The Determination of Ergosterol in Yeast

Part I. The Ultra-violet Absorption of Purified Ergosterol

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Most published records agree on the wavelengths of the three main peaks in the ultra-violet absorption curve of ergosterol, but there are discrepancies in the extinction values reported for the various maxima, apparently because of the difficulty of preparing pure ergosterol by direct recrystallisation of the commercial material and to a lesser extent because of small variations in the moisture content of the hydrated sterol. Simple recrystallisation does not always yield a pure product; the best means of purification is by recrystallising a suitable ester, with subsequent regeneration of the sterol.

The preparation and purification of ergosterol benzoate is described, and the physical properties and ultra-violet absorption (in various solvents) of a purified specimen of ergosterol are recorded.

Methods for determining small amounts of ergosterol are mostly based either on colour reactions or on measurements of ultra-violet absorption. A number of colour reactions are known[1,2,3] and some can be applied quantitatively to pure solutions. Similar colours, however, are given by many other sterols. The colorimetric method with acetic anhydride and zinc chloride,[4,5] modified by Pesez and Herbain,[6] appears to be specific for ergosterol amongst the yeast sterols, but this method was found to give insufficiently reproducible results, even when applied to purified ergosterol.

The most satisfactory method of detecting and determining small amounts of ergosterol is based on ultra-violet absorption measurements. The absorption of ergosterol is of high intensity and characteristic of Δ-5:7 unsaturated sterols, amongst which 7-dehydrocholesterol[7] is the only other one of importance known to occur naturally. Well-defined maxima occur at 271·5, 282 and 293·5 mμ in absolute alcohol, with a marked inflection at about 263 mμ

and a smaller inflection in the region of 253 mμ (Fig. 1). Most literature reports agree on the position of the maxima, but discrepant results are found for the extinction value of the pure compound at the main maximum (Table I).

The methods of purifying the samples used in obtaining the figures in Table I require some comment. No details of methods are given in the first three publications mentioned.

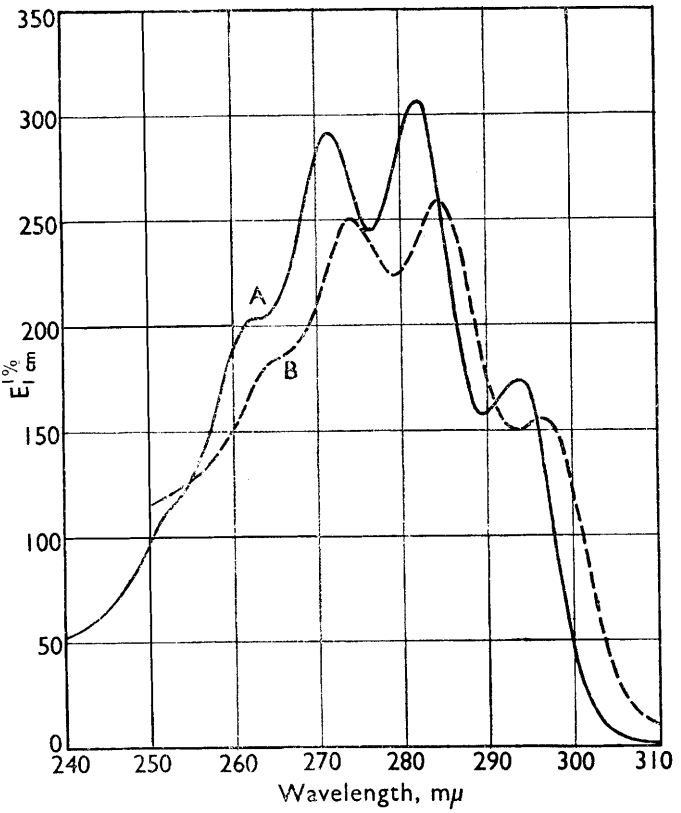

Fig. 1. Absorption curves. Curve A, ergosterol in absolute alcohol; curve B, ergosterol benzoate in chloroform. Concentrations approximately 0·002 per cent. w/v

Hogness *et al.*[11] recrystallised ergosterol from ethyl alcohol and benzene and then twice from *iso*octane. Subsequently, Huber *et al.*[7] showed that Hogness's values were too low and attributed this to decomposition during the several recrystallisations. They stated that the extinction values were highest on recrystallising the commercial material once only from a mixture of alcohol and benzene. Lamb, Mueller and Beach,[12] unable to confirm this, preferred recrystallisation from acetone containing 1 per cent. of water and obtained results about 4 per cent. higher. It thus appeared desirable to investigate again the extinction values in the solvents it was proposed to use subsequently for the determination of ergosterol.

