Lipid Formation in *Cryptococcus terricolus*

IV.   Separation of the Lipid Extract by Silicic Acid Column Chromatography

T. A. PEDERSEN*

Royal Institute of Technology, Division of Food Chemistry, Stockholm 70, Sweden

A chromatographic method has been developed which makes possible the separation of the lipids from the yeast *Cryptococcus terricolus*. The phospholipids were separated from the other lipid components by means of their greater adsorption values on a silicic acid column. By a subsequent elution of the adsorbed phospholipids with methanol, a separation of this material into three fractions was obtained. The free fatty acids were removed from the neutral lipids by adsorption on an Amberlite IRA-400 anion exchanger column. The neutral lipids could thereafter be fractionated into sterol esters, triglycerides, free sterols, diglycerides, and monoglycerides by elution with diethyl ether-petroleum ether mixtures from a silicic acid column.

The following composition of the total yeast lipids was established: phospholipids 1.8 %, free fatty acids 2.6 %, sterol esters 0.4 %, triglycerides 91.6 %, free sterols 0.6 %, diglycerides 2.5 %, and monoglycerides 0.3 %. As a contamination in the last part of the free sterol fraction (probably in the form of diglycerides), an amount equal to 0.3 % of the total material was found. The total amount eluted was 100.1 % of the material added. The phospholipid fraction was composed of 38 % of cephalins and 61 % of lecithin. The remaining 1 % consisted of phospholipids containing not only choline, but also nitrogen bases with free amino groups.

With their dominating content of triglycerides, the lipids of *C. terricolus* seem to differ from most other microbial lipids. The possible connection of this result with the extraction procedure used is discussed.

In earlier papers in this series, the effects of varying cultural conditions on the lipid synthesis in *Cryptococcus terricolus* have been investigated [1,2]. Under optimum conditions, the cells were found to contain more than 60 % of lipids on a dry weight basis. After the development of a mild extraction procedure for the yeast lipids [3], it was considered possible, by means of separations of the lipid mixture into its major fractions, to get an idea of the native

---

* Present address: Department of Microbiology, Agricultural College of Norway, Vollebekk, Norway.

composition of the cell lipids. Only a slight amount of knowledge has been accumulated concerning the composition of the lipids in yeasts. Recently, however, some investigations have been carried out to establish the total fatty acid composition of yeast lipids [4],[5]. The separation of the major lipid classes, followed by analyses of the fatty acid composition of the individual fractions, will probably yield some further information on the metabolism of the fatty acids.

The most widely employed adsorbent for lipid separations, when the column chromatographic technique is used, is silicic acid. A separation of the phospholipids from the other lipid components was obtained by Borgström [6], and a fractionation of the individual phospholipid components by Hanahan et al.[7] Using model compounds, Borgström [8],[9] found that silicic acid was also suitable for the fractionation of the neutral lipids. Barron and Hanahan [10] were able to obtain a separation of the neutral lipids of rat liver, beef liver, and yeast by elution with the non-polar solvent hexane in combinations with benzene and diethyl ether. However, they did not succeed in separating triglycerides and free fatty acids. Hirsch and Ahrens [11] investigated the adsorptive properties of silicic acid for various known lipids, when eluted with petroleum ether-diethyl ether mixtures. Using selected mixtures of this solvent system, they found that effective separations of complex lipid materials, e.g. human plasma, could be obtained by elution from a single column. Since this is of great practical advantage, the same method was tried in preliminary experiments, to obtain a separation of the complex C. terricolus lipids.

EXPERIMENTAL

The method for growing the organism, the extraction procedures, and the method used for the purification of the lipids have been described elsewhere [3].

Silicic acid powder ("Baker Analyzed" Reagent) was used by Borgström [6] after activation without any further standardizing treatments. To get a convenient flow rate through the column, Celite Analytical Filter-aid (Johns-Manville) has to be added to half the weight of the silicic acid. However, on elution with polar solvents, such as methanol, it was found in the present work that parts of the silicic acid was washed out from the column. This was avoided when the silicic acid was further prepared before use, according to the Hirsch and Ahrens [11] method. 200 g of silicic acid was placed in a 2 l glass cylinder. Absolute methanol was added to the 2 l mark and the silicic acid suspended by rapid agitation. After exactly 30 min settling, the methanol and the suspended silicic acid were decanted and discarded. This procedure was repeated once with methanol and twice with diethyl ether. As a result of the elimination of the part of the silicic acid that could be washed out with polar solvents, the flow rate also increased thus rendering the addition of Celite unnecessary.

