Phytochemistry, 1974, Vol. 13, pp. 1115 to 1121. Pergamon Press. Printed in England.

VARIATION IN THE LEVELS AND COMPOSITION OF THE STEROLS AND STEROL ESTERS OF *PHYCOMYCES BLAKESLEEANUS* WITH AGE OF CULTURE

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(*Received* 4 *September* 1973)

Key Word Index—*Phycomyces blakesleeanus*; Mucoraceae; sterols; sterol esters; fatty acids; triglycerides; phospholipids; age of culture.

Abstract—Comparisons of the fatty acid composition of the sterol esters, triglycerides and phospholipids and the sterol composition of the esterified and unesterified sterol fractions of *Phycomyces blakesleeanus* cultures of different ages have been made. The effect of transferring *P. blakesleeanus* mycelium to a starvation medium on the triglyceride level and the esterification of ergosterol has been studied.

INTRODUCTION

WE HAVE recently reported the results of a detailed analysis of the sterol and fatty acid moieties of the sterol esters of *Phycomyces blakesleeanus* and have compared their fatty acid composition with that of the triglycerides and phospholipids.[1] This study, however, was made on mycelium harvested from a 60 hr culture of the fungus grown at 24° and we felt that the values obtained might be more characteristic of the age of the culture than the organism itself. Consequently we have carried out an analysis of the same parameters on mycelium harvested from cultures grown under identical conditions for different lengths of time. We have also investigated the possibility that sterol esters constitute a reserve of either sterol or fatty acids which is mobilized when the organism requires it.

RESULTS

Variation on levels of sterol and sterol ester with age of culture

Sixty 250 ml conical flasks, each containing 100 ml of medium, were inoculated with 10 ml aliquots of a *P. blakesleeanus* spore suspension and cultured at 24° under constant illumination. At various times over a period of 54 hr five flasks were taken at random and the mycelium harvested, bulked and the dry weight determined, as was the pH of the spent medium. The lipid was then extracted and separated by alumina column chromatography into a 2% Et_2O in light petroleum (E/P) fraction containing the sterol esters and a 40% E/P fraction containing the unesterified sterols. The quantity of ergosterol in both fractions was determined spectrophotometrically. The sterol esters were then separated from the 2% E/P fraction by TLC (system 1), saponified and the resulting sterols and fatty acids

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[1] MERCER, E. I. and BARTLETT, K. (1974) *Phytochemistry* **13**, 1099.

TABLE 1. COMPOSITION OF THE STEROL MIXTURES DERIVED FROM THE STEROL ESTERS AND UNESTERIFIED STEROLS OF *Phycomyces blakesleeanus* CULTURES OF DIFFERENT AGES

	Percentage composition of sterols															
	Unesterified sterol							Sterol ester								
Sterol	14*	19	24	39	44	49	54	14*	19	24	29	34	39	44	49	54
Ergosterol	66·4	60·3	48·5	55·9	55·7	49·3	53·0	18·8	34·0	29·4	16·6	17·9	43·6	45·9	48·1	42·2
Episterol	30·6	34·7	36·5	37·5	36·3	36·0	36·8	55·5	54·5	60·0	65·7	75·8	54·3	47·8	45·0	44·5
Lanosterol	0·8	1·4	4·4	1·7	1·8	2·9	2·1	4·7	1·8	4·4	8·4	3·2	1·0	1·9	3·1	5·3
24-Methylene-24,25-dihydrolanosterol	2·7	3·6	10·6	5·3	4·3	11·8	8·1	20·9	9·7	6·3	9·3	3·1	1·1	3·4	3·8	8·0

* Hours of growth.

extracted. The fatty acids were then methylated and analysed by GLC. The sterols from the sterol esters and from the 40% E/P fraction were separated into 4-demethyl- and 4,4-dimethylsterols by TLC (system 2) and analysed by GLC.

Thus the following parameters were determined for the fungal mycelium harvested at each time during the experimental period; the dry weight, lipid weight and pH of the spent medium which are shown in Fig. 1; the quantity of ergosterol (total, esterified and unesterified) which are shown in Figs. 2 a and b and the percentage composition of the main 4-demethyl- and 4,4-dimethylsterols derived from the sterol esters and unesterified sterols which are shown in Table 1. Figure 3 shows the variation with age of culture of the percentage of ergosterol present as ester. The 3, 6 and 9 hr cultures contained no mycelium; centrifugation at 10000 *g* sedimented only spores.

