gave two fractions :-

(1) Melting point 155-157°, $[\alpha]_D$ -112°.

(2) Melting point 146-147°, $[\alpha]_D$ -91°.

Fractions (1) of A and B, owing to their practically identical physical constants and not giving a depression to a mixed melting point, were combined.

Spectroscopic examination showed the presence of 80% of ergosterol in Fraction (2) B, and this was therefore combined with Fraction (2) A.

Combined Fractions (1) were benzoylated. It was observed in this operation that, when pure pyridine was used as solvent, the reaction either did not proceed at all or that a low melting mixture of sterol and benzoate was produced. It was then found that the sterols were appreciably more soluble in technical pyridine than in the pure, and the benzoylations were carried out using technical pyridine as solvent. On addition of benzoyl chloride in excess of that required theoretically for the reaction, the solution developed an intense red colouration and became practically solid with precipitated matter. Whenever this red colouration was observed the product was found to contain no free sterol, which more than compensated for the higher content of tarry decomposition products.

The benzoylations were carried out using the following methods :-

Sterol (30 g.) was dissolved in technical pyridine (redistilled over solid caustic potash, boiling point 110-134°) (350 c.c.) and cooled in iced water, the temperature rising slowly from 18° to 25° on addition of benzoyl chloride (120 g.) during two hours. The solution turned dark red and was allowed to stand for six hours at room temperature. The reaction mixture was then poured into crushed ice and the precipitated benzoates filtered off. The solid was well washed, first with water to remove excess pyridine, and then with industrial alcohol to remove coloured impurity.

The benzoylation was carried out in three lots, the products of each being combined and crystallised from ethyl acetate, giving the following fractions :-

FRACTION (1)(Yield 70 g), melting at 161-166° and crystallising in long matted needles which contained a small quantity of plates.

FRACTION (2) (Yield 3.5 g.) melting at 175-181° was obtained after evaporation of the filtrate from Fraction (1) to small bulk, adding industrial alcohol and cooling in the ice chest. The solution was highly coloured and after filtration and evaporation no further

solid matter could be isolated. Fraction (2) crystallised in small soft plates, mixed melting point with ergosteryl benzoate (m.p. 168-169°) melted at 171-174°.

Both these Fractions were then fractionated, the solvent being changed to benzene (1000 c.c.)-alcohol (650 c.c.). The full course of the fractionations is shown in the following tables.

FRACTION 1. (70g)

161°-166°

BENZENE – ALCOHOL.

167°-70°	159°-63°	163°-5°	158°-60°
(27g)	(16g)	(10g)	(5g)
$[\alpha]_D$ -68°	$[\alpha]_D$ -68°	-	-
MATTED NEEDLES	CLEAR PLATES	GLISTENING PLATES.	GLISTENING PLATES.
Y.1.1.	Y.2.2.		
160°-8°	159°-63°	160°-5°	161°-168°
CLEAR PLATES	MIXTURE OF PLATES AND NEEDLES.	CLEAR PLATES	MIXTURE OF PLATES AND NEEDLES.
HYDROLYSE			
163°-4°			
$[\alpha]_D$ -130°.			

NEEDLES 167°-70°.

HYDROLYSE

163°-4°

$[\alpha]_D$ -126°

The two fractions Y 1.1. and Y 2.2. while differing considerably in melting point and crystalline structure have been proved dimorphous modifications of ergosteryl benzoate by the four following methods:-

(1) Spectroscopic examination.

Both benzoates showed the typical ergosterol absorption bands, there being no difference between the samples in either the position or the intensity of the bands. Using a 0.02% solution and a 2 mm. cell the extinction coefficient for 281 $\mu\mu$. is 1.4, pure ergosterol under the same conditions has an extinction coefficient of 1.2.

(a) Interchange of crystal structure and melting point.

A small quantity of the needles were dissolved in warm benzene-alcohol solvent, the solution cooled and seeded with plates. On cooling in the ice chest all the benzoate crystallised in plates; there was no sign of the very characteristic fibrous needles.

Melting point of needles 167-170°.

Melting point of plates 160-168°.

A hot concentrated solution of plates in benzene-alcohol solvent was seeded with needles. On cooling slowly the benzoate separated as needles containing a few plates.

Melting point of plates 159-163° (clear at 168°)

Melting point of needles 167-168°.