To prepare the column, 18 g of treated silicic acid was taken from a batch of adsorbent dried for 24 h at 120°C, and dusted into a glass tube (diameter 20 mm). The tube was furnished with a sintered glass plate, which was covered by a circular disk of diethyl ether-extracted filter paper. After packing the column, the silicic acid was further activated according to the method of Hirsch and Ahrens [11].

The anion exchanger Amberlite IRA-400 column was prepared according to the method described by Borgström [8]. A column 100 mm in height and 20 mm in diameter was used. The elution followed the procedure described by Borgström [8]. To achieve the best possible separation, the first eluate was passed once more through the column.

The yeast lipids were dissolved in a small volume of petroleum ether and pipetted on to the column. The total volume of solvent used for charging the column was always kept under 20 ml. The lipid amount added never exceeded 250 mg.

All solvents used for elution were of reagent grade, redistilled in glass. The diethyl ether was distilled over sodium and stored at 0—5°C. Large elution fractions were collected in Erlenmeyer flasks, while for smaller fractions an automatic collector was used.

The solvent was removed from the large fractions using a rotary vacuum evaporator. The small fractions collected in tubes were dried by a combination of moderate heating on a water bath and simultaneous bubbling with nitrogen. The samples were weighed after drying overnight in a vacuum desiccator. The resulting dry matter was redissolved in a known volume of solvent and aliquots taken out for analyses.

The methods used for the determinations of the phosphorus content, the free amino group content, and the carbohydrate content, as well as the qualitative test for free and esterified sterols, have been briefly described in a previous communication [3].

The content of free fatty acids was determined by titrating with 0.01 M tetramethyl ammonium hydroxide from a micrometer burette (graduated to 0.2 μl), using thymol blue as indicator [12].

The total, esterified fatty acid content was determined using the method modified by Morgan and Kingsbury [13]. This procedure is based on the formation of hydroxamic acids which react with ferric chloride to form coloured complexes having an absorption maximum at 515 mμ. The final, coloured product was compared with a glyceryl mono-stearate standard.

The quantitative estimation of glycerol followed mainly the method described by Hanahan and Olley [14]. However, the lipid material, usually dissolved in petroleum ether, was pipetted quantitatively into soda glass tubes, and evaporated to dryness. The dry material plus 1 ml of 2 N hydrochloric acid was enclosed in an ampoule and hydrolyzed for 48 h at 100°C. Thereafter, the oxidation of glycerol by periodate and the colorimetric measurement of the chromotropic acid-formaldehyde reaction at 570 mμ, followed strictly the method mentioned.

The sterol content was in some cases determined quantitatively according to the method used by Stadtman [15]. Principally the procedure followed that of Lieberman-Burchard, but, in this case, the colour was measured colorimetrically at 625 mμ. Ergosterol was used as a standard.

The presence of acetal types of phospholipids was investigated with Schiff's reagent, according to the method described by Marinetti et al.[16]

As will be shown in these investigations, free amino groups are absent from one fraction of the eluted phospholipids. Consequently, analyses for choline had to be performed. The choline-phosphomolybdate method of Wheeldon and Collins [17] was not sensitive enough for detecting the very small amounts present in the collected phospholipid fractions. Recently, a micro method has been worked out by Ackerman and Chou [18] for the determination of choline down to 1 μg, by precipitating choline reineckate from alkaline solutions, followed by measurements of the absorption at 303 mμ. A Beckman model DU spectrophotometer was used in all of the colorimetric determinations.

RESULTS AND DISCUSSION

To achieve an effective separation of the *C. terricolus* lipids, the method of Hirsch and Ahrens [11] was tried. Judging from Fig. 1, it can be concluded that an absolute separation of the phospholipids from the other components of the lipid mixture was obtained. The phospholipids were further separated into three fractions (Fig. 1A, right) by elution with absolute methanol alone. While the first and third peaks contained free amino groups in their phospholipid nitrogen bases, the material in the tubes 134—137 contained no such bases (Fig. 1B, right).

