Isolation from yeast of a metabolically active water-soluble form of ergosterol

B. G. ADAMS and L. W. PARKS

Department of Microbiology, Oregon State University, Corvallis, Oregon 97331

ABSTRACT A water-soluble complex containing ergosterol together with a component of yeast has been isolated. The complex can be isolated from commercial yeast extract to which ergosterol has been added or directly from whole yeast cells. The complexing component has the properties of a large polysaccharide and the binding between the sterol and the polysaccharide appears to be noncovalent. The complex is easily prepared and is stable in aqueous solution; ergosterol in this solution is metabolically available to yeast cells to which it is added.

KEY WORDS ergosterol · soluble form · complex · yeast extract · digitonide · dimethyl sulfoxide

If a soluble, metabolically active form of sterol which could be incorporated into growth media or cell-free reaction mixtures without the imposed deleterious effects of the solubilizing vehicle were available, it would greatly facilitate investigations of the physiological functions of sterols. We recently observed that various culture media would retain ergosterol in solution. Some components of these media were capable of binding exogenously supplied sterol in such a way that it could no longer be extracted with organic solvents. This paper describes the isolation of the complexing agent from artificial mixtures of ergosterol and yeast extract and from whole yeast cells. The agent has been tentatively characterized as a polysaccharide.

MATERIALS AND METHODS

Materials

The following materials were commercial products: ergosterol from Sigma Chemical Company, St. Louis, Mo.; blue dextran, Sephadex G-50, and glass chromatography columns from Pharmacia Fine Chemicals Inc., Piscataway, N.J.; Silica Gel G from Brinkmann Instruments Inc., Westbury, N.Y.; digitonin from Calbiochem, Los Angeles, Calif.; reagent grade dimethylsulfoxide from J. T. Baker Chemical Co., Phillipsburg, N.J.; and yeast extract from Difco Laboratories, Detroit, Mich. Nitrocellulose membrane filters were type B 6, obtained from Schleicher & Schuell Co., Keene, N.H.

Preparation of the Complexing Agent from Yeast Extracts

90 g (wet weight) of Fleischmann's dry yeast was aerated in an aeration medium consisting of 0.1 M KH_2PO_4 and 1% glucose for 4 hr, centrifuged, washed with distilled water, resuspended in distilled water (total volume 400 ml), and refluxed for 4 hr. The suspension was filtered while still hot through Whatman No. 2 filter paper and through a nitrocellulose filter to give a clear yellow solution which was lyophilized and resuspended in distilled water to give a 15% w/v solution. 12 ml of this solution was chromatographed on a Pharmacia glass column, 2.5 cm I.D., packed to height of 31 cm with Sephadex G-50. The column had a void volume of 54 ml as determined with blue dextran. The column was eluted with 0.1 M phosphate buffer (6.53 g of K_2HPO_4 and 8.51 of KH_2PO_4 per liter, final pH 6.6) at a flow rate of 3.0 ml/min. After collection of the first 40 ml of eluent, samples were collected at 1 min intervals for 15 min. Each sample was combined with an equal volume of 95% ethanol, and the precipitate was sedimented by low-speed centrifugation. This high molecular weight fraction was dissolved in distilled water and reprecipitated with an equal volume of 95% ethanol. The precipitate was dried by lyophilization.

Preparation of Ergosterol Complex

To "15% yeast extract," prepared as described above, enough of a 1 mg/ml solution of ergosterol in absolute

Abbreviations: DMSO, dimethyl sulfoxide; TLC, thin-layer chromatography.

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ethanol was added to give a final concentration of 150 μg/ml. Uncombined sterol was removed by hexane extraction, and 50 ml of solution was passed through a column of Sephadex G-50 (4 cm I.D.; height; 51 cm). Elution was performed with 0.1 M phosphate buffer, pH 6.6. The opalescent solutions emerging just after the void volume of the column were examined and found to contain ergosterol.