Comparison of the fatty acid composition of sterol esters, triglycerides and phospholipids at different ages of culture.

Acetone-Et_2O lipid extracts of 1 l. batches of mycelium grown from spores for 24, 36, 48, 60 and 84 hr at 24° under constant illumination were separated by alumina chromatography into 2% E/P, 8% E/P, 20% E/P and MeOH fractions. The mycelium from each batch was then re-extracted with MeOH to complete the removal of phospholipids.

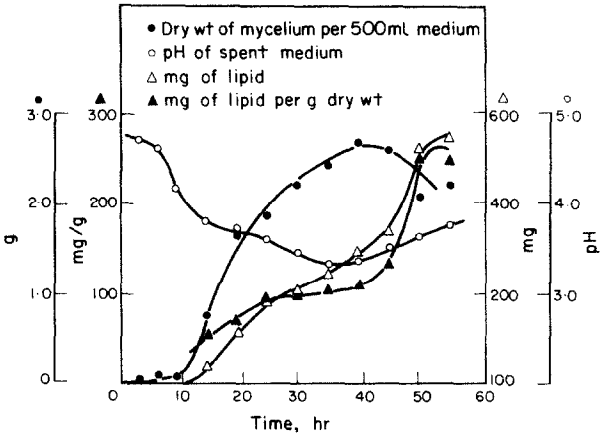

FIG. 1. GROWTH CHARACTERISTICS OF *Phycomyces blakesleeanus*.
Phycomyces blakesleeanus was grown from spores at 24° under constant illumination; the following parameters were measured at intervals; dry weight (g) of mycelium per 500 ml of medium; pH of spent medium; mg of lipid; mg of lipid per g dry wt.

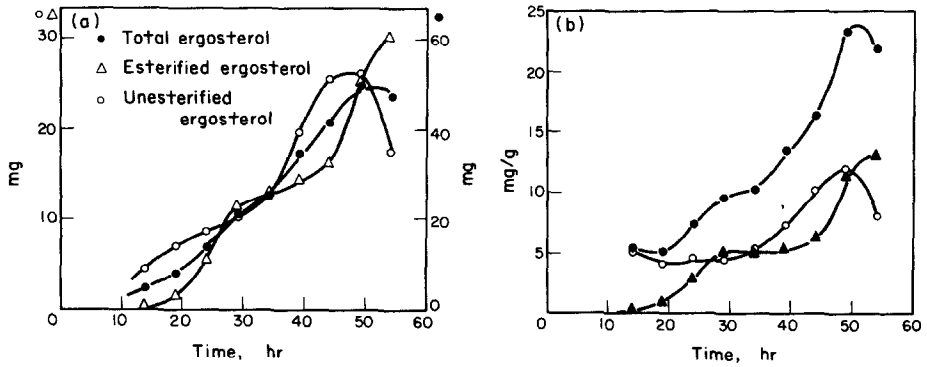

FIG. 2. VARIATION IN THE LEVEL OF TOTAL, ESTERIFIED AND UNESTERIFIED ERGOSTEROL WITH AGE OF CULTURE.

(a) Total ergosterol, esterified ergoesterol and unesterified ergosterol in mg per 500 ml of *P. blakesleeanus* culture.

(b) Total ergosterol, esterified ergosterol and unesterified ergosterol in mg per g dry wt of *P. blakesleeanus* mycelium.

Sterol esters were isolated from the 2% E/P fraction by TLC (system 1), saponified and the resulting fatty acids extracted. Triglycerides were isolated from the 8% E/P fraction by TLC (system 1; R_f 0·28 co-chromatographing with tripalmitin), saponified and the resulting fatty acids extracted. The MeOH fraction from the column chromatogram and the MeOH extract were shown to be composed of phospholipids by TLC (system 3). When sprayed with the Vaskovsky reagent[2] both showed zones at R_f values of 0·05, 0·10, 0·13, 0·30, 0·34, 0·40, 0·57, 0·63 and 0·84 of which the largest were those at R_fs 0·57 (co-chromatographing with egg lecithin), 0·30 and 0·84. Both phospholipid fractions were saponified and the resulting fatty acids extracted.