By multiplying the phosphorus content by the approximate factor 25, the total phospholipid content was found to be 3.0 mg, or 1.5 % of the total lipid material. By multiplying the sum of the serine contents by the mean factor 7.8, it was concluded that half of the phospholipids contained nitrogen bases with free amino groups.
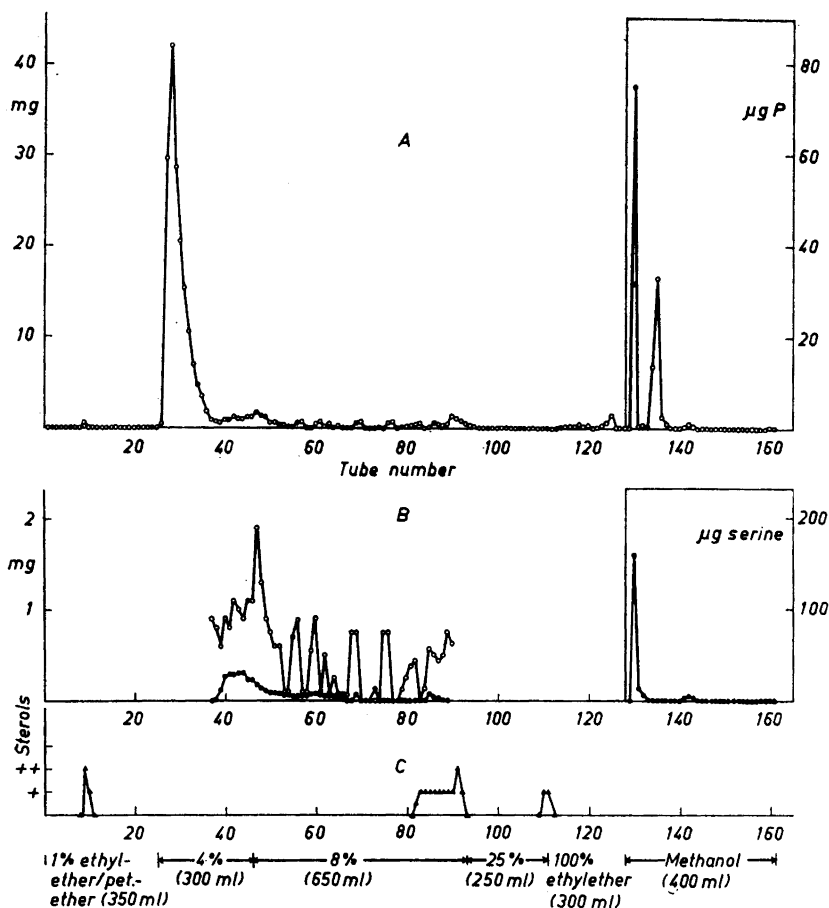
Fig. 1. Separation of *Cryptococcus terricolus* lipids on silicic acid column. The abscissas show the tube number collected fractionally, and the various solvents used to effect elution. Charge: 206.9 mg of lipids. Eluted: 206.2 mg of lipids.

A. O Amount eluted in each tube. Phosphorus test negative in the left part of the figure.
B. Left: ● Amount of titratable fatty acids expressed as oleic acid. O Total amount eluted in each tube.
   Right: ● Amount of free amino groups expressed as serine.
C. ▲ Peaks showing the presence of sterols. Amounts approximately measured by visual readings.

The elution of the neutral lipids and the free fatty acids resulted in a great many peaks (Fig. 1A, left).

All fractions containing esterified fatty acids will be further analyzed at a later stage of this investigation. However, it is convenient to mention the different fractions by their supposed names already in the following.

A single fraction was eluted with 1 % diethyl ether in tubes 9 and 10. This material gave a positive Lieberman-Burchard test (Fig. 1C). As found by

Hirsch and Ahrens [11], the sterol esters were eluted by this solvent mixture, while the free sterols were more strongly adsorbed on the column. The total amount of sterol esters was found to be below 0.5 % of the total material.

The dominant fraction of the lipid material was eluted with 4 % diethyl ether in petroleum ether. Tubes 26—37 contained in all 165.3 mg, or 79.9 % of the lipid material. As shown below, this fraction was composed of triglycerides. The elution of the free fatty acids started in tube 38 and proceeded more or less continuously in the subsequent 50 tubes (Fig. 1B, left). In addition, it will be seen that the amount of free fatty acids present did not constitute the total content of material in these particular tubes. The elution of triglycerides was probably not finished before the elution of the free fatty acids began.

The lipid material contained at least two different free sterols (Fig. 1C). The last peak seemed to be eluted separately but in inweighable amounts. The sterol contents in tubes 81—92 were also very low and could not account for the total contents of eluate in these tubes.

Finally, judging from the elution pattern found by Hirsch and Ahrens [11], it was considered likely that the peak eluted with 100 % diethyl ether was composed of monoglycerides.