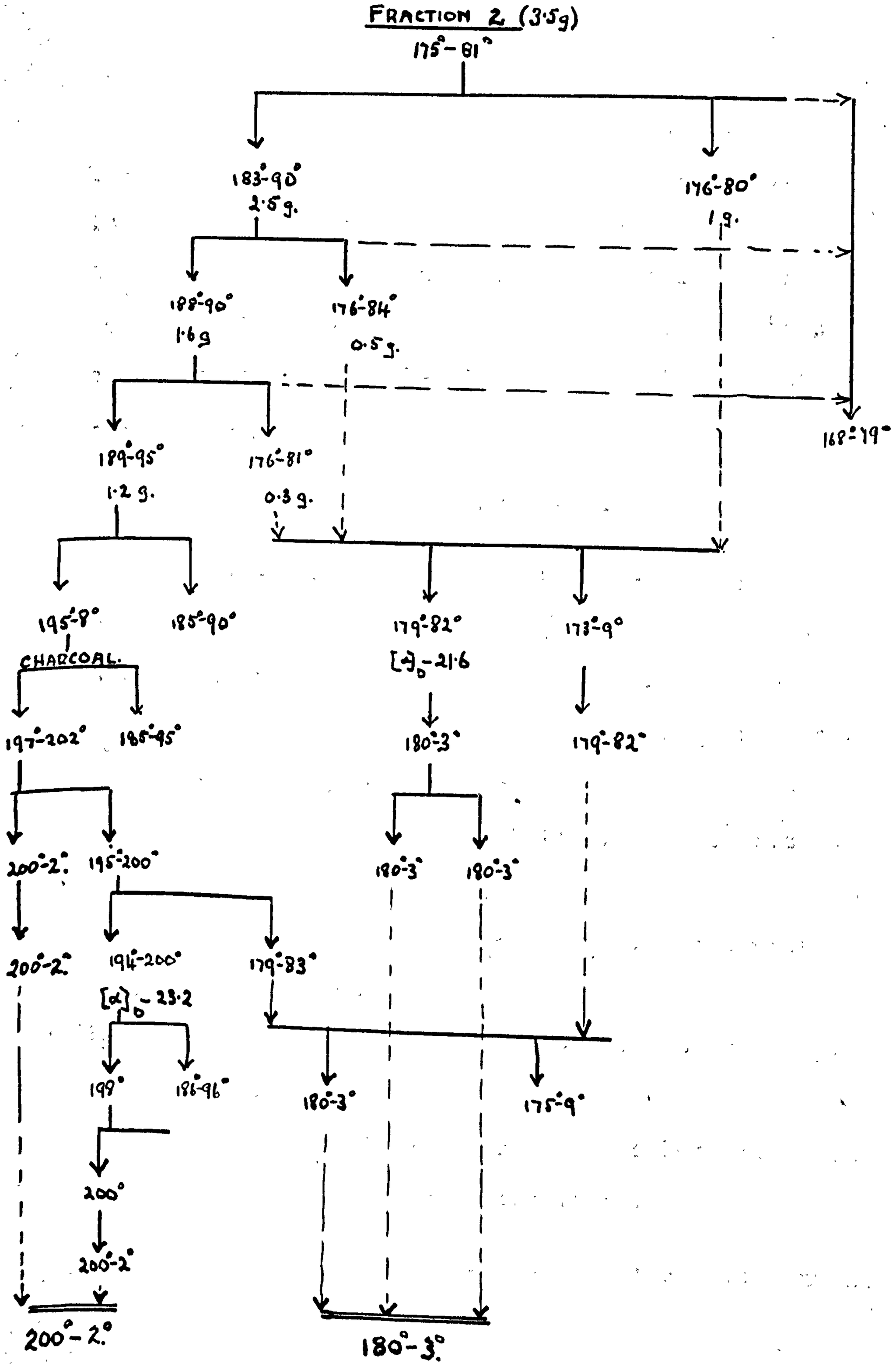
(3) Hydrolysis to the free sterol

The benzoates (5 g.) were refluxed with 3% alcoholic caustic potash (150 c.c.) for one hour. The sterols were precipitated with water and crystallised from benzene-alcohol solvent. Both crystallised in small white needles of melting point 163-164° and gave no depression to a mixed melting point. Both sterols were spectroscopically examined and showed the typical ergosterol bands with a very small increase in intensity, Extinction coefficient for 281 μμ. is 1.35. The two samples were indistinguishable.

(4) Specific rotation of benzoates and sterols.

Benzoate (needles)	$[\alpha]_D$ -68.4°
Benzoate (plates)	$[\alpha]_D$ -68.3°
Sterol from needles	$[\alpha]_D$ -126° (C_{CHCl_3} 2.98, α -3.75°)
Sterol from plates	$[\alpha]_D$ -130° (C_{CHCl_3} 2.56, α -3.32°)

Ergosteryl benzoate	$[\alpha]_D$ -70.5° (Wieland)
Ergosterol	$[\alpha]_D$ -133° (Wieland)


FRACTION 2 (3·5g)
175°-81°
183°-90°
2·5g.
176°-80°
1g.
188°-90°
1·6g
176°-84°
0·5g.
168°-79°
189°-95°
1·2 g.
176°-81°
0·3 g.
195°-8°
185°-90°
179°-82°
[α]D -21·6
173°-9°
CHARCOAL.
197°-202°
185°-95°
180°-3°
179°-82°
200°-2°
195°-200°
180°-3°
180°-3°
200°-2°
194°-200°
179°-83°
[α]D -23·2
199°
186°-96°
180°-3°
175°-9°
200°
200°-2°
200°-2°
180°-3°

Following the scheme of fractionation as shown in Table 3 two constant melting fractions were isolated. The most insoluble fraction had a melting point of 200-202° and possessed a specific rotation of -23.3° (C_{CHCl_3} 2.15, α -0.50); the more soluble fraction had a melting point of 180-183° and a specific rotation of -21.6° (C_{CHCl_3} 2.50, α -0.54°).

High Melting Fraction.

The benzoate of high melting point crystallised from benzene-alcohol solvent in glistening plates, and gave the following colour reactions :-

With the antimony trichloride reagent, a faint pink changing to brown.

With the modified Tortelli-Jaffe reaction a brilliant olive-green ring.

With the Rosenheim reaction no colouration was observed.

Mixed melting point with pure α-dihydro ergosteryl benzoate 197°.

The yield of pure benzoate was less than 0.1 g.

α-Dihydro ergosterol.

The benzoate (50 mm.) was refluxed with 3% alcoholic

caustic potash (25 c.c.) for one hour. The α-dihydroergosterol was precipitated with water and crystallised from methyl alcohol. It crystallised in characteristic fern-like needles and melted at 177-178°. Specific rotation -27.5° (C_{CHCl_3} 1.67, α -0.46°).

Mixed melting point with an authentic specimen of pure α-dihydroergosterol was 177°.

The sterol was spectroscopically examined and was estimated to contain less than 2.5% of ergosterol. With the exception of the bands due to this content of ergosterol the specimen showed complete transparency.

α-Dihydroergosteryl acetate.

The α-dihydroergosterol (40 mg., melting point 177°) was refluxed for 15 minutes with acetic anhydride (5 c.c.). The excess acetic anhydride was destroyed by refluxing with methyl alcohol and the acetate was precipitated with water. The crude acetate was twice crystallised from industrial alcohol, the melting point remaining constant at 184-185°.. The acetate crystallises from this solvent in small transparent laminae.

(Found (micro): C,81.9; H,10.8. F_2 $C_{29}H_{46}O_2$ requires C,81.6; H, 10.8%).

Low Melting Fraction.

The second fraction had a constant melting point of 180-183° and a specific rotation of -21.6°. The benzoate crystallises from benzene-alcohol solvent in glistening plates and gives no depression to a mixed melting point with ergosteryl benzoate.

(Found (micro): C,83.4; H, 9.7. $C_{34}H_{48}O_2F_2$ requires C,83.5; H,9.9%).

Yield of pure benzoate was 0.8 g.

Neosterol.

The benzoate (0.46 g.) was refluxed with 3% alcoholic caustic potash for half-an-hour. The sterol was precipitated with water and recrystallised from industrial alcohol until constant melting point 167-169° was reached. The sterol crystallised in colourless leaves and possessed a specific rotation of -62.5° (C_{CHCl_3} 2,59, α -1.62°). The colour reactions of this sterol are identical with, but less intense than, the colour reactions of ergosterol.

Antimony trichloride reagent, pink to brown.

Modified Tortelli-Jaffe, brilliant green ring

Rosenheim's reagent, faint pink to colourless to blue.

On spectroscopic examination of the sterol, bands characteristic of ergosterol at approximately 50% intensity

