Microbiology, Vol. 69, No. 5, 2000, pp. 530–535. Translated from Mikrobiologiya, Vol. 69, No. 5, 2000, pp. 636–641.
Original Russian Text Copyright © 2000 by Galanina, Konova.

EXPERIMENTAL ARTICLES

Effect of Exogenous Sterols on the Growth and Fatty Acid Composition of the Oomycete *Pythium debaryanum*

L. A. Galanina and I. V. Konova

Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117811 Russia

Received October 4, 1999; in final form, March 23, 2000

Abstract—Exogenous ergosterol and cholesterol were found to affect the growth and lipogenesis of the oomycete fungus *Pythium debaryanum*, which is unable to synthesize de novo steroid compounds. These sterols stimulated the growth of the fungus during its submerged cultivation in glucose–peptone medium. This was accompanied by the shortening of the lag phase, the lengthening of the period of active growth, and by a 3.7- or 4.3-fold increase in the maximum biomass in response to the addition of ergosterol or cholesterol, respectively. In the presence of ergosterol, the cellular content of polyenoic fatty acids increased, and the relative content of eicosapolyenoic fatty acids reached 31.4% of the total amount of fatty acids in cells. Conversely, cholesterol decreased the cellular content of polyenoic acids, and the relative content of eicosapolyenoic acids fell to 19.6% of the total amount of fatty acids. It may be inferred that exogenous sterols enhance the yield of pharmacologically active polyenoic acids because of the growth stimulation.

Key words: oomycetes, sterols, lipids, fatty acids, eicosapolyenoic acids

The ability of oomycetes of the genus *Pythium*, family *Pythaceae*, to synthesize eicosapolyenoic fatty acids [1] attracts the attention of researchers to these fungi as potential producers of pharmacologically active lipids [2]. Publications exist concerning the ecology, phytopathogenic activity [3, 4], and the absence of functionally active steroid components in their membranes [5]. Being plant parasites, oomycete fungi utilize the sterols of their host plants.

Oomycetes cultivated in synthetic media in the absence of sterols lose the capability for sexual reproduction and for the formation of oogonia and antheridia, but retain the ability to form these organs if the growth medium is supplemented with individual sterols or sterol-containing substances (such as plant oils) [6, 7]. Different sterols possess different abilities to stimulate sexual reproduction (the formation of oogonia and then oospores): cholesterol and β-sitosterol are the most active in this respect, whereas ergosterol is the least [7]. Nelsen *et al.* [8] studied the effect of soybean lecithin and other nutritional factors of a lipid nature on reproductive processes in *Pythium ultimum* [8]. The genetic properties of oomycetes were studied by Nes *et al.* [9].

Unlike sexual reproduction, the vegetative growth of oomycetes does not require to exogenous sterols [10, 11], although their beneficial effect on the growth of *Pythium debaryanum* colonies on agar medium has been reported [7]. To the best of our knowledge, there is no published information about the influence of exogenous sterols on the submerged growth of pythiums, their lipogenic activity, and fatty acid composition.

In the present study, we investigated these problems using the oomycete fungus *P. debaryanum*, whose lipids show a pronounced hypocholesteremic effect in rabbits [12, 13].

MATERIALS AND METHODS

The *Pythium debaryanum* strain used in this study was obtained from our laboratory collection. The fungus was maintained on wort agar. The effect of sterols on the reproduction and growth of the fungus was studied by cultivating it at 26°C on wort agar plates supplemented with particular sterols in an amount of 20 mg/l (sterols were added to the agar medium in the process of its preparation in the form of alcohol solutions). The presence of reproductive organs was examined microscopically. Fungal growth was evaluated by measuring the diameter of 15-day-old colonies.

The fungus was cultivated in a submerged mode at 26°C on a shaker (200–220 rpm) in 250-ml Erlenmeyer flasks with 50 ml of medium containing (%) glucose, 4.0; bactopeptone, 2.0; KH_2PO_4, 0.14; $MgSO_4 \cdot 7H_2O$, 0.025; and yeast extract, 0.1. Material for inoculation was obtained by washing off fungal mycelium from wort agar plates.