TABLE I

RECORDED EXTINCTION VALUES OF ERGOSTEROL AT 281·5 TO 282 mμ (MAX.)

Authors	$E^{1\%}_{1\,cm}$	ε
Morton, Heilbron and Kamm[8]	—	10,200
Morton and Gillam (quoted by Bacharach, Smith and Stevenson[9])	333	13,800*
Morton and de Gouveia[10]	—	11,700
Hogness, Sidwell and Zscheile[11]	—	10,600
Huber, Ewing and Kriger[7]	—	11,500
Lamb, Mueller and Beach[12]	289	12,000*

* ε calculated, assuming the monohydrate composition.

The chief impurities likely to be encountered in commercial ergosterol are zymosterol and 5-dihydroergosterol. Callow[13] stated that the former, but not 5-dihydroergosterol, could be eliminated by recrystallisation from an alcohol - benzene mixture. By recrystallisation of the benzoate and subsequent regeneration of the sterol he obtained a purified product, but unfortunately did not record its ultra-violet absorption values. Our experience supports the view that recrystallisation of the sterol does not invariably yield a pure product and that purification is most satisfactory after recrystallisation of a suitable ester.

Ergosterol crystallises from hydrated solvents with one molecule of water of crystallisation ($C_{28}H_{44}O.H_2O$ requires 4·3 per cent.), but the moisture content as found by analysis normally differs a little from the theoretical figure, and it appears necessary to carry out this determination on any material to be used as standard. To avoid any confusion, our results, unless otherwise stated, are expressed throughout in terms of anhydrous ergosterol.

Experimental

Spectrophotometers—The greater part of this work was carried out with a Unicam spectrophotometer (model SP 500), the more important results being confirmed on a second Unicam or a Uvispek instrument. The wavelength scale was checked as a routine with the 486·1-mμ hydrogen line and occasionally with the mercury lines at 313·2 mμ. The maximum error in wavelength near the ergosterol maxima appeared to be about 0·2 mμ.

The optical density scale and cell thickness (1 cm) were checked by means of 0·006 per cent. w/v potassium dichromate in slightly acid solution, in the manner described by Cama, Collins and Morton,[14] with whose figures ours were in close agreement ($E^{1\%}_{1cm}$ equal to 124·6, 144·8, 48·8 and 106·8 at 235 mμ (min.), 257 mμ (max.), 313 mμ (min.) and 350 mμ (max.), respectively).

The two main maxima on the ergosterol absorption curve (Fig. 1) are sharp and the band-width used has some effect on the measured optical density at the maxima for any given solution. The maximum permissible band-width does not have to be found when a spectrophotometer designed to be operated at a constant band-width of the order of 0·5 mμ is used. Nevertheless, spectrophotometers requiring a constant energy level should be operated at the narrowest slit-width (and hence band-width) compatible with adequate electrical sensitivity of the instrument at the particular wavelength used. In practice, on a Unicam instrument, slit-widths up to 0·5 mm at 282 mμ and 0·6 mm at 271·5 mμ may be used without perceptibly lowering the observed optical densities. These slit-widths correspond, according to the manufacturer's formula, to nominal band-widths of 1·8 and 1·9 mμ, respectively. To satisfy these requirements the hydrogen lamp must be of high emission at the wavelengths used and any solvent used must be of good transmittancy.

Solvents—

Absolute alcohol—Usually the laboratory reagent grade of absolute alcohol is satisfactory without further treatment.

cyclo*Hexane*—The commercial grade of *cyclo*hexane usually contains traces of benzenoid impurities that cause a marked decrease in transmittancy below 282 mμ. As a rule these are insufficient to affect readings at the 282-mμ ergosterol maximum, but, when additional readings are required at the 271·5-mμ maximum, it is necessary to use *cyclo*hexane containing as little as possible of these impurities. Alternatively, *cyclo*hexane specially purified for spectroscopy can be used.

Chloroform—Analytical reagent grade chloroform usually requires no further treatment.