Isolation of Sterol

We carried out "methanolic pyrogallol saponification" by adding 0.5% pyrogallol in absolute methanol–60% KOH–absolute methanol 3:2:3 to precipitated or lyophilized column fractions in extraction tubes fitted with condensers, and by refluxing for 1.5 hr. "Methanolic pyrogallol acid hydrolysis" was performed in the same manner except for the substitution of an equal volume of 1 N HCl for the 60% KOH. Simple "acid hydrolysis" consisted of making samples 0.1 N with respect to HCl by addng 0.2 N HCl, heating for 1 hr in an Arnold sterilizer, adjusting the pH to 10, and extracting the lipid with *n*-hexane.

Sterol was recovered from the Sephadex column fractions either by precipitation with digitonin or cleavage with DMSO. Precipitation with digitonin consisted of combining the column fractions containing sterol with different ratios of 0.5% digitonin in 50% ethanol. The digitonides were stored at −20°C overnight, centrifuged, washed twice with acetone–diethyl ether 1:1 and once with diethyl ether, and dried under nitrogen. They were dissolved in a 3.0-ml volume of glacial acetic acid, and the Liebermann-Burchard color was measured against a reagent blank and standard solutions of ergosterol digitonide.

When cleavage with DMSO was used, the column fractions containing sterol were first combined with equal volumes of 95% ethanol. The samples were well mixed and stored overnight at −20°C. The tubes were then centrifuged at 14,000 *g* for 15 min, and the supernatant solutions were decanted and discarded. 5 ml of DMSO was added to each sample. The tubes were covered with aluminum foil, heated for 1 hr in an Arnold sterilizer, and cooled. The contents were extracted with 3 × 10 ml of *n*-hexane, and the extracts were evaporated to dryness under nitrogen. Their sterol content was determined by the Liebermann-Burchard reaction after the residues had been dissolved in chloroform (1).

TLC and Detection of Sterol

TLC was carried out on silica gel G (25 g/55 ml distilled water) spread to a thickness of 0.375 mm on 20 × 20 cm glass plates with a Desaga-Brinkmann spreader. The plates were developed in benzene–ethyl acetate 5:1. Sterol was detected on thin-layer plates either by heating the plates at 100°C for 5 min and spraying them with 20% perchloric acid (2) or by spraying the plates at room temperature with 20% antimony pentachloride in chloroform (3).

Yeast Culture

Yeast mutant strain KD46, which requires either ergosterol or oleic acid for growth (4), was cultured on agar plates of Wickerham's defined medium (5) and on plates containing Wickerham's defined medium to which column fractions containing 50 μg/ml of ergosterol had been added to give a final concentration of 12.5 μg/ml. Additional plates of Wickerham's medium containing an equal amount of complexing agent but without ergosterol were also utilized. All cultures were incubated at 30°C, and the time of appearance of colonies was noted. Purity of the resultant growth was determined by microscopic examination.

RESULTS

That a water-soluble ergosterol complex is formed with yeast extract and that it is not dialyzable through ordinary dialysis membranes was shown as follows. A 5% w/v solution of yeast extract to which ergosterol has been added to a final concentration of 50 μg/ml was extracted with petroleum ether to remove the uncombined sterol. The resulting aqueous solution was extensively dialyzed against 0.1 M phosphate buffer and was then lyophilized. The dried powder was redissolved in a small volume of water, subjected to pyrogallol saponification, and extracted with petroleum ether. The Liebermann-Burchard color test and TLC showed that the extract contained sterol. There was no significant loss of sterol through the dialysis membrane. The complexing material appears to be of large molecular weight, since it could not pass through the membrane.

When the solution of sterol complex was applied to thin-layer plates which were then developed in benzene–ethyl acetate 5:1, the carrier component remained at the origin while the ergosterol separated as the free sterol. These results indicated that the binding was noncovalent and suggested the possibility of separating the sterol from the complexing agent with the aid of digitonin. Table 1 shows that the sterol can indeed be precipitated from the complex with digitonin. The addition of the ethanolic digitonin solution, however, introduces a complication in that too much alcohol precipitates the yeast complex, and this makes the estimation of sterol in aqueous solutions of complex where the amount is unknown less satisfactory. The sterol is recoverable from the precipitated sterol complex with DMSO as described below.