0026-2617/00/6905-0530$25.00 © 2000 MAIK "Nauka/Interperiodica"

Table 1. Effect of exogenous sterols on the growth of the oomycete *P. debaryanum* in glucose–peptone medium and parameters of its lipogenesis

Parameter	Control (without sterols)				Ergosterol				Cholesterol			
	Cultivation time, days											
	2	3	4	7	2	3	4	7	2	3	4	7
Dry biomass, g/l	1.2	3.0	6.3	5.4	4.6	8.6	13.0	23.4	5.6	11.5	16.4	26.9
Lipids, % of dry biomass	7.8	6.5	6.8	9.1	5.6	6.7	7.8	12.5	5.5	6.1	8.0	10.1
Fatty acid, * % of total fatty acids												
$C_{14:0}$	7.5	8.2	7.9	9.7	4.4	6.8	6.5	8.2	7.8	7.6	8.8	9.0
$C_{16:0}$	17.0	16.8	16.7	18.8	15.0	14.8	15.2	15.0	17.8	17.0	16.2	16.7
$C_{16:1}$	4.5	2.1	3.0	1.0	1.9	1.0	2.0	0.8	2.0	1.5	1.0	1.0
$C_{18:0}$	3.8	3.8	4.2	2.5	4.6	5.0	4.0	3.7	8.0	9.3	8.0	7.2
$C_{18:1}$	23.8	22.2	19.8	20.4	15.2	16.2	15.6	21.0	23.0	23.4	22.0	23.5
$C_{18:2}$	16.6	16.8	18.8	19.1	21.9	23.8	27.3	23.2	20.7	19.6	20.1	19.6
$C_{18:3}$	3.1	3.2	2.1	2.4	4.2	2.9	2.0	2.1	4.2	2.8	2.9	3.0
$C_{20:3}$	Traces	1.2	Traces	Traces	1.8	1.0	1.5	1.0	0.4	0.9	0.8	Traces
$C_{20:4}$	12.6	14.7	14.0	13.5	14.7	15.0	13.0	12.2	8.3	9.1	11.0	9.0
$C_{20:5}$	10.7	11.0	12.8	10.7	14.9	13.2	12.2	11.4	6.5	7.4	7.8	9.2

* Along with the fatty acids shown, the trace amounts of $C_{12:0}$, $C_{14:1}$, and four other unidentified fatty acids were also detected.

Ergosterol and cholesterol, purchased from Sigma, were added to the medium prior to inoculation in the form of alcohol solutions (the final concentration of sterols was 20 mg/l). The control medium was supplemented with an equivalent volume of alcohol (actually, alcohol at the concentrations used exerted no noticeable effect on growth or lipogenesis). Culture growth was evaluated by weighing the biomass dried at 94°C to a constant weight.

Lipids were extracted by the Bligh and Dyer method [14]. The methyl esters of fatty acids prepared by acidic methanolysis were analyzed on a Model 3700 gas–liquid chromatograph equipped with a flame ionization detector and a column packed with Chromosorb WAW-DMCS-HP80 (1 mesh) containing 17% diethylglycol succinate. The column temperature was 180°C. The carrier gas was argon at a flow rate of 50 ml/min.

RESULTS AND DISCUSSION

Cholesterol added to wort agar enhanced the growth and size of *P. debaryanum* colonies. However, we failed to reveal any reproductive structures (oogonia, antheridia, or oospores) in the mycelium of *P. debaryanum* grown in the presence of ergosterol or cholesterol, which are known as stimulators of reproductive processes. This failure can be explained by the fact that fungi of the genus *Pythium* are capricious with respect to sexual reproduction under laboratory conditions. In particular, only fresh *P. debaryanum* isolates exhibit an ability to form reproductive structures [7]. As for the fungal strain used in our experiments, it had been maintained under laboratory conditions for a long time.

Most data on the effect of exogenous sterols on the growth of *P. debaryanum* and its lipid composition were obtained in experiments with submerged cultures cultivated for 7 days. In the medium without sterols, the fungus grew most actively between the second and fourth days of cultivation (Table 1): daily increase in the biomass within this time period comprised 31.7 and 52.4%, respectively. The maximum biomass was observed on the fourth day of cultivation (6.3 g/l). By the seventh day of cultivation, the biomass decreased to 5.4 g/l. On the second day of cultivation in the medium containing ergosterol or cholesterol, the fungal biomass made up, respectively, 309 and 406% of its level in the control medium without sterols. In this case, the lag phase shortened, and the phase of active growth lengthened (Fig. 1), so that the biomass continued to increase until the seventh day of cultivation, reaching, respectively, 23.4 and 26.9 g/l in the media with ergosterol and cholesterol.