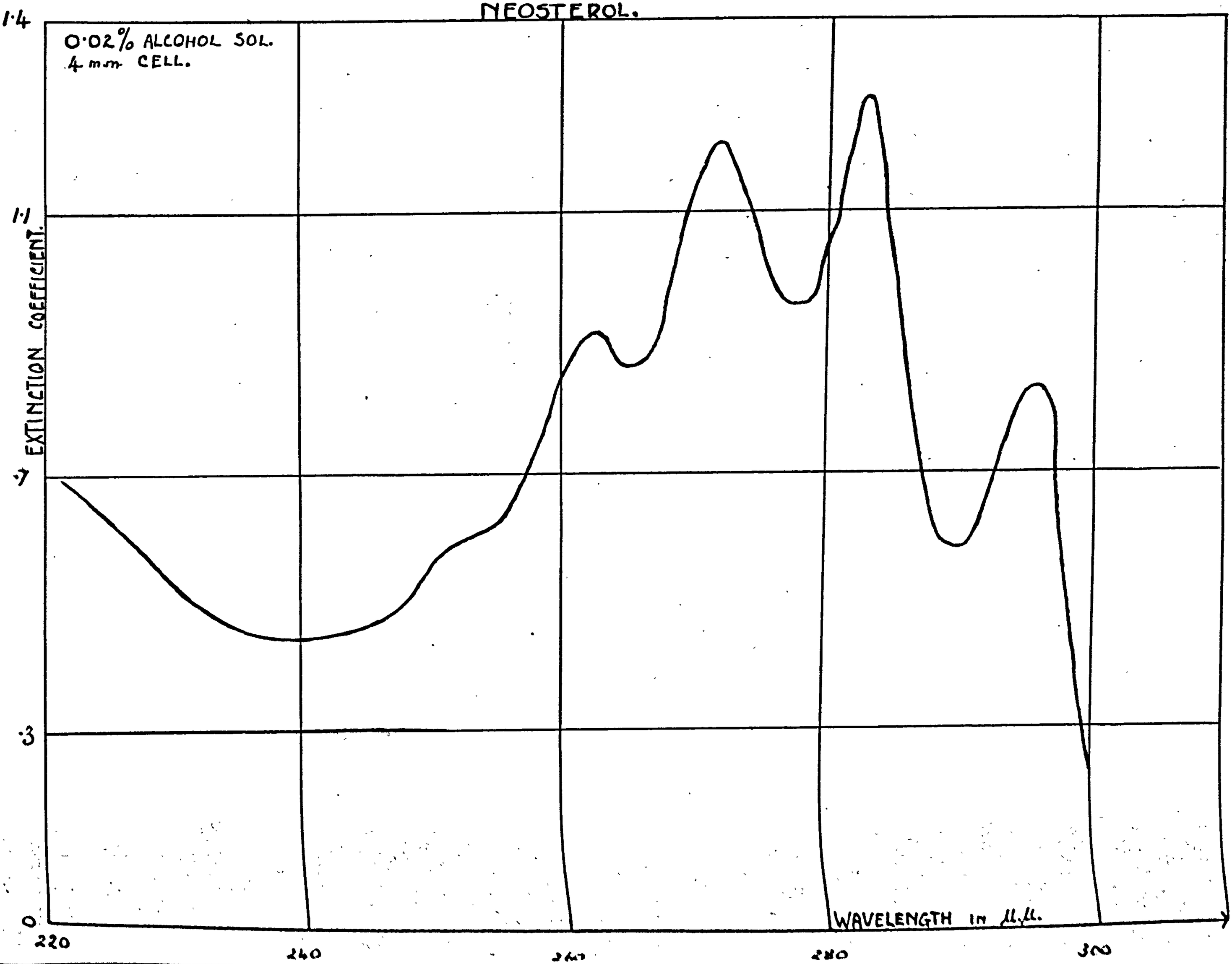
were observed. In order to prove the purity of the sterol and that the bands observed were not due to contamination by ergosterol, a small quantity of sterol was refluxed in permanganate stable acetone with a little potassium permanganate. Slow reduction of the permanganate took place, the solution losing the purple colouration. The sterol was then refluxed with charcoal and, after filtration, was crystallised from acetone. The sterol crystallised in feathery plates and melted unchanged at 168-169°; the colour reactions did not diminish in intensity.

Neosteryl acetate.

Neosterol, melting point 167-169°, (40 mg.) was refluxed for 15 minutes with acetic anhydride (5 c.c.). The crude acetate was crystallised twice from benzene-methyl alcohol solvent, the melting point remaining unchanged at 178-180°. The acetate crystallises from this solvent in large soft plates.

(Found (micro): C,81.6; H, 11.1. $C_{29}H_{46}O_2$ F_2. requires C,81.6; H, 10.9%).

NEOSTEROL.

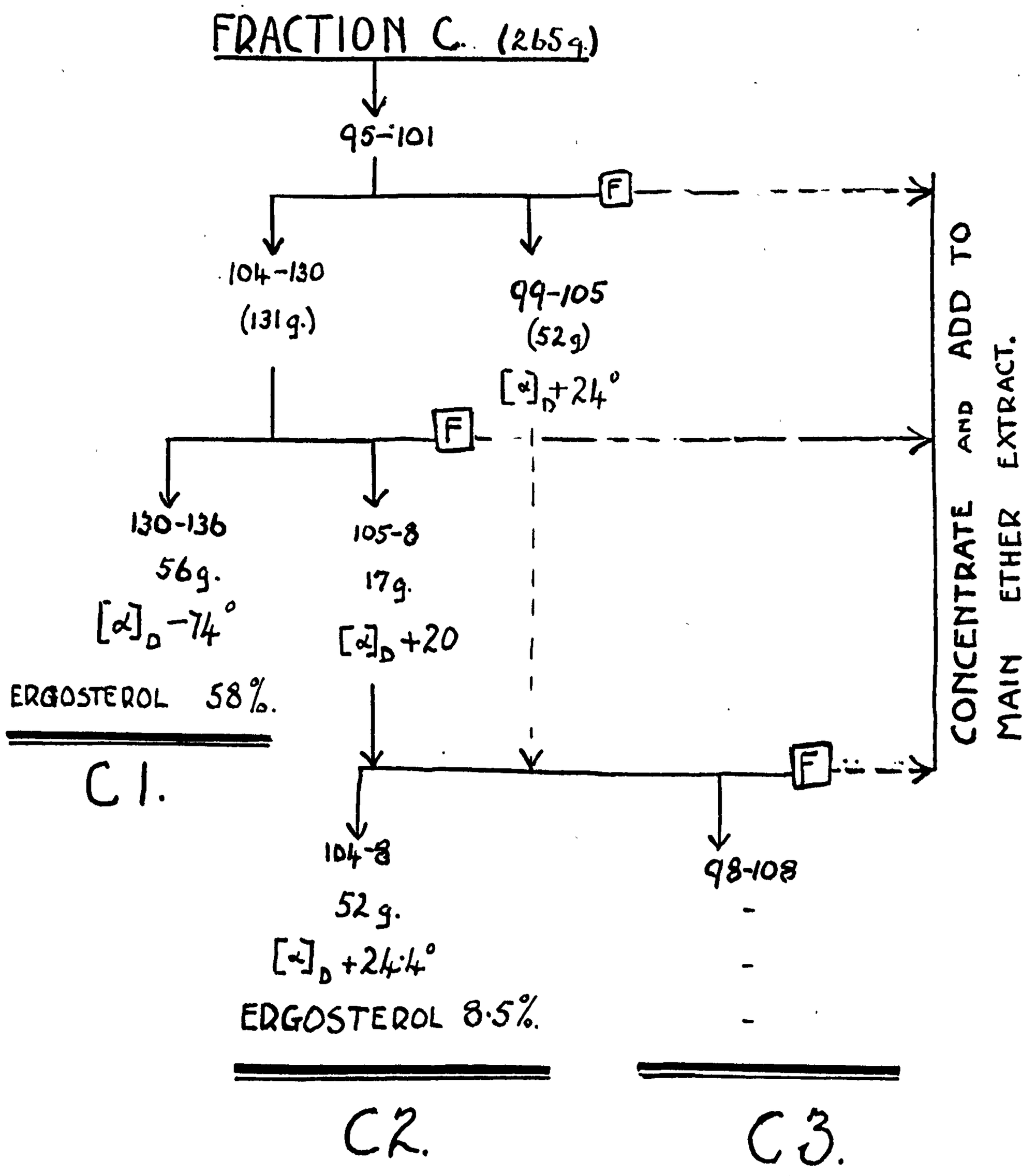

0·02% ALCOHOL SOL.
4 mm CELL.
1·4
1·1
·7
·3
0
EXTINCTION COEFFICIENT.
220
240
260
280
300
WAVELENGTH IN μμ.

PRELIMINARY PURIFICATION OF FRACTION C.

(m.p. 95-101°).