The possible difficulties in obtaining effective separations by this elution system were briefly pointed out by Hanahan [19]. The successful separation obtained by Hirsch and Ahrens [11] was due to the comparatively simple composition of the human plasma lipids investigated. It was to be expected that more complex mixtures, especially those with a higher content of unsaturated derivatives, would complicate the fractionation. However, by modifying the
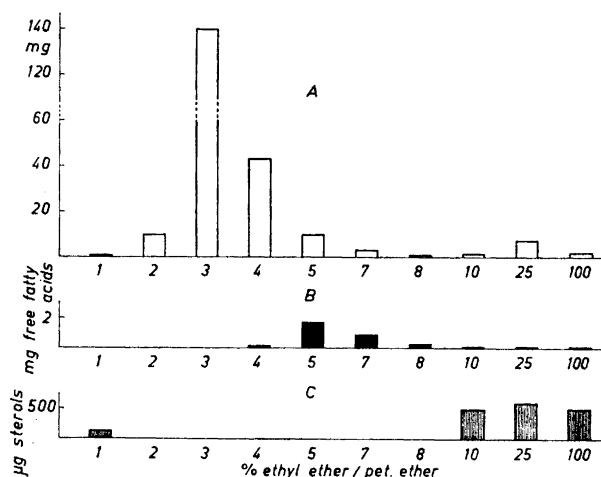

Fig. 2. Separation of *Cryptococcus terricolus* lipids on silicic acid column. Lipids eluted with 200 ml portions of different diethylether/petroleum ether mixtures. Phospholipids removed before start. Charge 215.3 mg of lipids. Eluted: 214.7 mg of lipids.
A. Weight in mg of each fraction.
B. Amount of titratable fatty acids expressed as mg of oleic acid.
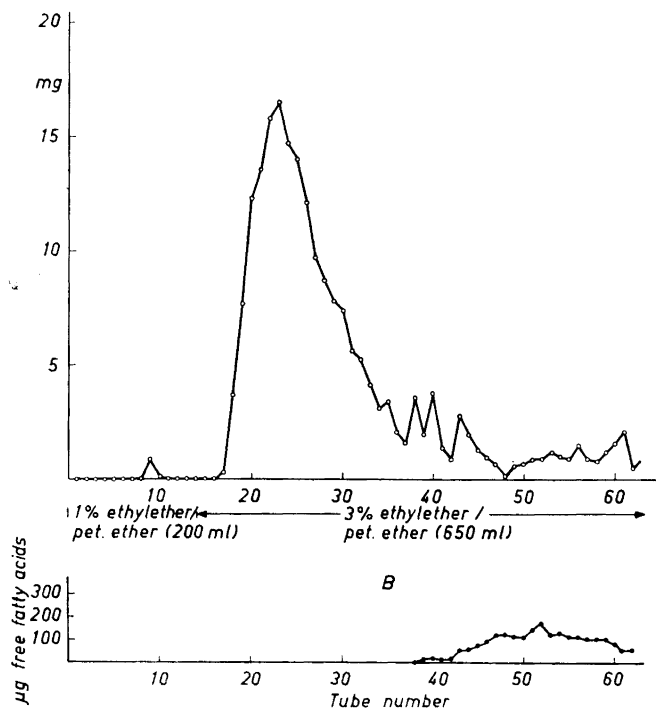C. Content of sterols expressed as µg of ergosterol.

Fig. 3. Separation of *Cryptoccocus terricolus* lipids on silicic acid column. Lipids eluted
with 1 and 3 % solvent mixtures.
A. ○ Weight in mg of each fraction.
B. ● Amount of titratable fatty acids expressed as μg of oleic acid.

solvent system, it was thought possible to obtain a better separation of the
triglycerides from the free fatty acids.