The content of total lipids in the mycelium changed insignificantly in the growth process and did not exceed 12% (Table 1). The maximum content of lipids in the 7-day mycelia grown in the presence of sterols was slightly greater than in the mycelium grown in the control medium.

The fatty acid composition of fungal lipids was qualitatively identical in all experimental variants, even though there were some quantitative differences associated with the effect of exogenous sterols (Tables 1 and 2).

Table 2. Effect of exogenous sterols on the relative content (% of total fatty acids) of particular fatty acid groups synthesized by the oomycete *P. debaryanum*

Sterol	Cultivation time, days	$C_{14:0}$ and $C_{16:0}$ acids	C_{18} acids		Eicosapolyenoic $C_{20:3}$, $C_{20:4}$, and $C_{20:5}$ acids	The sum of mono- and polyenoic acids	The sum of polyenoic acids
			monoenoic $C_{18:1}$ acid	$C_{18:2}$ and $C_{18:3}$ acids			
Control (without sterols)	2	29.0	23.8	19.7	23.3	66.8	43.0
	3	27.1	22.2	20.0	26.9	69.1	46.9
	4	27.6	19.8	20.9	26.8	67.5	47.7
	7	30.0	20.4	21.5	24.2	66.1	45.7
Ergosterol	2	21.3	15.2	26.1	31.4	72.7	57.5
	3	22.6	16.2	26.7	29.2	72.1	55.9
	4	23.6	15.6	29.3	26.3	71.2	55.6
	7	24.0	21.0	25.3	24.6	70.9	49.9
Cholesterol	2	27.6	23.0	24.9	15.2	63.1	40.1
	3	26.1	23.4	22.4	17.4	63.2	39.8
	4	26.0	22.0	23.0	19.6	64.6	42.6
	7	26.9	23.5	22.6	18.7	64.3	40.8

Table 3. Effect of exogenous sterols on the yield of lipids (g/l) and C_{18} and C_{20} polyenoic fatty acids (mg/l) synthesized by the oomycete *P. debaryanum*

Sterol	Cultivation time, days	Lipids, g/l	Eicosapolyenoic acids			The sum of C_{18} and C_{20} polyenoic acids
			total	$C_{20:4}$ acid	$C_{20:5}$ acid	
Control (without sterols)	2	0.09	15.7	8.5	7.2	29.0
	3	0.195	39.2	21.4	16.0	68.4
	4	0.428	86.0	44.9	41.1	153.1
	7	0.491	89.1	49.7	39.4	168.4
Ergosterol	2	0.257	60.6	28.4	28.9	111.1
	3	0.576	126.1	64.8	57.0	241.5
	4	1.014	203.0	98.9	92.8	422.8
	7	2.925	539.7	267.6	250.1	1094.6
Cholesterol	2	0.308	36.2	19.2	15.0	92.7
	3	0.701	91.5	47.8	38.9	209.3
	4	1.312	192.8	108.2	76.7	419.2
	7	2.717	370.7	183.3	187.4	831.1

As can be seen from these tables, the content of short-chain fatty acids synthesized at early stages of biogenesis (predominantly myristic ($C_{14:0}$) and palmitic ($C_{16:0}$) acids) was somewhat lower in the mycelia grown in the presence of sterols (especially ergosterol) than in the control medium. Variations in the content of C_{18} and C_{20} fatty acids, which are synthesized at later stages of biogenesis with the involvement of elongases and desaturases, were more pronounced. For instance, the $\left(\frac{\sum C_{18}}{C_{16:0}}\right)$ ratio of the total amount of C_{18} fatty acids to the content of palmitic acid, from which the former are synthesized, was equal to 3.1–3.4 and 3.2–3.3 for the mycelia grown in the presence of ergosterol and cholesterol, respectively, while this ratio was 2.4–2.8 for the control mycelium. These data suggest that the sterols stimulated the elongation processes $C_{16} \longrightarrow C_{18}$.