Fraction C (265 g. containing tarry matter) was crystallised from ethyl acetate. The first crop (131 g.) melted broadly at 104-130°. This crop was again crystallised and gave as the most insoluble portion a sterol melting at 130-136° (56 g.), having a specific rotation of -74° and showing spectroscopically a content of 58% of ergosterol. From the filtrates of both of these recrystallisations crops were obtained possessing positive rotations of 24° and 20° respectively. They were therefore combined and recrystallised, yielding a crop of medium solubility, m.p. 104-108° (52 g.). $[\alpha]_D$ +24.4° containing an estimated percentage of 8.5 ergosterol, and a very soluble crop of melting point 98-108°, for which, owing to contaminating tarry matter and high colour no further physical characteristics could be obtained. The total combined filtrates were concentrated but the concentrate contained too much tarry matter to crystallise, and it was therefore added to the main ether extract before the separation of Fraction D.

The course of this preliminary purification is shown graphically in Table 4.


FRACTION C. (265g.)
95-101
F
104-130
(131g.)
99-105
(52g)
[α]D+24°
F
130-136
56g.
[α]D-74°
ERGOSTEROL 58%.
C1.
105-8
17g.
[α]D+20
104-8
52g.
[α]D+24·4°
ERGOSTEROL 8·5%.
C2.
F
98-108
-
-
C3.
F
CONCENTRATE AND ADD TO MAIN ETHER EXTRACT.

Further Purification of Fraction C 1.

Fraction C 1 (56 g.) was benzoylated in the usual manner and gave a benzoate melting approximately at 151-156°. The crude benzoate was first crystallised from ethyl acetate and then fractionated from benzene alcohol solvent, the full details of the fractionation are given in the following table.

CRUDE BENZOATE.

Ethyl acetate.

149°-56°

108°-112°

Benzene Alcohol

Acetone-Ethyl acetate.

163°-6° | 160°-2° | 155°-60° | 119°-124° | 110°-122°.

Acetone.

163°-5° | 165°-70° | 123°-30° | 111°-22° | 116°-22°.

$[\alpha]_D$ -63·7 | $[\alpha]_D$ -50·8

164°-7° | 165°-6° | 169°-75° | 168°-70° | 125°-37° | 119°-120°.

HYDROLYSE.

STEROL

158°-62°

$[\alpha]_D$ -133-8

ERGOSTEROL.

174°-7°

177°-81°

128°-39°

HYDROLYSE PERMANGANATE.

COMBINE WITH SIMILAR FRACTIONS FROM FOLLOWING FRACTIONATION.

Bromine in PYRIDINE

191°-94°

196°-7°

α-DI HYDRO ERGOSTERYL BENZOATE.

150°-4° | 139°-41°

154°-9° | 148°-50°

CRUDE FAECOSTEROL

The top fraction melting point 164-167° (5 g.) was refluxed with 3% alcoholic caustic potash (150 c.c.) for one hour. The free sterol crystallised in short needles from benzene-alcohol solvent and melted at 158-162°, and had a specific rotation of -133.8°. There was no depression to a mixed melting point with pure ergosterol and this fraction was therefore considered to be ergosterol.

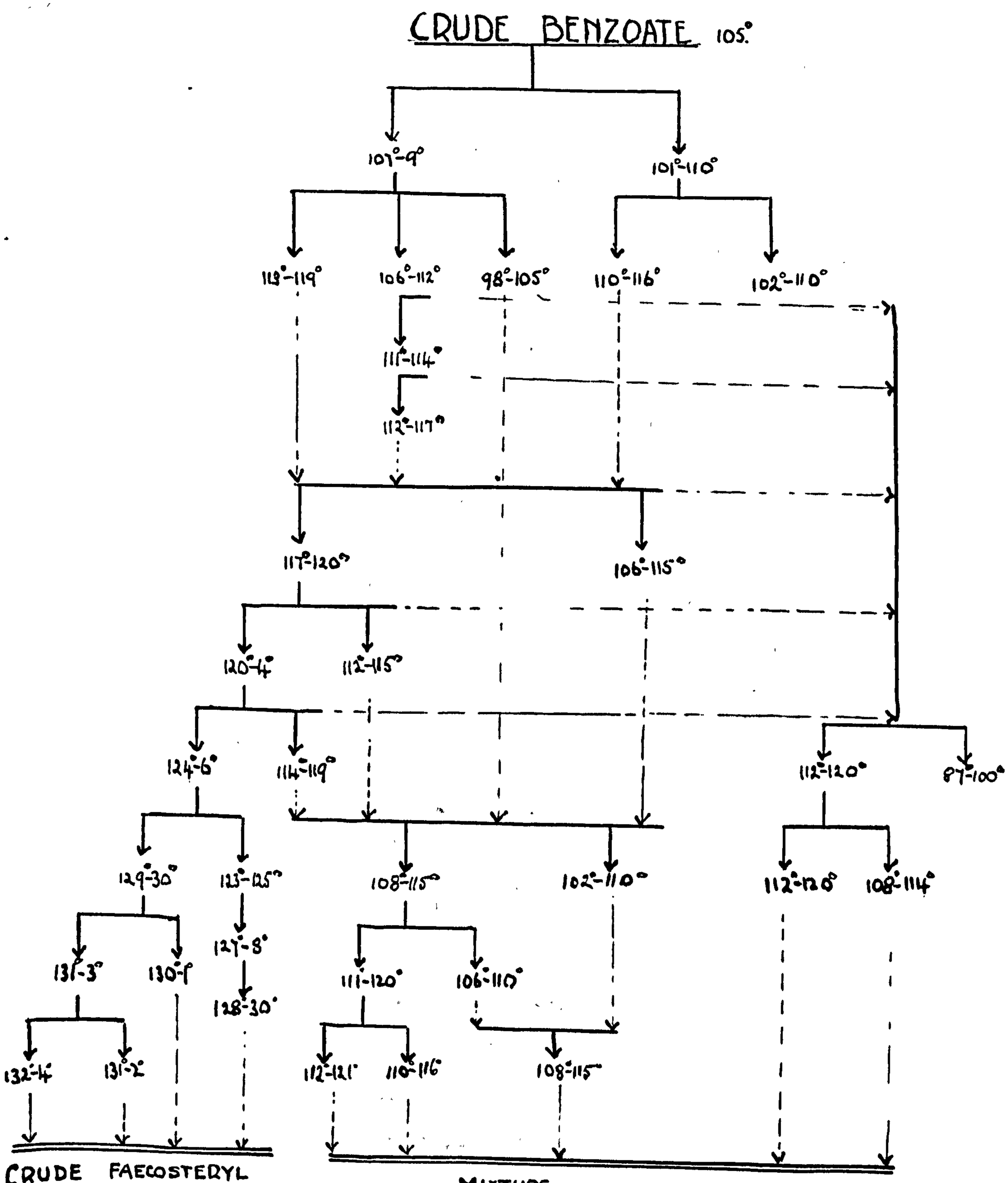
The second fraction, the melting point of which tended towards that of the previously isolated α-dihydro ergosteryl benzoate, was treated in pyridine solution with 3-4 drops of bromine. This treatment was intended to oxidise any ergosterol still present. The product after two crystallisations from benzene-alcohol solvent melted at 196-197°, which corresponds closely to that of pure α-dihydroergosteryl benzoate. There was no depression to a mixed melting point of these two benzoates.

The third fraction had not reached a constant melting point and the separation was slow. The benzoate was therefore hydrolysed with alcoholic caustic potash and refluxed in acetone with potassium permanganate and charcoal to destroy any ergosterol present. The sterol so purified crystallised from acetone in small plates melting at 150-159°. Following a further crystallisation the melting

point rose to 154-159°. Since this sterol was expected to be isolated from the next impure main fraction no further purification was attempted. For this reason also, no further work was done on the collected crops making up the fourth fraction.