The fatty acids derived from the sterol esters, triglycerides and two phospholipid fractions were methylated and analysed by GLC. The identity of the fatty acid methyl esters was established by co-chromatography with standards and by hydrogenation followed by re-chromatography. The results are shown in Table 2.

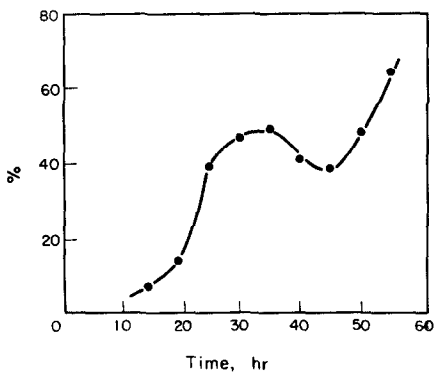

FIG. 3. VARIATION OF THE PERCENTAGE OF ERGOSTEROL PRESENT AS ESTER WITH RESPECT TO THE AGE OF THE CULTURE.

[2] VASKOVSKY, V. E. and KOSTETSKY, E. Y. (1968) *J. Lipid Res.* **9**, 396.

TABLE 2. VARIATION OF FATTY ACID COMPOSITION OF STEROL

Fatty acid*	Percentage composition of fatty acids: Sterol esters 24 hr	36 hr	48 hr	60 hr	84 hr	Triglycerides 24 hr	36 hr	48 hr	60 hr	84 hr
12:0	0·0	0·0	0·0	0·0	0·4	0·6	0·3	0·3	0·1	0·3
12:1	0·0	0·0	0·0	0·0	0·3	0·0	0·0	0·0	0·1	0·2
13:0	3·1	0·7	0·0	0·0	2·9	0·0	0·0	0·0	0·0	0·0
13:1	1·5	0·0	0·0	0·0	0·6	0·0	0·0	0·0	0·0	0·0
14:0	1·2	0·2	1·1	1·9	1·8	3·2	2·5	2·1	1·2	1·8
14:1	1·6	0·0	0·8	0·4	0·9	1·5	0·6	0·0	0·6	0·9
15:0	0·4	1·3	0·6	0·8	0·9	1·5	1·2	0·9	0·4	0·6
16:0	28·9	8·3	12·7	13·7	18·1	29·7	31·5	37·9	32·0	39·8
16:1	8·0	1·9	4·6	4·1	11·5	8·2	4·8	5·5	5·3	9·1
16:2	0·0	0·0	0·0	0·0	0·0	1·3	0·0	0·9	0·7	0·9
17:0	2·2	6·6	1·1	1·1	0·8	3·4	0·9	1·1	0·3	0·5
17:1	0·4	0·2	0·2	0·5	0·6	0·4	0·4	0·3	0·3	0·4
18:0	6·5	8·9	2·9	5·5	6·1	6·7	8·3	3·9	8·4	8·3
18:1	16·9	5·6	31·2	37·9	35·3	17·4	25·6	25·8	28·1	25·7
18:2	5·8	2·8	14·7	13·6	8·5	6·0	8·7	10·5	11·2	6·9
†18:3(γ)	7·1	12·7	14·2	14·2	2·3	7·3	12·3	9·3	8·9	0·6
‡19:1?	1·5	0·9	2·6	0·5	0·0	1·5	0·2	0·5	0·2	2·3
20:0	2·5	10·2	1·5	1·7	0·3	2·7	0·6	0·5	0·7	0·1
21:0	4·2	10·3	2·3	0·5	2·6	2·7	0·2	0·2	0·1	0·4
22:0	3·3	9·0	3·3	0·9	0·7	2·1	0·6	0·0	0·7	0·1
>22:0	4·8	20·0	4·8	2·5	3·6	4·9	1·1	0·9	1·0	0·6

* The figure before the colon is the number of carbon atoms in the fatty acid and the figure after the colon is the number of double bonds.

† γ-Linolenic acid (octadeca-6,9,12-trienoic acid).

‡ This is a C_{19} unsaturated fatty acid, probably with one double bond.

(1) Phospholipids from the MeOH fraction of the Al_2O_3 column chromatography.

(2) Phospholipids from MeOH extract.