The $\frac{\sum C_{20}}{\sum C_{18}}$ ratio, which was equal to 0.4–0.5 for the control mycelium and to 0.5–0.7 and 0.3–0.4 for the mycelia grown in the presence of ergosterol and cholesterol, respectively, gives an idea of the degree of conversion of C_{18} fatty acids into eicosapolyenoic

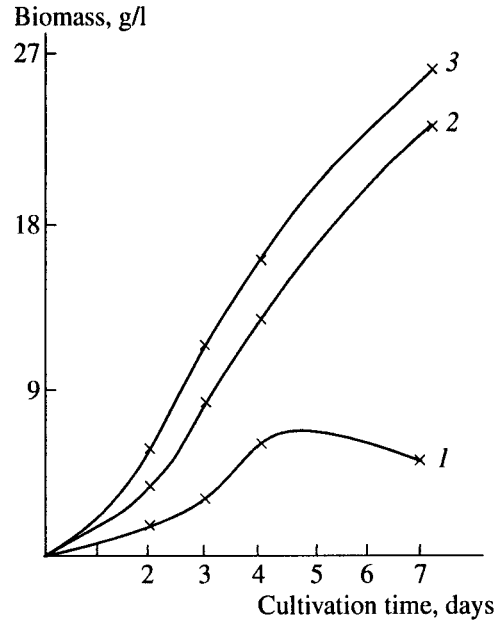

Fig. 1. Effect of sterols on the growth of *P. debaryanum*: (*1*) control medium, (*2*) medium with ergosterol, and (*3*) medium with cholesterol.

(C_{20} polyunsaturated) acids. As follows from these data, unlike cholesterol, ergosterol stimulated the synthesis of eicosapolyenoic acids.

Long-chain fatty acids were dominated by C_{18} unsaturated acids. Stearic (C_{18} saturated) acid was present in the mycelium in minor amounts (2.5–5.0%). However, its content in the mycelium grown in the presence of cholesterol was noticeably higher (9.3%) (Tables 1 and 2).

The maximum content of C_{18} and C_{20} unsaturated fatty acids was observed in the mycelium grown in the presence of ergosterol (this was due to an increased content of polyenoic acids). At the same time, a minimum content of polyenoic acids was observed in the mycelium grown in the presence of cholesterol (this was due to a decreased content of eicosapolyenoic acids, especially eicosapentaenoic acid).

In the mycelium grown without exogenous sterols, the rise in the polyenoic acid level coincided with the period of active growth (2–3 days of cultivation), when the increase in the biomass was maximal and, hence, the concentration of membrane lipids (the substrates of desaturases) was high. On the seventh day of cultivation, when growth processes almost ceased, and the level of cellular lipids somewhat increased, the content of polyenoic acids showed a decrease, evidently because of a diminishing content of eicosapolyenoic acids.

In the presence of ergosterol, the increase in the biomass in the first two days of growth was 12%, i.e., lower than in the control medium. At the same time, the maximum biomass accumulated in the medium with ergosterol was three times as great as in the control medium. The 14.3% increase in the cellular content of polyenoic acids was probably associated with the activation of growth processes and the related increase in the level of membrane lipids. By the seventh day of cultivation, the content of polyenoic acids decreased.

Exogenous cholesterol exerted a still stronger stimulatory effect on the growth of *P. debaryanum* than ergosterol. Indeed, in the presence of cholesterol, the increase in the biomass in the first two days of growth comprised 19%, and the maximum biomass accumulated in the cholesterol-containing medium was four times as great as in the control medium. In this case, the level of polyenoic acids was the least because of the diminished content of eicosapolyenoic acids, amounting to no more than 19.6% of the total fatty acids (the content of the major eicosapolyenoic acids, arachidonic and eicosapentaenoic, was 9.3 ± 0.8 and 7.7 ± 0.8%,