Ethylene dichloride—Commercial ethylene dichloride can be freed from traces of acid and other impurities by shaking it twice with a small volume of 50 per cent. w/w aqueous potassium hydroxide. The aqueous washings are discarded and the solvent is filtered through a dry paper, dried with phosphorus pentoxide, filtered again and distilled; the first and last tenths are discarded.

Preparation and purification of ergosterol—

Direct recrystallisation—The three methods generally used for the direct purification of ergosterol involve recrystallisation from a mixture of 1 part of 95 per cent. alcohol and 2 parts of benzene,[7] 95 per cent. alcohol[13] or acetone containing 1 per cent. of water.[12] To test the efficiency of these methods of purification, 50-g portions of commercial ergosterol

of good quality ($E^{1\%}_{1cm}$ (anhyd.) at 282 mμ = 294 in absolute alcohol) were recrystallised a number of times from each solvent. After each recrystallisation the ergosterol was dried *in vacuo* over calcium chloride and the moisture determined in a "pistol"-type apparatus with boiling toluene (b.p. 110° C) in the outer jacket. The ultra-violet absorption in absolute alcohol was determined after each recrystallisation; the results are recorded in Table II.

TABLE II

RECRYSTALLISATION OF ERGOSTEROL

Solvent	Number of recrystallisation	Amount lost at 105° C *in vacuo*, %	$E^{1\%}_{1cm}$ (calculated as anhydrous) 271·5 mμ (max.)	282 mμ (max.)	293·5 mμ (max.)
Alcohol (95 per cent.) - benzene mixture (1 + 2)	1	5·0	283·0	297·3	169·5
	2	4·9	283·3	298·4	169·7
	3	4·8	284·5	299·0	170·3
	4	4·9	285·1	300·5	170·4
	5	5·0	287·2	303·1	172·3
	6	4·9	285·8	301·0	171·1
Alcohol (95 per cent.)	1	4·5	279·5	294·3	167·5
	2	4·5	280·3	295·0	167·9
	3	4·2	279·2	294·6	167·6
	4	4·6	282·7	298·0	168·8
	5	4·8	282·8	297·2	169·0
Acetone containing 1 per cent. of water	1	4·8	280·1	295·4	167·7
	2	3·7	281·4	296·7	168·6
	3	4·9	283·0	298·9	170·3
	4	4·9	284·3	299·3	170·1
	5	4·9	282·5	297·9	169·4

They show that alcohol - benzene appears to be the most satisfactory solvent, although acetone with 1 per cent. of water is only slightly less efficient. Extinction values, however, are 1 to 2 per cent. lower than those subsequently obtained after purification by means of the benzoate.

Purification through ergosterol benzoate—A 50-g portion of the same commercial sample as that used for the recrystallisations just recorded was benzoylated in dry pyridine, substantially as described by Callow.[13] The resulting material (46 g) was recrystallised five times from ethyl acetate to give a product having physical constants (Table III) close to those quoted by Callow. The ultra-violet absorption curve for the purified benzoate in chloroform is recorded in Fig. 1.

TABLE III

RECRYSTALLISATION OF ERGOSTEROL BENZOATE FROM ETHYL ACETATE

Recrystallisation	Melting point, ° C	$(\alpha)^{20}_{D}$	$(\alpha)^{20}_{546\cdot1\,m\mu}$	$E^{1\%}_{1cm}$ (max.) at 284 to 284·5 mμ in chloroform approx. 0·002% w/v
1	169·8 to 170·4	−66·5	−87·5	255·0
2	170·8 to 171·5	−67·7	−87·1	259·0
3	170·8 to 171·3	−67·6	−87·5	257·4
4	170·8 to 171·3	−67·7	−87·2	257·5
5	171·0 to 171·3	−68·0	−88·0	258·9*

* $E^{1\%}_{1cm}$ (max.) at 273·5 to 274 mμ = 250·5, $E^{1\%}_{1cm}$ (max.) at 296 mμ = 156.

The purified benzoate (10·4 g) was hydrolysed with 3 per cent. w/v alcoholic potassium hydroxide; the regenerated sterol was washed with alcohol and water and then recrystallised once from 95 per cent. alcohol (yield 7 g). All recrystallisations and the hydrolysis were carried out in an atmosphere of nitrogen to prevent oxidation.