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TABLE 1 DIGITONIN PRECIPITATION OF ERGOSTEROL OUT OF THE YEAST COMPLEX

Conditions		Yield of Sterol	Ratio Sterol: Complex
Complex*	Digitonin		
ml		*μg*	*μg/ml*
3.0	1.0	190	63
3.0	1.0	190	63
3.0	2.0	194	65
3.0	2.0	199	67
5.0	2.5	291	58
5.0	2.5	266	53

* "Complex," aqueous solution of purified complex from commercial yeast extract.

Ergosterol digitonide, when similarly applied to thin-layer plates after exhaustive washing with acetone–ether 1:1 to remove free sterol, also gives rise to a free sterol spot, but in this case some sterol also remains at the origin. This may mean that the noncovalent bonding of ergosterol to digitonin is "stronger" than to the yeast component, or it may merely reflect the lower solubility of the digitonide in the developing solvent, benzene–ethyl acetate 5:1.

It was determined that the complex was precipitated with 50% alcohol and could be easily redissolved in water. In solution it gave a red color when treated with iodine solution. When applied to the surface of thin-layer plates or to filter paper and dried, the complex gave a negative ninhydrin reaction but gave an intensely positive reaction when sprayed with periodate and then benzidine. The presence of a compound with vicinal hydroxyl groups is indicated.

Lyophilized hot-water extracts of whole dry yeast were assayed to determine if the sterol complex could be isolated from that source. A 15% w/v solution of this extract was compared to a similar solution to which 150 μg/ml ergosterol had been added. Aliquots of the two solutions were analyzed for total sterol prior to gel filtration, the fractions from which were also analyzed for sterol by three different procedures. The results of these analyses are given in Table 2; they demonstrate that the sterol is in fact present in the alcohol-precipitable fractions from the columns and that a soluble form of sterol occurs naturally in yeast and can be isolated from hot-water extracts of it.

The ability of DMSO to cleave sterol from the complex was investigated because it has this action on diginotides (6). The data in Table 3 show that sterol is extracted by hexane from the DMSO-treated complex in a yield directly proportional to the initial amount of complex. This technique was used for determining the amount of ergosterol present in the solutions of complex eluted from a Sephadex G-50 column. The elution profile presented in Fig. 1 shows that the sterol is eluted immediately after the void volume of the column without appreciable tailing and is separated from the bulk of the small molecular weight components contained in the yeast extract.

The UV absorption spectrum of ergosterol in the complex was determined on column fractions, first diluted four times with distilled water. The spectrum exhibited shoulders at 282 and 297 mμ and peaks at 262 and 271 mμ. These are similar to literature values for ergosterol (7) and to curves obtained on the same instrument with ergosterol in methanol which show a shoulder at 262 mμ and peaks at 271, 282, and 294 mμ.

Metabolic availability of ergosterol in the soluble complex was demonstrated by the use of the yeast mutant strain KD46. This organism requires either ergosterol or oleic acid for growth (4); it was obtained from Doctors M. A. Resnick and R. K. Mortimer. Cells of this strain were cultured on agar plates of Wickerham's defined medium (5) and on plates containing Wickerham's defined medium to which material from the Sephadex column had been added to give a final ergosterol concentration of 12.5 μg/ml. An additional control plate of Wickerham's medium contained complexing agent but no ergosterol. After 4 days colonies appeared on the Wickerham's medium supplemented

TABLE 2 RECOVERY OF STEROL FROM YEAST EXTRACT COMPLEX BY HYDROLYSIS

Sample	Hydrolysis Method	Sterol Recovered
		μg
1 A	Pyrogallol saponification	86
1 B	Pyrogallol saponification	201
2 A	Acid hydrolysis	21
2 B	Acid hydrolysis	98
3 A	Pyrogallol saponification	60
3 B	Pyrogallol saponification	223
4 A	Pyrogallol acid hydrolysis	36
4 B	Pyrogallol acid hydrolysis	41

A and *B* respectively refer to hot-water extracts of whole yeast and to commercial yeast extract to which ergosterol had been added. Samples 1 A and 1 B were the untreated yeast extract preparations; the remaining samples were corresponding preparations that had been subjected to column chromatography for purification of the ergosterol complex.