Effect of a starvation medium on the triglyceride level and the esterification of ergosterol

The mycelium from a 3 l. culture of *P. blakesleeanus* grown from spores on the usual medium for 40 hr was harvested and a portion taken for analysis whilst the rest was resuspended in 1·5 l. of sterile phosphate (1·5 g KH_2PO_4/l.) containing $MgSO_4$ (0·5 g/l). It was then incubated for 30 hr at 24° under constant illumination and aliquots removed aseptically at various time for analysis. The mycelium from each sample was harvested, the dry weight determined and the lipid extracted. The lipid was then separated by alumina column chromatography into 2% E/P, 8% E/P and 20% E/P fractions. The ergosterol ester content of the 2% E/P fraction and the unesterified ergosterol content of the 20% E/P fraction were determined spectrophotometrically. Triglyceride was isolated from the 8% E/P fraction by TLC (system 1) and determined gravimetrically. The experiment was repeated using a 4 l. culture harvested after 20 hr growth and incubated in the phosphate–$MgSO_4$ medium for 72 hr; however, only the ergosterol levels, in esterified and unesterified form, were determined. The results of these two experiments are shown in Fig. 4.

DISCUSSION

The measurement of the growth of a mycelial fungus like *P. blakesleeanus* in liquid culture is notoriously difficult since no measurable parameter fully expresses growth. Indeed, as Bu'Lock has pointed out,[3] even the term "growth" in this context is not always easy

[3] BU'LOCK, J. D. (1967) *Essays in Biosynthesis and Microbial Development*, p. 46. Wiley, New York.

ESTERS, TRIGLYCERIDES AND PHOSPHOLIPIDS WITH AGE OF CULTURE

Percentage composition of fatty acids									
Phospholipids(1)					Phospholipids (2)				
24 hr	36 hr	48 hr	60 hr	84 hr	24 hr	36 hr	48 hr	60 hr	84 hr
0·1	0·0	0·0	0·2	0·2	0·3	1·1	0·5	0·2	0·3
0·2	0·0	0·0	0·2	0·1	0·3	0·1	0·2	0·2	0·1
0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·2	0·0	0·0
0·0	0·0	0·0	0·0	0·0	1·3	0·6	0·8	0·0	0·0
0·5	4·4	0·8	1·0	1·3	2·9	4·3	0·4	0·4	0·5
1·6	0·3	0·0	0·8	0·5	0·0	0·4	0·6	0·5	0·4
0·2	0·5	0·4	0·6	0·5	0·5	0·1	0·0	0·4	0·0
12·8	18·8	23·8	26·4	18·2	14·4	21·6	26·2	27·3	24·3
4·0	6·3	3·6	6·2	11·2	4·8	5·3	4·6	4·3	6·9
0·0	0·0	0·0	0·0	0·0	1·3	3·5	0·0	0·3	0·6
0·7	1·5	0·3	0·3	0·5	1·1	1·4	0·9	0·4	1·0
0·2	0·4	0·3	0·4	0·4	0·7	0·5	0·5	1·1	0·7
3·1	4·8	0·4	4·2	2·7	2·1	2·3	3·6	2·7	1·8
32·3	23·8	28·6	31·0	31·1	33·3	19·0	32·9	30·3	32·5
15·7	14·4	17·7	13·9	15·9	14·5	17·0	15·1	16·6	18·3
18·6	16·2	18·6	12·1	14·2	18·0	18·6	12·5	14·4	11·7
0·3	0·5	0·6	0·8	0·5	0·2	0·9	0·9	0·8	0·4
0·7	2·2	0·5	0·4	0·5	2·2	0·9	0·0	0·0	0·0
0·8	2·2	0·7	0·3	0·5	1·0	0·7	0·0	0·0	0·0
0·4	1·8	0·4	0·6	0·1	0·0	0·9	0·0	0·0	0·0
2·3	5·7	1·4	0·0	1·3	0·0	0·0	0·0	0·0	0·0

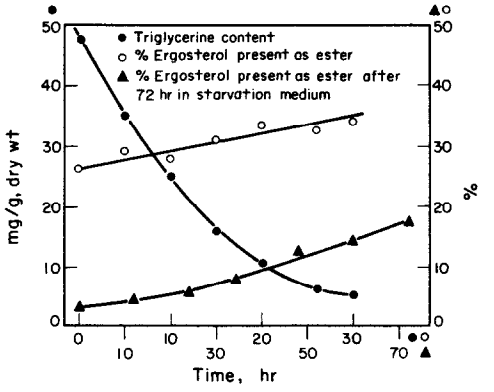

FIG. 4. CHANGES IN THE LEVEL OF TRIGLYCERIDE AND THE PERCENTAGE OF ERGOSTEROL IN ESTER FORM IN 20 AND 40 hr CULTURES OF *Phycomyces blakesleeanus* INCUBATED FOR A FURTHER PERIOD IN A STARVATION MEDIUM.