The purified hydrated sterol was obtained as colourless crystals, m.p. 163·6 to 164·4° C, $(\alpha)^{20}_{D}$ −129·5° (−136·0° as anhydrous), $(\alpha)^{20}_{546\cdot1\,m\mu}$ −165·6° (−174·0° as anhydrous), loss *in vacuo* at 105° C, 4·8 per cent. ($C_{28}H_{44}O.H_2O$ requires 4·3 per cent.).

Portions of this material were stored in sealed nitrogen-filled ampoules kept in a refrigerator. The absorption curve in absolute alcohol is shown in Fig. 1 and the specific

and molecular extinction coefficients in the solvents mentioned above are recorded in Table IV.

Discussion of results

The discrepancies among the extinction values of ergosterol found in the literature appear to have arisen through failure to prepare pure samples by direct recrystallisation and, to a lesser extent, through small differences in moisture content of the material after recrystallisation. By recrystallisation of commercial samples, notably from a mixture of alcohol and benzene, almost pure ergosterol can be obtained, but the most reliable method involves purification through a suitable ester such as the benzoate. Huber *et al.*[7] prepared several nitrobenzoyl esters, but did not use them as means of purifying ergosterol. The extinction values in absolute alcohol for the hydrated sterol shown in Table IV are about 5 per cent. higher than those recorded by Huber *et al.*[7] and very slightly higher than those of Lamb, Mueller and Beach,[12] with whose results we are in substantial agreement. For the exceptionally high result obtained in 1933 by Morton and Gillam, and quoted by Bacharach, Smith and Stevenson,[9] we have no explanation to offer.

Table IV

Mean extinction values for purified ergosterol

Solvent	Number of determinations	Wavelengths of maxima ($\pm 0{\cdot}5$ mμ), mμ	$E^{1\%}_{1cm}$ of hydrated ergosterol*	$E^{1\%}_{1cm}$ of anhydrous ergosterol	Molecular extinction coefficient, ϵ
Absolute alcohol ..	9	271·5	276·3	290	11,500
		282·0	291·1 ($\pm 0{\cdot}34$)†	306	12,150
		293·5	165·9	174	6,900
Chloroform	2	274·5	240·7	253	10,000
		284·5	260·2	273	10,850
		296·0	158·3	166	6,600
Ethylene dichloride ..	4	273·5	258·9	272	10,800
		283·5	274·7	289	10,450
		295·5	159·0	167	6,650
*cyclo*Hexane	4	271·5	268·6	282	11,200
		282·0	284·7	299	11,900
		294·0	162·4	171	6,750

* Loss at 105° C *in vacuo*, 4·8 per cent. Results for hydrated ergosterol are given to four figures, as observed. The remaining extinction values have been rounded off to be consistent with the precision of the measurements.

† Standard error of the mean.

In view of these discrepancies we thought it advisable to have the main extinction values of our material checked by Professor Morton, who kindly undertook an independent examination of a portion of the purified hydrated sterol. He reported a maximum at 282 mμ, $E^{1\%}_{1cm}$ of 291, in absolute alcohol and a maximum at 282·5 mμ, $E^{1\%}_{1cm}$ of 286, in *cyclo*hexane (not corrected for moisture). He also observes that $E^{1\%}_{1cm}$ at 281·5 mμ must be taken as between 286 and 290 for the monohydrate of ergosterol, which would correspond with 299 to 303 for the anhydrous material, if the hydrate contains ergosterol and water in molar proportions.

He adds that, in the Liverpool laboratories, the maximum absorption is generally found to be at 281·5 mμ, compared with 281·8 mμ estimated on our particular instrument.

It will be seen that agreement between the values obtained at Liverpool and by us for purified ergosterol is excellent. Confidence in the results is increased when it is remembered that agreement was also good for potassium dichromate solutions measured by different observers with photo-electric instruments of different types.

Any analyst proposing to use ultra-violet absorption methods for determining ergosterol should check the wavelength scale of his particular instrument by means of a suitable light source emitting in the region of 282 mμ and check the optical density readings against dichromate in the manner described. Further calibration with specially purified ergosterol should not then be necessary.

In certain instances an analyst may desire to work against a "standard preparation" of the purest ergosterol available. Provided that the examination of a reference standard such as dichromate does not reveal any instrumental errors and the extinction values of

his material do not depart significantly from the values given in Table IV, he can safely use his own figures for analytical purposes.

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