The distribution of the lipid fraction, when eluted with 200-ml portions of
ten different diethyl ether/petroleum ether mixtures with increasing diethyl
ether contents, is shown in Fig. 2. As before, the sterol esters were eluted with
1 % diethyl ether in petroleum ether. By multiplying the amount of free
sterols, computed as ergosterol after hydrolysis, by the factor 1.4, it was
possible to calculate approximately the content of sterol esters. It will be
seen that the sterol ester content was not sufficient to account for the total
material eluted in this fraction (Figs. 2A and 2C). The elution of the triglyceride
fraction began with the 2 % solvent mixture. The main amount, however,
was eluted with 3 % diethyl ether in petroleum ether. The triglyceride frac-
tion had so far been obtained free from titratable fatty acids. When the diethyl
ether content in the solvent mixture was further increased, however, the elu-
tion of the free fatty acids began. Approximately 80 % of the total free fatty
acid content was eluted with the 5 and 7 % mixtures, but traces were found pre-
sent in all subsequent fractions. With this elution method, approximately 70 %
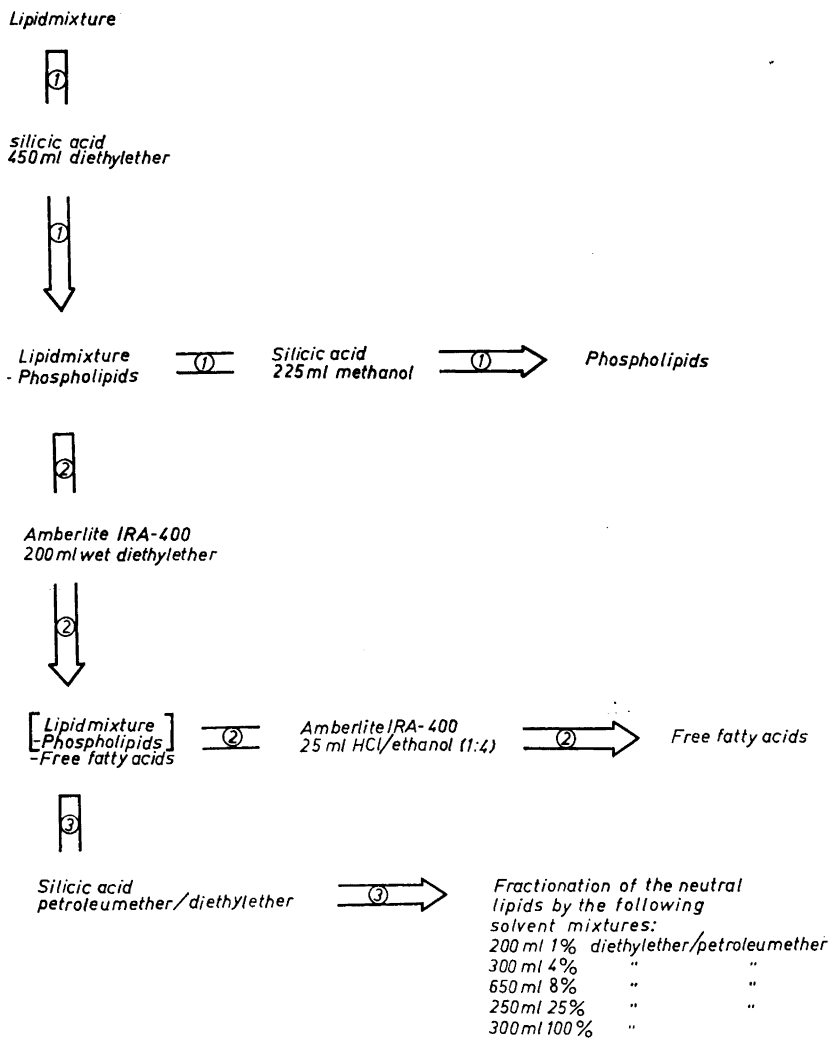
Fig. 4. Elution scheme for fractionation of *Cryptococcus terricolus* lipids. As shown by the numbers, three different columns are used.

of the lipid material was obtained without any content of free fatty acids. Free sterols were present in the last three fractions.

It was assumed that part of the lipid material eluted with 4 % diethyl ether might be obtained free from titratable fatty acids by increasing the amount of the 3 % solvent mixture added to the column. As a result of this procedure (Fig. 3), 81 % of the lipid material was obtained completely free from titratable fatty acids. However, even with the 3 % mixture, the elution of the free

fatty acids began, and continued, as shown in Fig. 2, during the elution of the other neutral lipid components. It was, therefore, assumed that, with this solvent system it would be impossible to obtain a total separation of the free fatty acids from the different glycerides.

It was considered necessary to achieve a total removal of the free fatty acids from the remaining neutral lipids by means of special procedures. Borgström[8] successfully used the Amberlite anion exchanger IRA-400 for this purpose. After passing the material, dissolved in wet petroleum ether, twice through the column, less than 0.1 % of the free fatty acids could be detected in the eluted neutral lipids. No changes in the fatty acid composition were detected, and the elution could be performed quantitatively. However, before adding the lipids to the ion exchanger column, the phospholipids had to be removed, to avoid the simultaneous adsorption of the polar phospholipids. As a consequence of this special removal of both the phospholipids and the free fatty acids, the separations had to be performed on three different columns, two of which, however, were identical (Fig. 4). Three different organic solvents were used throughout the separations, except in the special treatment to release the adsorbed fatty acids from the ion exchanger.