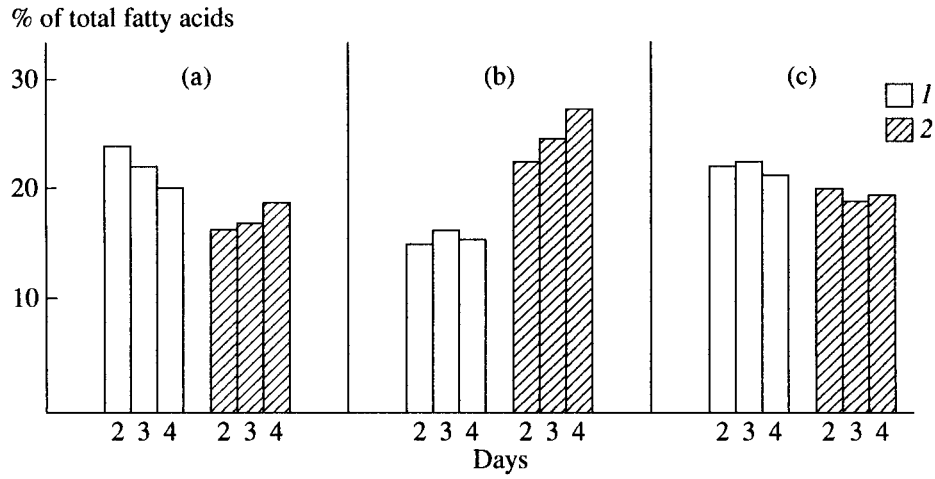

Fig. 2. Effect of sterols on the relative content of oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids in the fatty acids of *P. debaryanum*: (a) control medium, (b) medium with ergosterol, (c) medium with cholesterol: (*1*) $C_{18:1}$, (*2*) $C_{18:2}$.

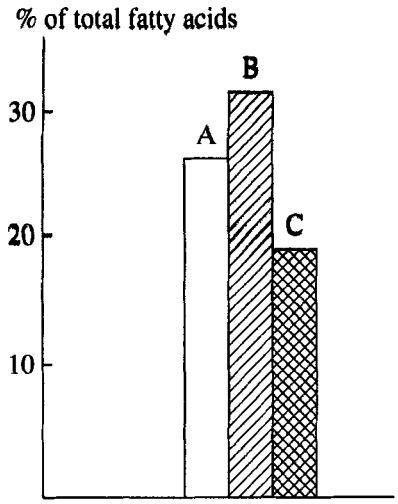

Fig. 3. Effect of sterols on the yield of eicosapolyenoic acids synthesized by *P. debaryanum*: (A) control medium, (B) medium with ergosterol, and (C) medium with cholesterol.

respectively). For comparison, in the mycelium grown in the presence of ergosterol, the content of eicosapolyenoic acids was 31.4% and that of arachidonic and eicosapentaenoic acids comprised 13,7 ± 1.1 and 12.9 ± 1.1%, respectively.

These data suggest that ergosterol stimulated the activity of the desaturase complex responsible for the formation of C_{18} and C_{20} polyenoic fatty acids. This suggestion is supported by the data presented in Fig. 2, showing that ergosterol stimulated the conversion of oleic acid into linoleic acid, and, in Fig. 3, showing that ergosterol stimulated the formation of eicosapolyenoic acids. It should be noted that the data presented can also be interpreted as follows: ergosterol may activate an antioxidant system preventing lipid peroxidation.

Some authors explain the stimulatory effect of sterols on yeast growth by their biological activity related to the presence of the double bond in the cyclic ring system of their molecules [15]. The different effects of ergosterol and cholesterol on the lipid metabolism of *P. debaryanum* probably reflect differences in the structure of their molecules: the ergosterol molecule differs from the cholesterol molecule in having an additional CH_3 group and two double bonds in its aliphatic side chain and in the cyclic ring. The presence of the latter double bond provides the structure reminding the one of provitamin D.

The structural difference of ergosterol and cholesterol may be responsible for their different activities in the membranes of oomycetes [5]. Oomycetes of the genera *Lagenidium* and *Phytophthora*, incapable of the de novo sterol synthesis, incorporate exogenous cholesterol in their lipoproteins, which are shown to be involved in cellular metabolism [17]. In animals, exogenous cholesterol affects the incorporation of fatty acids into phospholipids [18].

The results presented in this paper show that exogenous sterols affect the growth of *P. debaryanum* and its lipogenesis, especially the processes of elongation and desaturation of fatty acids. The effects of ergosterol and cholesterol on desaturation processes and, hence, on the entire lipid metabolism are different.

As can be seen from the data presented in Table 3, both sterols positively affect the yield of pharmacologically active polyenoic C_{18} and C_{20} fatty acids synthesized by the oomycete fungus. This may be of interest for researchers concerned with the biotechnological production of pharmacologically active lipids with the aid of *P. debaryanum*.