TABLE 3 EXTRACTION OF STEROL FROM THE COMPLEX BY MEANS OF DMSO

Complex*	Yield of Sterol†
ml	*μg*
2.0	112 ± 3
3.0	154 ± 5
4.0	197 ± 3
5.0	241 ± 5

* See Table 2.
† Mean of two determinations and actual range.

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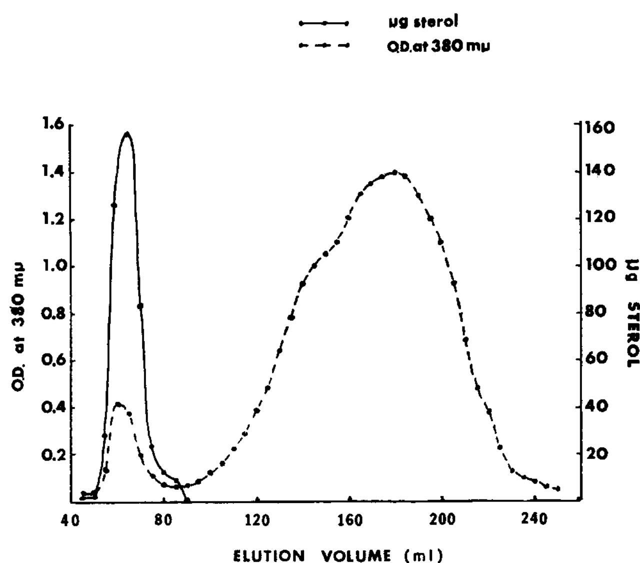

Fig. 1. Chromatography of a 12 ml solution of 15% yeast extract containing 150 μg/ml ergosterol on a Pharmacia glass column, 2.5 cm I.D., packed to a height of 31 cm with Sephadex G-50. 5-ml samples were eluted with 0.1 M phosphate buffer and analyzed for sterol by the DMSO procedure and for nonspecific absorbancy at 380 mμ.

with ergosterol complex; no growth at all was observed in the control, even after 2 wk of incubation. Thus, the ergosterol complex is able to provide the ergosterol required for growth by this mutant.

DISCUSSION

A component of yeast extract is evidently capable of binding ergosterol. The results of dialysis and the elution behavior of the complex from Sephadex are evidence that the component is a large molecule. The negative reaction to ninhydrin, the red color with iodine, the precipitation with alcohol, and the positive test with periodate–benzidine indicated that the complexing agent is a polysaccharide. Since the main carbohydrate reserve in yeast is glycogen (8), the complexing agent may in fact be glycogen. The possibility is not ruled out, however, that the complexing agent is a minor polysaccharide component, of which several are known in yeast.

Experiments are in progress to define more precisely the nature of the complexing agent and the mechanisms of sterol complex formation. Although an extensive literature survey has been made, no report of such a water-soluble form of sterol has been found.

It can also be concluded that the complex can be isolated as such from yeast cells, although the amount of sterol present in the cell in this water-soluble form cannot be accurately judged because of the inefficiency of extraction with hot water.

The discovery of a water-soluble form of ergosterol with proven metabolic activity will greatly facilitate the investigation of the role of ergosterol and other sterols in the physiology of yeast in both whole cell and cell-free systems and may eliminate the need to include surface-active agents in growth media and reaction mixtures.

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References

1. Parks, L. W., and P. R. Starr. 1963. *J. Cell. Comp. Physiol.* **61:** 61.
2. Lepage, M. 1964. *J. Chromatog.* **13:** 99.
3. Katsuki, H., and K. Bloch. 1967. *J. Biol. Chem.* **242:** 222.
4. Resnick, M. A., and R. K. Mortimer. 1966. *J. Bacteriol.* **92:** 597.
5. Wickerham, L. J. 1946. *J. Bacteriol.* **52:** 293.
6. Issidorides, C. H., I. Kitagawa, and E. Mosettig. 1962. *J. Org. Chem.* **27:** 4693.
7. Fieser, L. F., and M. Fieser. 1959. Steroids. Reinhold Publishing Corp., New York. 93.
8. Edwards, T. E. 1965. *Methods Carbohydrate Chem.* **5:** 176.

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