The results of the fractionation obtained with this "three column" technique are shown in Fig. 5. By this procedure the phospholipids were also separated into two large fractions (tubes 2—4 and 4—11) and one very small fraction (tube 15) (Fig. 5A, left). Because of the strong adsorption of the phospholipids to the silicic acid, addition of a larger volume of non-polar solvents had no effect on the elution results. The total phosphorus content was found to be 110.5 μg, corresponding to approximately 2.8 mg of phospholipids. This amount, as well as the fractionation pattern obtained, agrees fairly well with the results given in Fig. 1. The presence of free amino groups (computed as serine) was found in the first and the last fraction of the eluted phospholipids (Fig. 5B, left). The material in each of the three phospholipid fractions was combined and choline determined. No choline was found to be present in the first elution peak of Fig. 5 (tubes 1—3). The next peak (tubes 4—11) contained 290 μg of choline, corresponding to approximately 1 600 μg of lecithin, which agrees very well with the amount of material eluted. The third peak (tubes 14—16) contained 43 μg of choline, indicating that a mixture of lecithin and, perhaps, sphingomyelin, together with some free amino group-containing substance(s) (the serine reaction was also positive) was included in this peak. No further attempts were made to identify these minor substances.

Acetal types of lipids are often found in various phospholipids. No significant results were obtained, however, by using Schiff's test on the three different phospholipid fractions, and these compounds were thus at most present only in trace amounts. The same could be said of the presence of sugars in the eluted phospholipid fractions.

The phospholipid contents of different yeasts have often been found to be very high, varying from 13 up to 80 %, if calculated on the basis of the lipid content [20]. It is essential to point out here that these investigations were performed with yeasts containing only a few per cent of lipids. Nilsson and Nielsen [21] stated that the phospholipid content decreased in proportion to the increase of lipids in *Rhodotorula gracilis*. With 53.6 % of lipids in their
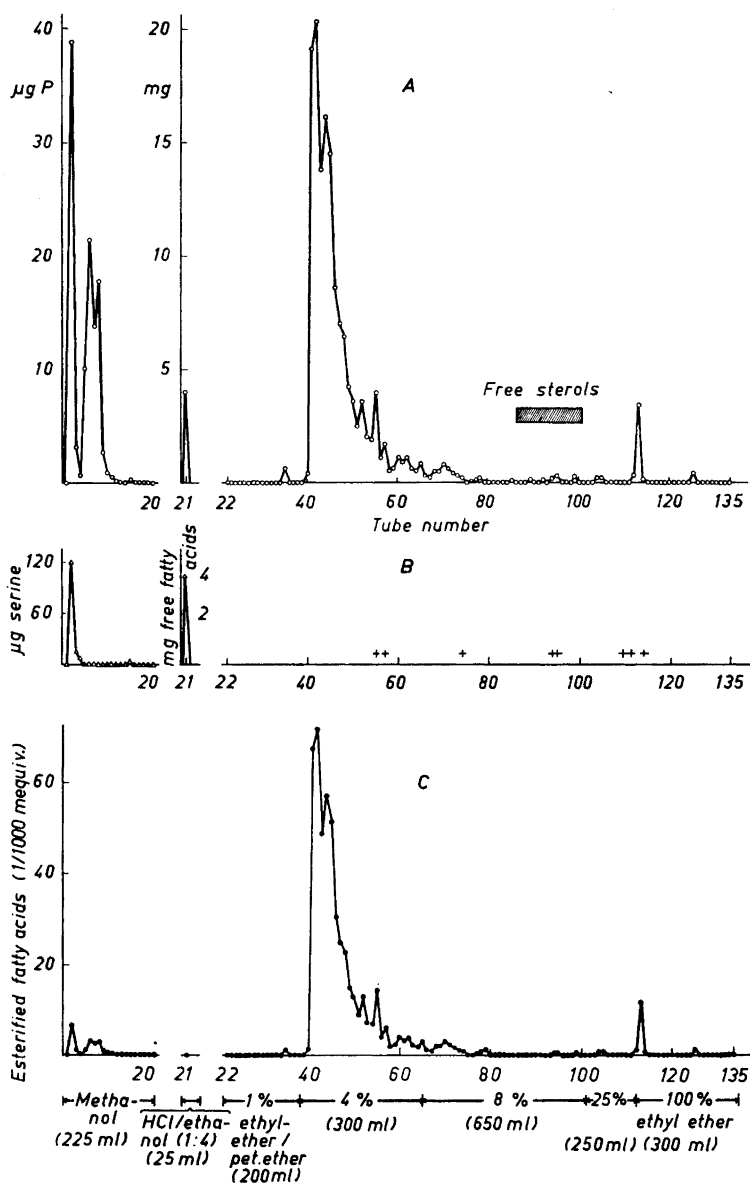
Fig. 5. Separation of *Cryptococcus terricolus* lipids. Charge: 154.0 mg. Eluted: 154.4 mg.
Tubes 1—20: Phospholipid fraction. Tube 21: Fatty acids. Tubes 22—135: Neutral lipids.
A. O Amount eluted in each tube.
B. Left: △ Amount of free amino groups expressed as serine.
   Right: △ Amount of titratable fatty acids expressed as oleic acid. + indicates traces
   of free fatty acids.
C. ● 1/1000 mequiv. of esterified fatty acids.

yeast, they found a phospholipid content of the same magnitude as that found for *Cryptococcus terricolus*.