REFERENCES

1. Haskins, R.H. and Tulloch, A.P., Host Range and Fatty Acids of a Species of *Pythium*, *Amer. J. Bot.*, 1963, vol. 50, pp. 626–631.
2. Solov'eva, N.L., Konova, I.V., Galanina, L.A., and Babanova, N.K., About the Lipids of *Pythium debaryanum*, *Mikrobiologiya*, 1997, vol. 66, no. 4, pp. 475–480.
3. Pystina, K.A., Ecological Characterization of Fungi of the Genus *Pythium*, *Mikol. Fitopatol.*, 1993, vol. 27, no. 4, pp. 52–57.
4. Hendrix, F.E. and Campbell, W., Pythiums as Plant Pathogens, *Annu. Rev. Phytopathol.*, 1973, vol. 11, pp. 77–98.
5. Elliott, C.G., Sterols in Fungi: Their Function in Growth and Reproduction, *Adv. Microbiol. Physiol.*, 1977, vol. 15, pp. 121–166.
6. Haskins, R.H., Tulloch, A.P., and Mičetich, R.G., Sterols and the Stimulation of Sexual Reproduction of a Species of *Pythium*, *Can. J. Microbiol.*, 1964, vol. 10, no. 2, pp. 187–195.
7. Pystina, K.A., Effect of Sterols and Plant Oils on the Growth and Sexual Reproduction of Fungi of the Genus *Pythium* Pringh, *Mikol. Fitopatol.*, 1973, vol. 7, no. 6, pp. 493–501.
8. Nelson, E.B. and Hsu-Jst, Nutritional Factors Affecting Responses of Sporangia of *Pythium ultimum* to Germination Stimulants, *Phytopathology*, 1994, vol. 34, no. 7, pp. 677–683.
9. Paul, B., Galland, D., and Masih, J., *Pythium prolatun* Isolated from Soil in the Burgundy Region: A New Record for Europe, *FEMS Microbiol. Lett.*, 1999, vol. 173, no. 1, pp. 69–76.
10. Nes, W.D., Saunders, G.A., and Heftmann, E., Role of Steroids and Triterpenoids in the Growth and Reproduction of *Phytophthora cactorum*, *Lipids*, 1982, vol. 17, pp. 178–182.
11. Elliott, C.G., Influence of the Structure of the Sterol Molecule on Sterol-Induced Reproduction in *Phytoph-*

thora cactorum, J. Gen. Microbiol., 1979, vol. 115, pp. 117–121.

12. Solov'eva, N.L., Babanova, N.K., Konova, I.V., Galanina, L.A., and Kaptsov, V.V., Liposomal Medicinal Preparation Containing Eicosapentaenoic Acid, *Tez. dokl. III Rossiiskogo nats. kongressa "Chelovek i lekarstva"* (Proc. III All-Russia Congr. "Human and Remedies"), Moscow, 1996, p. 49.
13. Solov'eva, N.L., Preparation and Investigation of Microbial Lipids Containing Eicosapentaenoic Acid, *Cand. Sc. (Biol.) Dissertation*, Moscow, 1996.
14. Kates, M., *Techniques of Lipidology. Isolation, Analysis and Identification of Lipids*, Amsterdam: Elsevier, 1972. Translated under the title *Tekhnika lipidologii*, Moscow: Mir, 1975.
15. Gal'tsova, R.D., *Sterinoobrazovanie u drozhzhevykh organizmov* (Synthesis of Sterols in Yeasts), Moscow: Nauka, 1980, pp. 25–54.
16. Gurr, M.J. and James, A.T., *Lipid Biochemistry: An Introduction*, 3rd ed., New York: Chapman and Hall, 1980.
17. Warner, S.A., Eierman, D.F., Domnas, A.J., and Dean, D.D., A Novel Lipoprotein from Oomycete Fungi, *Exp. Mycol.*, 1986, vol. 10, no. 4, pp. 315–322.
18. Morin, R.J., The Influence of Cholesterol on the Incorporation of [1-^{14}C] Acetate into Phosphatidyl Choline and Phosphatidyl Ethanolamine of Rat-Liver Mitochondria, *Biochim. Biophys. Acta*, 1967, vol. 144, no. 3, pp. 594–604.