Further Purification of Fraction C 2.

Fraction C 2 (52 g.) was benzoylated in the usual manner, and gave a benzoate melting approximately at 105°. The benzoate was first recrystallised from ethyl acetate and then fractionated from benzene-alcohol; the details of the fractionation are given in the following table.


CRUDE BENZOATE 105°
101°-9°
101°-110°
113°-119°
106°-112°
98°-105°
110°-116°
102°-110°
111°-114°
112°-117°
117°-120°
106°-115°
120°-4°
112°-115°
124°-6°
114°-119°
112°-120°
87°-100°
129°-30°
123°-125°
108°-115°
102°-110°
112°-120°
108°-114°
131°-3°
130°-1°
127°-8°
128°-30°
111°-120°
106°-110°
132°-4°
131°-2°
112°-121°
110°-116°
108°-115°
CRUDE FAECOSTERYL BENZOATE
MIXTURE.
SLOW SEPARATION.

CRUDE FAECOSTERYL BENZOATE.

130°

↓ HYDROLYSE.

STEROL

↓ PERMANGANATE.

148-50°

< ACETONE.

152-4° | 149-50° | 147-50°

RE BENZOYLATE.

↓

140°-2°

↓

141-3°

< BENZENE – ALCOHOL

143°-4° | 140°-2°

< ACETONE- ETHYL ACETATE.

143°-4°

↓ HYDROLYSE

157°-60°

< ACETONE

161°-2°

< ACETONE.

161°-2° | 161°-2°

PURE FAECOSTEROL.

Faecosteryl benzoate.

The top fraction (Table 6), a benzoate melting approximately at 130°, was hydrolysed with 5% alcoholic caustic potash. Since this product contained ergosterol it was first refluxed with potassium permanganate in acetone solution and then with charcoal. After this treatment the sterol melted at 148-150°, and on crystallising from acetone the melting point rose to 152-154°. During this operation it was observed that the sterol was dimorphous, the crystals appearing as long flattened needles or small plates depending upon the strength and temperature of the solution at the crystallising point.

This crude faecosterol was combined with a similar fraction obtained from a previous separation, and further purification was effected by re-benzoylation and re-fractionation.

Faecosterol (1.4 g.) was dissolved in dry pyridine (20 c.c.) and benzoyl chloride (6 g.) was added over 20 minutes; the mixture was left at room temperature for 6 hours and the benzoate precipitated with methyl alcohol (30 c.c.). The benzoate was crystallised from acetone-ethyl acetate solvent until constant melting point 144-145° was reached. Faecosteryl benzoate crystallised in thin

glistening plates and is easily soluble in benzene and chloroform, sparingly soluble in ethyl acetate, and insoluble in acetone and alcohol.

$[\alpha]_D$ + 28.2° (C_{CHCl_3} 2.62, α +0.74°).

(Found (micro): C, 82.9; H, 10.1. $C_{34}H_{50}O_2$ requires C, 83.2; H, 10.1%).

Ergosteryl benzoate melting point 168-170°)
Faecosteryl benzoate melting point 144-145°) Mixed 137°.

Faecosterol.

Faecosteryl benzoate, melting point 144-145°, (0.9 g.) was refluxed with 2.5% alcoholic caustic potash (50 c.c.) and the sterol crystallised from acetone until constant melting point 163-164° was reached. The sterol crystallises from acetone in long flattened needles, and is easily soluble in benzene, chloroform and ethyl acetate, sparingly soluble in acetone and alcohol.

$[\alpha]_D$ +33.5° (C_{CHCl_3} 4.42, α 1.48°).

(Found (micro): C, 83.8; H, 12.0. $C_{27}H_{46}O$ requires C, 83.9; H, 11.9%).

Spectroscopic Examination.

Using a concentration of sterol 50 times greater than that used in ergosterol work, no trace of any selective absorption was detected.

Colour Reactions.

With the antimony trichloride reagent a very faint pink colouration was developed slowly in concentrated solution.

The modified Tortelli-Jaffe reaction gave a brilliant green ring which persisted on shaking.

The Rosenheim reaction was negative.

Faecosteryl acetate.

Faecosterol, melting point 150-152°, (0.2 g.) was refluxed with acetic anhydride (10 c.c.) for 15 minutes. The crude product melted at 149-152°. It was recrystallised from ethyl acetate-acetone solvent until constant melting point 158-160° was reached.

Faecosterol acetate crystallises in large clear plates and is easily soluble in benzene and ethyl acetate, sparingly soluble in acetone.

(Found (micro): C, 81.7; H, 11.6. $C_{29}H_{48}O_2$ requires C, 81.3; H, 11.2%).

Purification of crude ascosterol.

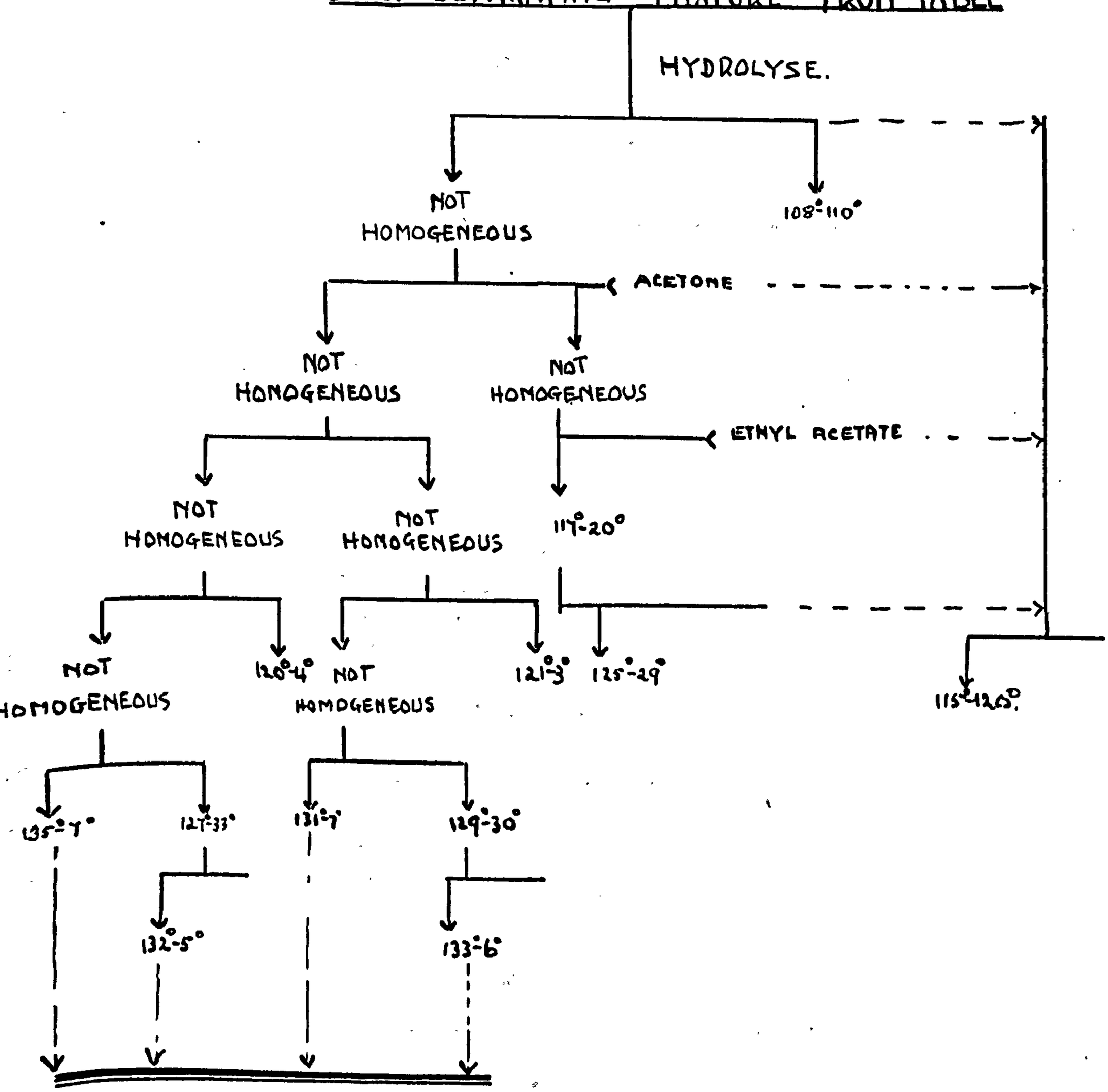
The inseparable mixture of benzoates of melting point 108-121° (Table 6) was hydrolysed and the sterol crystallised from acetone. The first crop was very