The content of free fatty acids which comprised only 4.0 mg, or 2.6 % of the total eluted lipid material, was collected in tube 21 (Fig. 5A). As pointed out by Kleinzeller [20], there seems to be some clear indications that a considerable amount of free fatty acids may be present in microorganisms. However, as far as yeasts are concerned, the results reviewed by Kleinzeller [20] were obtained by extractions of acid-hydrolyzed cell materials. The possible hydrolytic release of esterified fatty acids as a result of such treatments, should of course be kept in mind. Barron and Hanahan [10] found the free fatty acids to constitute *ca.* 60 % of the total lipids in *Saccharomyces cerevisiae* cells. In their experiments, the lipids were extracted with ethanol-diethyl ether without any pretreatment of the cells. However, repeated 8-hour extraction periods were used, and the presence of fatty acids may perhaps be partly explained as a result of an enzymatic action. It is well known that lecithinases are solvent activated during the extraction procedure. Consequently, in phospholipid-rich materials, or if the same activation phenomenon occurs for the lipolytic enzymes in fat-rich materials, an extensive production of free fatty acids would be possible. By means of the short-time technique used for the extraction of the *Cryptococcus terricolus* lipids, the enzymatic degradation is kept at a minimum. Small amounts of free fatty acids occur in all native lipid mixtures. The amount found in the present material is thus probably not the undesired result of the extraction treatments.

However, the results obtained recently by Hartman *et al.*[4] do not fit in with this view. The high acid value found by them for *Rhodotorula graminis* lipids is of a magnitude often found in earlier investigations with other "fat" yeasts. They supposed that no enzymatic splitting of the neutral fats occurred during their extraction procedure, but, according to the present author, some reservations should be made. The extractions were performed by heating in Soxhlet extractors, or by refluxing, and certain parts of the lipid mixtures (12—21 %) were obtained after acid hydrolysis of the yeast material. These methods do not exclude the possibility of a release of free fatty acids.

After the removal of the free fatty acids, the elution of the neutral lipids resulted in a clearer and more easily identifiable fractionation pattern than that found earlier (Fig. 5A, right). As before, the sterol esters were eluted with 1 % diethyl ether in petroleum ether. The content of esterified fatty acids in this fraction is shown in Fig. 5C. In agreement with the results obtained by Hirsch and Ahrens [11], the free sterols were removed from the column after the elution of the triglyceride fraction. The materials in tubes 86—100 gave a positive Lieberman-Burchard reaction. These tubes contained, in all, approximately 1.0 mg of lipid materials. Using the quantitative sterol test, this amount could be accounted for if the sterols were computed as ergosterol. The free sterol fraction was probably composed of a complex of different sterols, as indicated by the several peaks obtained on elution. Judging from Fig. 5C, however, the sterols in tubes 94—100 seemed to be impure, since, in addition, esterified acids were found. The complete separation of ergosterol and diglycerides was found by Barron and Hanahan [10] to be impossible in their diethyl ether-hexane elution system, although cholesterol and diglycerides were easily

Table 1. Presence of different glyceride types in lipids from *Cryptococcus terricolus*. The italicized alternatives show the actual glyceride in each fraction tested.

| Material | | EFA 1/1000 mequiv. | EFA mg | EFA per mg of material | mg of glyceride calculated per mg of material | | |
Tubes	Weight in mg				MG	DG	TG
41— 48	105.8	377	95.76	0.905	1.23	1.07	*1.01*
104—114	3.2	10.65	2.71	0.847	1.15	*1.00*	0.94
125	0.4	1.14	0.29	0.725	*0.99*	0.85	0.81

Abbreviations: EFA- esterified fatty acids, MG-monoglyceride, DG- diglyceride, TG-triglyceride.
Average molecular weight of fatty acids, calculated: 271.

separated. The difficulties in obtaining effective separations when ergosterol is present are due to the larger polarity of ergosterol, as compared with that of cholesterol, resulting in too small chromatographic differences between the ergosterol and the diglycerides. The free sterols amounted to ca. 0.6 % and the sterol esters to ca. 0.4 % of the total lipid material. These amounts are within the limits usually found for lipid-rich yeasts.