A culture of *P. blakesleeanus* was grown for 40 hr from spores and the mycelium then transferred aseptically to phosphate buffer and incubated for a further 30 hr. The triglyceride content and percentage of ergosterol present as ester were determined at intervals. The experiment was repeated with mycelium from a 20 hr culture incubated for 72 hr in the starvation medium; the percentage of ergosterol present as ester was determined at intervals.

to define or relate to what is usually measured. Borrow *et al.*[4] and Bu'Lock *et al.*,[5] on the basis of detailed studies of *Gibberella fujikuroi* and *Penicillium urticae* respectively, have

[4] BORROW, A., JEFFERYS, E. G., KESSEL, R. H. J., LLOYD, E. C., LLOYD, P. B. and NIXON, I. S. (1961) *Can. J. Microbiol.* **7**, 227.

[5] BU'LOCK, J. D., HAMILTON, D., HULME, M. A., POWELL, A. J., SHEPHERD, D., SMALLEY, H. H. and SMITH, G. N. (1965) *Can. J. Microbiol.* **11**, 765.

introduced terms to describe fungal growth in submerged cultures. The "balanced growth" phase of Borrow *et al.*[4] and "trophophase" of Bu'Lock *et al.*[5] are roughly equivalent and describe that phase of rapid, though not necessarily logarithmic, balanced growth which occurs before one of the major nutrients is exhausted. This is then followed by a period in which growth rate declines and the secondary metabolites characteristic of the fungus are produced. This is referred to as "idiophase" by Bu'Lock *et al.*[5] and is probably equivalent to a combination of the "storage" and "maintenance" phases of Borrow *et al.*[4]

The growth curve of *P. blakesleeanus* (Fig. 1), based on dry weight determination, would suggest that trophophase commences after a lag phase of 9 hr and lasts for about 30 hr after which it is followed by idiophase. The pH of the spent medium almost exactly mirrored the dry weight changes. The total lipid production is relatively low in trophophase but increases dramatically in idiophase; this is particularly apparent when lipid is expressed in terms of dry weight and is characteristic of idiophase.[3,5] Ergosterol ester production mirrored lipid production more closely than did that of unesterified ergosterol or total ergosterol (Figs. 2 a and b). The percentage of ergosterol present in ester form (Fig. 3) rose sharply to 48 during the first 25 hr of trophophase and then declined to 39 in the last 5 hr only to rise again as idiophase began, reaching 67 after 54 hr of growth. The peak in ergosterol ester production in trophophase is difficult to explain; it could be that sterol esters fulfil an overspill function such that any ergosterol produced in excess of immediate metabolic or structural requirements is stored temporarily as ester and that the rapid rise and fall in ester production reflect this function. The percentage composition of the major 4-demethyl- and 4,4-dimethylsterols in the unesterified state did not change markedly during the growth of the culture (Table 1). Conversely the percentage composition of these sterols in the esterified form varied to a considerable degree. Thus episterol varied from 44·5 to 75·8% of the total esterified sterol whilst remaining almost constant at an average of 35·3% of the total unesterified sterol. It is quite clear from Table 1 and Fig. 2, a and b that both sterols and sterol esters are produced during trophophase and idiophase and so cannot be regarded as secondary metabolites.

The fatty composition of the sterol esters showed a general increase in the percentage of unsaturated fatty acids with increasing age of culture (Table 2); the proportion of the most abundant saturated fatty acid, palmitic, decreased whilst those of the most abundant unsaturated fatty acids, oleic, linoleic and γ-linolenic, increased. The fatty acid composition of the triglycerides showed no dramatic change as the culture aged. The percentage of saturated fatty acids remained at 45–50% throughout the period studied whilst that of both the main fatty acids, palmitic and oleic, showed a small rise. The fatty acid composition of the phospholipids also did not vary markedly with age of culture, the percentage of saturated fatty acids only fluctuating between 25 and 30%.