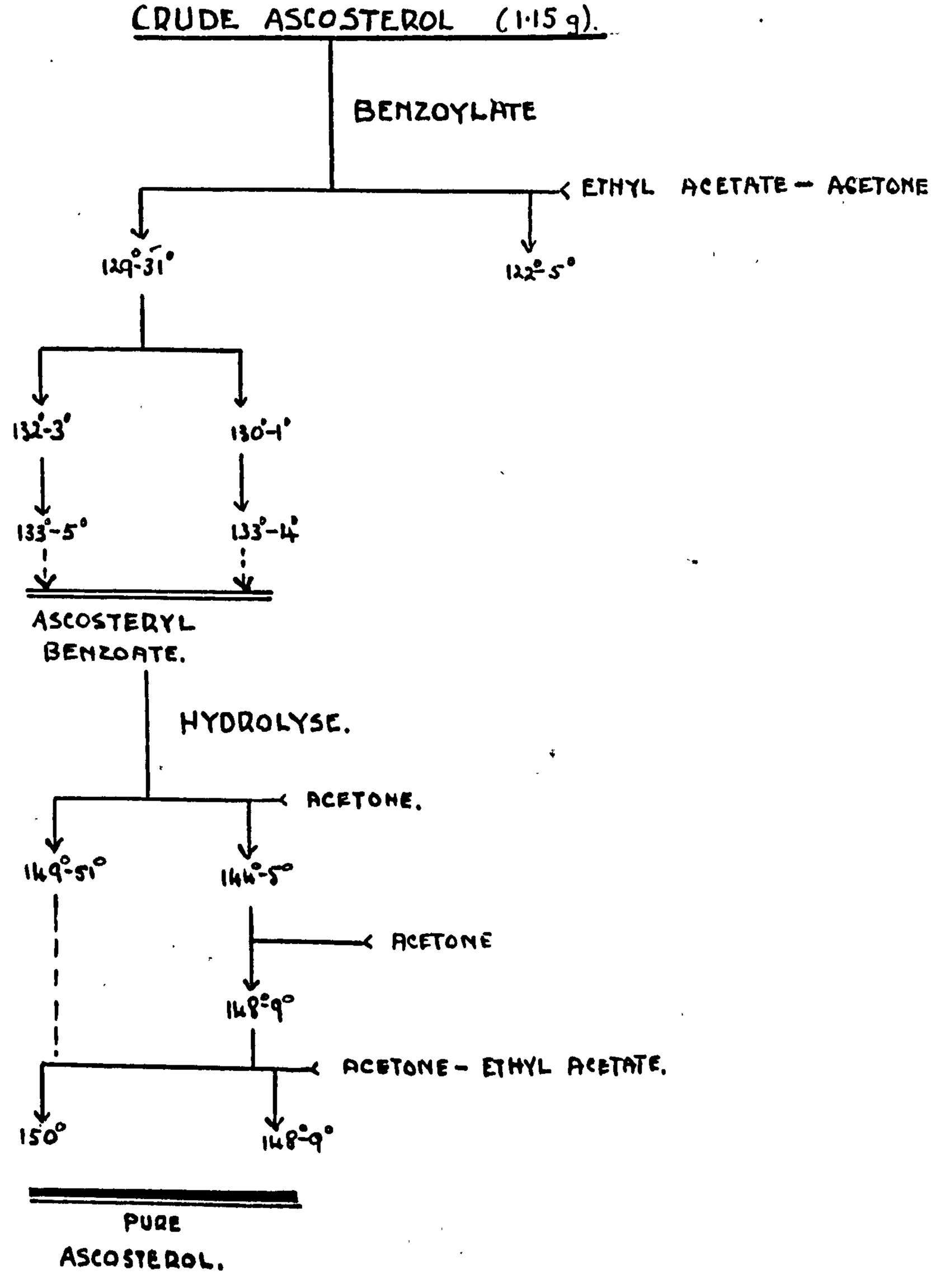
characteristic, consisting of long flattened needles covered with spots, which were seen under the microscope to be compact clusters of small opaque crystals. The solvent was changed to ethyl acetate, and after four recrystallisations a clear insoluble fraction was obtained melting approximately at 130-137°. This crop was then benzoylated. The crude sterol (1.15 g.) was dissolved in dry pyridine (20 c.c.) and benzoyl chloride (5 g.) was added over 15 minutes. The mixture was allowed to stand several hours and the benzoate was precipitated with methyl alcohol (30 c.c.). The benzoate melted after crystallising from ethyl acetone at 130-132°, and after many recrystallisations melted constant at 134-135°.

Ascosteryl benzoate crystallises in small plates and is easily soluble in benzene and chloroform, sparingly soluble in ethyl acetate and acetone.

$[\alpha]_D$ +29° (C_{CHCl_3} 2.07. α +0.60°).

SLOW SEPARATING MIXTURE FROM TABLE




CRUDE ASCOSTEROL (1·15 g).
BENZOYLATE
ETHYL ACETATE – ACETONE
129°-31°
122°-5°
132°-3°
130°-1°
133°-5°
133°-4°
ASCOSTERYL BENZOATE.
HYDROLYSE.
ACETONE.
149°-51°
144°-5°
ACETONE
148°-9°
ACETONE – ETHYL ACETATE.
150°
148°-9°
PURE ASCOSTEROL.

Ascosterol.

Pure ascosteryl benzoate (0.9 g.), m.p. 134-135° (corr.) was hydrolysed with 5% alcoholic caustic potash (20 c.c.). The ascosterol was precipitated with water and twice crystallised from acetone; it melted constant at 151° (corr.). The sterol crystallises in small feathery plates and is easily soluble in chloroform and benzene, soluble in ethyl acetate, and sparingly soluble in acetone and alcohol.

Colour reactions.

Antimony trichloride reagent, faint pink to brown in concentrated solution.

Modified Tortelli-Jaffe reaction, brilliant olive-green ring.

$[\alpha]_D$ +32.8° (C_{CHCl_3} 2.56, α +0.84°)

Faecosterol m.p. 163-164°)
) Mixed m.p. 153-158°.
Ascosterol m.p. 151°)

Ascosteryl acetate.