The triglycerides were eluted in tubes 39—79. Many peaks were obtained in this fraction, indicating to a certain extent the complex composition of the triglyceride mixture. The total weight of the contents in these tubes amounted to 141.4 mg, or 91.6 % of the eluted material. By this quantitative dominance of the triglyceride fraction, *Cryptococcus terricolus* differs markedly from other "fat" yeast species studied so far. Barron and Hanahan [10], in one of the few investigations on the separation of yeasts lipids, found their "triglyceride" fraction to constitute 71.0 % of the total cell lipids. However, upon further analysis, 81 % of this fraction was found to be composed of free fatty acids. On the other hand, in tissues of living plants and animals, free fatty acids are generally considered to occur in very small amounts or to be completely lacking. Judging from the above results, the same seems to be the case with *C. terricolus* cells.

The most direct method to determine whether the three main peaks (tubes 40—79, 104—114, and 125, respectively) in the fractionation scheme in Fig. 5A (right) were composed exclusively of tri-, di-, and monoglycerides, respectively, would be to determine the total content of fatty acids and glycerol of each peak individually. However, it was found difficult to obtain reproducible results, within the limits necessary here, with the method used for the glycerol analysis [14]. The varying results were probably due to difficulties encountered in obtaining quantitative hydrolysis of the lipid materials in the different experiments. Instead of performing further investigations concerning this method, the content of esterified fatty acids in each fraction was determined [13]. The material in tubes 41—48 was collected and used as a sample of the proposed triglyceride fraction, while tubes 104—114 and 125 were supposed to contain diglycerides and monoglycerides, respectively. When expressing the results as mg of esterified fatty acids, it was assumed that the various fatty

acids found by preliminary gas-chromatographic analyses were equally distributed over all the fractions. On the basis of this suggestion, the average molecular weight of the fatty acids was calculated at 271.

On the basis of the amounts of esterified fatty acids found per mg of material in each of the three different samples, three alternatives were calculated, *viz.* that the material in question be composed exclusively of either mono-, di-, or triglycerides (Table 1). With the great precision in the experimental techniques used (coefficient of variation far below 1 %), it could be proved that tubes 41—48 contained triglycerides, tubes 104—114 diglycerides, and the last tube (125) monoglycerides. Judging from these results, it may be concluded that the separation of the fat component of the *C. terricolus* lipids into tri-, di-, and monoglycerides can be performed by means of the technique described.

REFERENCES

1. Pedersen, T. A. *Acta Chem. Scand.* **15** (1961) 651.
2. Pedersen, T. A. *Acta Chem. Scand.* **16** (1962) 359.
3. Pedersen, T. A. *Acta Chem. Scand.* **16** (1962) 374.
4. Hartman, L., Hawke, J. C., Shorland, F. B. and diMenna, M. E. *Arch. Biochem. Biophys.* **81** (1959) 346.
5. Deinema, M. H. *Meded. Landbouwhogeschool, Wageningen* **61** (1961) 1.
6. Borgström, B. *Acta Physiol. Scand.* **25** (1952) 101.
7. Hanahan, D. J., Dittmer, J. C. and Warashina, E. *J. Biol. Chem.* **228** (1957) 685.
8. Borgström, B. *Acta Physiol. Scand.* **25** (1952) 111.
9. Borgström, B. *Acta Physiol. Scand.* **30** (1954) 231.
10. Barron, E. J. and Hanahan, D. J. *J. Biol. Chem.* **231** (1958) 493.
11. Hirsch, J. and Ahrens, Jr., E. H. *J. Biol. Chem.* **233** (1958) 311.
12. Entenman, C. in Colowick, S. P. and Kaplan, N. O. *Methods in Enzymology*, Vol. III, Academic Press, New York 1957.
13. Morgan, D. M. and Kingsbury, K. J. *Analyst* **84** (1959) 409.
14. Hanahan, D. J. and Olley, J. N. *J. Biol. Chem.* **231** (1958) 813.
15. Stadtman, T. G. in Colowick, S. P. and Kaplan, N. O. *Methods in Enzymology*, Vol. III, Academic Press, New York 1957.
16. Marinetti, G. V., Erbland, J. and Kochen, J. *Federation Proc.* **16** (1957) 837.
17. Wheeldon, L. W. and Collins, F. D. *Biochem. J.* **70** (1958) 43.
18. Ackerman, C. J. and Chou, M. *Analyt. Biochem.* **1** (1960) 337.
19. Hanahan, D. J. *Lipide chemistry*, Wiley, New York 1960.
20. Kleinzeller, A. *Advances in Emzymol.* **8** (1948) 299.
21. Nilsson, N. G. and Nielsen, N. *Acta Chem. Scand.* **7** (1953) 1067.