The most abundant fatty acids in the three classes of lipid studied were palmitic, palmitoleic, stearic, oleic, linoleic and γ-linolenic acids. However, the proportions of several of these fatty acids in these lipids were significantly different; average values for the percentage of the following fatty acids in the sterol esters, triglycerides and phospholipids were respectively: palmitic acid, 16·4, 34·2 and 22·8; stearic acid, 6·0, 7·1 and 2·5; linoleic acid, 9·1, 8·7 and 16·3, and γ-linolenic acid, 10·1, 7·6 and 15·1. These results indicate that random esterification is not taking place, a fact which may be explained by there being different pools of fatty acids within the organism or by some of the enzymes taking part in the formation of these lipids exerting a degree of specificity with respect to fatty acids or fatty acid-containing substrates.

The possibility that sterol esters constitute a reserve of either sterol or fatty acid which is mobilized when the organism needs it appears to have been negated by the results of the starvation medium experiments (Fig. 4). When the 40 hr culture was transferred to a medium containing no organic nutrients there was a marked and progressive drop in the triglyceride level from 47·5 to 6·0 mg/g dry weight over a period of 30 hr. During this time the ergosterol ester remained virtually constant and the percentage of ergosterol present as ester rose from 26·7 to 33·8. A similar result was obtained with a 20 hr culture transferred to and maintained on the starvation medium for 72 hr; the percentage of ergosterol present as ester rose from 3·7 to 16·8. Had sterol ester been acting as a reserve of either fatty acid or sterol the level of ergosterol ester and the percentage of ergosterol present as ester would have been expected to decline as was shown to occur with the triglyceride level.

EXPERIMENTAL

Organism and cultural conditions. Phycomyces blakesleeanus Burgeff, (−) strain was maintained on slopes of saboraud dextrose agar. Spore suspensions used to inoculate liquid cultures were made by irrigating slopes well endowed with sporangiophores, under aseptic conditions, with sterile water. After inoculation the medium[6] was incubated at 24° under constant illumination (3750 lx) in a gyrotary shaker.

Extraction of lipid. Lipid was extracted with Me_2CO and Et_2O as described previously.[1] When complete extraction of phospholipids was required the mycelial residue remaining after extraction with Me_2CO and Et_2O was extracted several times with MeOH.

Column chromatography. This was carried out in the manner described previously.[1] TLC. System 1: silica gel G (0·25 mm) impregnated with Rhodamine 6G[7] developed with C_6H_6–light petrol. (2:3). System 2: silica gel G (0·25 mm) impregnated with Rhodamine 6G developed with $CHCl_3$. System 3: silica gel G (0·25 mm) developed with $CHCl_3$–MeOH–H_2O (65:24:4).

GLC. Sterols were analysed on 183 cm × 4 mm i.d. glass columns packed with 3% OV-1 on 100–120 mesh Gas Chrom Q operated isothermally at 240°. The carrier gas was argon flowing at 40 ml/min and detection was by FID. Cholestane was chromatographed with each sample and peaks were identified by their retention times relative to cholestane determined previously.[1] Fatty acid methyl esters were prepared and analysed as described previously.[1]

Saponification of sterol esters, triglycerides and phospholipids. This was carried out as described previously.[1]

Spectrophotometric determination of ergosterol. Samples containing esterified or unesterified ergosterol were dissolved in spectroscopically pure EtOH and their UV spectra taken. The quantity of ergosterol present was then calculated using a $E^{1\%}_{1cm}$ of 310 after correction for irrelevant absorption by the Morton and Stubbs procedure[8] using the correcthon equation quoted by Glover.[9]

[6] GOULSTON, G., GOAD, L. J. and GOODWIN, T. W. (1967) *Biochem. J.* **102,** 15C.
[7] AVIGAN, J., GOODMAN, D. S. and STEINBERG, D. (1963) *J. Lipid Res.* **4,** 100.
[8] MORTON, R. A. and STUBBS, A. L. (1946) *Analyst* **71,** 348.
[9] GLOVER, J. (1964) *The Determination of Sterols,* Monograph No. 2, Society for Analytical Chemistry, p. 10.