Pure ascosterol, m.p. 149-150° (corr.) (25 mg), was refluxed with acetic anhydride (2 c.c.). The acetate was precipitated with water(m.p. 151-152°)(mixed m.p. with original sterol 128°), separating on recrystallisation from ethyl acetate in large thin plates, m.p. 153-154°.

(Found: C,81.2; H, 11.2. $C_{29}H_{48}O_2$ Fl requires C,81.3; H, 11.2%).

Further purification of fraction C 3.

This fraction, the most soluble portion of the main fraction C, was contaminated with tarry matter and was highly coloured; consequently, with the exception of the melting point of 98-108°, complete physical identification was impossible. The crude sterol gave the three colour reactions typical of ergosterol and was still obviously a rather complex mixture. The sterol was benzoylated in the usual manner; sterol (50 g.) was dissolved in dry pyridine (150 c.c.) and benzoyl chloride (60 g.) was added over one hour, the solution being cooled in ice. The mixture was allowed to stand at room temperature for 16 hours; the benzoate was then precipitated by the addition of methyl alcohol (300 c.c.).

Fractionation of the benzoate, m.p. 93-101°, was attempted from both ethyl acetate and acetone but the separation was far too slow and ill-defined to be of practical value. The melting point of the top fraction rose during this operation from 93-101° to 103° (indefinite). The benzoate was therefore hydrolysed to the free sterol, which was twice crystallised from ethyl acetate. The

melting point of the sterol remained approximately constant in the neighbourhood of 108°, and from this it was assumed that the sterol was an impure specimen of zymosterol, and Reindel's (23) method for its complete purification was attempted. The sterol, m.p. 107-110°, (5 g.) was acetylated by refluxing with acetic anhydride (25 c.c.); the acetate was crystallised from ethyl acetate and gave three crops melting respectively at 127-128°, 117°, and 111-112°. The top fraction was dissolved in a large volume of ethyl acetate and the solution allowed to stand seven days. Soft star-like crystals were deposited, melting at 162-163° and having a specific rotation of -20.8° (C_{CHCl_3} 2.69, α 0.56°).

The acetate gave the following colour reactions :-

Antimony trichloride, pink to deep red.

Rosenheim's reaction, faint pink to green to intense blue

Modified Tortelli-Jaffe, intense green ring.

A mixed melting point of the acetate and ergosteryl acetate (172-173°) melted at 164-168°. That this crop was ergosteryl acetate was confirmed by hydrolysis, the free sterol melting at 160° after three crystallisations from ethyl alcohol, and gave no depression to a mixed melting point with ergosterol (m.p. 159-160°).

The melting point of the remaining crops ruled out any possibility of their being the zymosterol, as reported by Reindel (22), but since it had been observed, in the previous work of Messrs. Berk's sterol residues, that a steryl acetate of melting point 123-124° could be prepared from a stable dibromide, the acetate was brominated. The acetate, m.p. 117°, (1 g.) was dissolved in dry ether (20 c.c.) and a 5% solution of bromine in glacial acetic acid containing 10% of ammonium acetate (20 c.c.) was slowly added. The solution remained perfectly colourless, and after standing 5 minutes was extracted with water to remove acetic acid and ammonium acetate; the final washings contained sodium bicarbonate to remove the last traces of acetic acid. On evaporation three crops were obtained melting at 165-166°, 154-155°, 130°. Of these crops only the final one was coloured.

Finally, all the sterol crops were acetylated, and the acetates possessing similar melting points were combined and brominated. The products of bromination were found to fall into three distinct and well defined groups :- (1) m.p. 164° (approximately), (2) m.p. 155°, (3) m.p. 130°. These fractions were therefore combined and a complete fractionation was carried out as shown in the following table.

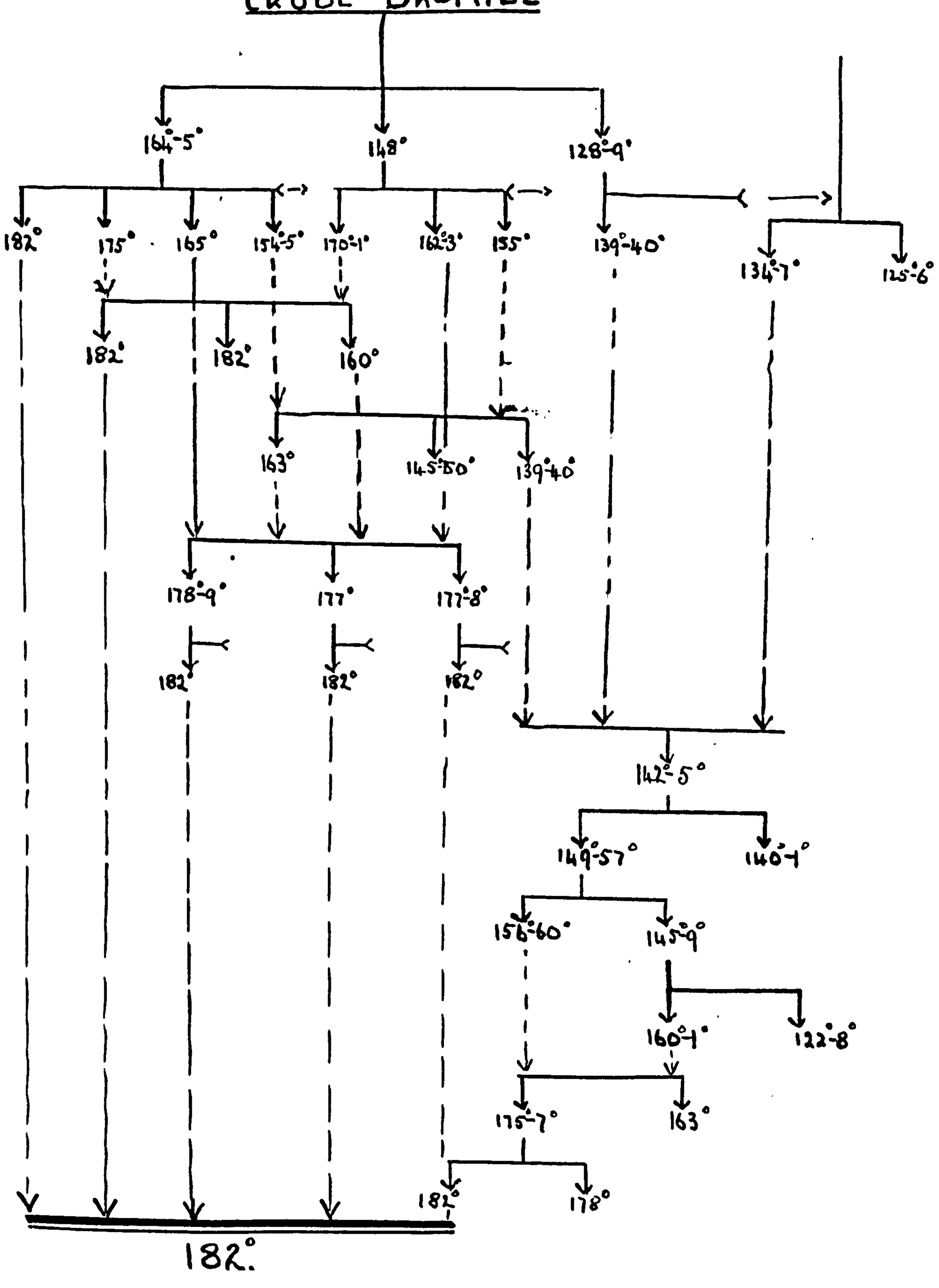
PURIFICATION OF FRACTION D.

Owing to the gelatinous fat-like residues occuring in the final filtrates of previous fractions, and also to the impossibility of obtaining good yields of steryl benzoates, the assumption was made that the original sterol residues had been incompletely saponified. Fraction D was therefore saponified before any separation was attempted. The alkaline solution rapidly darkened and finally became quite black, the greater portion of the colour, however, was removed in the aqueous extract.

Fraction D (75 g.) was refluxed for two hours with a 5% solution of sodium ethoxide (250 c.c.). Any excess sodium ethoxide was destroyed by the addition of an equal volume of water and the alcohol then distilled off. The residue was extracted with ether and the soap removed by repeated aqueous extractions. When free from alkali the ethereal solution was dried over anhydrous sodium sulphate and then evaporated to small bulk. Methyl alcohol (150 c.c.) was added and the solution left in the ice chest over-night. The nonsaponifiable matter which crystallised in small plates was removed and washed thoroughly with methyl alcohol. A yield of 35 g. of sterol melting at 90-95^{o} was obtained.

The sterol prepared as above was acetylated, twice crystallised from ethyl acetate and brominated. The acetate,

m.p. 115°, (17 g.) was dissolved in dry ether (340 c.c.) and a 5% solution of bromine in glacial acetic acid containing 10% anhydrous ammonium acetate (170 c.c.) was added. The solution remained perfectly colourless and after standing a short while was washed well with water to remove acetic acid and ammonium acetate. The last traces of acetic acid were removed by washing finally with a dilute solution of sodium bicarbonate. The ether solution was dried over anhydrous sodium sulphate and evaporated. The resulting bromide was fractionated as shown in the following table.


CRUDE BROMIDE
164°-5°
148°
128°-9°
182°
175°
165°
154°-5°
170°-1°
162°-3°
155°
139°-40°
134°-7°
125°-6°
182°
182°
160°
163°
145°-50°
139°-40°
178°-9°
177°
177°-8°
182°
182°
182°
142°-5°
149°-57°
140°-1°
156°-60°
145°-9°
160°-1°
122°-8°
175°-7°
163°
182°
178°
182°

PURIFICATION OF FRACTIONS E AND F.

Both fraction E and F contained large quantities of oily matter which was thought to be unchanged glyceryl esters of fatty acids and they were therefore saponified by refluxing in an atmosphere of nitrogen with 10% sodium ethoxide (500 c.c.) for two hours. The resulting solution was diluted with water to decompose unchanged sodium ethoxide and the excess alcohol removed under reduced pressure. The residue was extracted with ether and the ethereal extract washed with water until free from alkali and soaps; in this operation it was observed that the greater part of the colouring matter passed into the aqueous extract. The ethereal solution was dried over anhydrous sodium sulphate and a hard resinous solid was obtained on removal of the ether.

Since, as was observed in the previous fractionation, teh separation of the soluble sterols by means of the steryl benzoates was not successful, the method given by Wieland and Gough (loc.cit.) was applied to this fraction.

The crude sterol (22 g.) was dissolved in dry pyridine (75 c.c.) and a solution of p-nitro benzoyl chloride (30 g.) dissolved in dry benzene (50 c.c.) was added. The mixture was well stirred and then allowed to stand. After remaining for four hours at room temperature the almost solid mass was treated with ice-water to decompose any additive compound of the p-nitro benzoyl chloride and the pyridine. The

resulting mixture was extracted with benzene and the benzene layer washed, first with water to remove the greater portion of the pyridine, then with dilute hydrochloric acid and dilute caustic soda solution, and finally with water to remove any trace of the caustic soda. The benzene was distilled off under reduced pressure and the tarry residue taken up in ether, which solvent only dissolves the steryl p-nitro benzoates. The ethereal solution was decanted, evaporated to small bulk and the p-nitro benzoates precipitated by addition of methyl alcohol.

An attempt was made to fractionate the steryl-p-nitro benzoates from various solvents, but no definite crystals could be obtained and the melting point of the more insoluble portion rose very slowly from 100-110^{0} to 118-120^{o}. As it appeared that this method could not yield a pure product the still impure p-nitro benzoate mixture was hydrolysed with alcoholic potassium hydroxide and the sterol acetylated in the usual manner.

The melting point of the acetate, after three re-crystallisations from ethyl acetate, rose from 110-113^{o} to 119-^{o}, and as this melting point was tending to that of the acetate obtained by the bromine separation method it was brominated following the usual experimental procedure.

After two recrystallisations from acetone-chloroform solvent the acetyl bromide melted constant at 182°. This acetyl dibromide gave no depression to a mixed melting point with the acetyl dibromide obtained from the previous fractionation, and they were therefore combined.

Acetyl debromide m.p. 182°

The insoluble fraction, m.p. 182°, was sparingly soluble in ether and was better crystallised from chloroform-methyl alcohol solvent. It was obtained from this solvent in small thin plates.

Analysis. (Found (micro): C,60.1; H,7.9; Br.27.3. $C_{29}H_{46}O_2Br_2$ requires C,59.7; H,7.9; Br,27.3%).

Hydrolysis of acetyl dibromide.

Acetyl dibromide, m.p. 182°, (1 g.) was suspended in cold 5% alcoholic caustic potash (50 c.c.) and shaken for 24 hours. Water was added until there was no further precipitation of bromide. The crude product melted at 167-168°. It was recrystallised from chloroform-methyl alcohol and melted constantly at 168°. There was no depression to a mixed melt with the dibromide obtained in the first section of this work.

Acetylation of the bromide m.p.168°

Sterol dibromide, m.p. 168°,(1 g.),obtained in the first section of this work by bromination of the free sterol, was refluxed with acetic anhydride (5 c.c) for 15 minutes. The excess of acetic anhydride was destroyed by refluxing with methyl alcohol, and the solution was allowed to crystallise. The crude product melted at 157°; it was recrystallised from chloroform-methyl alcohol solvent and then melted at 179-180°. A mixed melt with acetyl dibromide, m.p. 182°, prepared hy bromination of the steryl acetates, melted at 180°.

Debromination of the acetyl dibromide (Reindel).

Acetyl dibromide, m.p. 182°, (1 g.) was suspended in 95% acetic acid (75 c.c.) and shaken for 20 hours with oxide-free zinc dust (5 g.). The solution was filtered to remove the unchanged zinc and the steryl acetate was precipitated by addition of water. M.p. of crude product 103-104°. The acetate was recrystallised from ethyl acetate. It crystallises from this solvent in feathery plates melting unchanged at 103-104°.

Analysis

(Found (micro): C,80.9; H,10.9. $C_{29}H_{48}O_2$ requires C,81.3; H,10.9%).

Debromination of acetyl dibromide, m.p. 182 (Heilbron)

The acetyl dibromide (0.5 g.) was refluxed in 95% alcohol (25 c.c.) with oxide-free zinc dust for four hours. The solution was filtered to remove the unchanged zinc and the steryl acetate precipitated by the addition of water. The crude product, m.p. 109-110°, was crystallised from ethyl acetate and separated from this solvent in large plates, m.p. 118-119°. On recrystallisation from the same solvent the melting point rose to 122-123°; there was no depresion to a mixed melting point with the pure zymosteryl acetate obtained in the first section.

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