Adenosine 3':5'-Cyclic Monophosphate in *Chlamydomonas reinhardtii*: Isolation and Characterization

(protein kinase assay/aminophylline/cyclic nucleotide phosphodiesterase)

NIKOLAUS AMRHEIN* AND PHILIP FILNER

MSU/AEC Plant Research Laboratory, Michigan State University, East Lansing, Mich. 48823

Communicated by Anton Lang, February 9, 1973

ABSTRACT *Chlamydomonas reinhardtii* contains a factor that can replace adenosine 3':5'-cyclic monophosphate (cAMP) in the stimulation of rabbit-muscle protein kinase. The factor cochromatographs and coelectrophoreses with authentic cAMP, and is inactivated by beef heart cyclic nucleotide phosphodiesterase. When *C. reinhardtii* is exposed to aminophylline (theophylline, ethylenediamine), the concentration of the factor in the cells increases within 1 hr, from about 25 pmol of cAMP equivalents per g dry weight to more than 250 pmol. Cyclic nucleotide phosphodiesterase activity is present in crude extract of *C. reinhardtii* and is inhibited by theophylline. We conclude that cAMP occurs in *C. reinhardtii* and that the endogenous concentration is governed at least in part by a theophylline-sensitive cyclic nucleotide phosphodiesterase. These findings provide a sound basis for attributing the effects of methylxanthines on flagellar function and regeneration in *C. reinhardtii* to the resultant elevation of endogenous cAMP.

We have recently obtained evidence (1, 2) that methylxanthines can inhibit the function and regeneration of flagella of the green alga, *Chlamydomonas reinhardtii*. These compounds are known to increase the amount of adenosine 3':5'-cyclic monophosphate (cAMP) in mammalian cells by inhibiting cyclic nucleotide phosphodiesterase activity (3).

To our knowledge, there is no published evidence concerning the occurrence of cAMP or a methylxanthine-sensitive cyclic nucleotide phosphodiesterase in *C. reinhardtii* or any other alga. Furthermore, the evidence for their occurrence in higher green plants is tenuous. Lettuce is the only species that has been reported to contain cAMP, by means of an assay involving conversion of cAMP to ATP (4). Other reports provide circumstantial evidence for the possible occurrence of cAMP in green plants. These reports describe effects of exogenously supplied cAMP (6-11), or chromatographic similarities between authentic cAMP and a plant metabolite derived from adenine (12-14), or the presence of enzyme activities that may function in cAMP metabolism (5, 15-17).

Theophylline-sensitive activity of cyclic nucleotide phosphodiesterase was recently detected in cultured *Vinca rosea* tumor cells (5).

Our interpretation of the effects of methylxanthines on *Chlamydomonas* flagella depends upon two assumptions: (i) that the organism contains cAMP, and (ii) that methylxanthines bring about an increase in endogenous cAMP by

inhibiting cyclic nucleotide phosphodiesterase in this organism. By means of the cAMP assay of Wastila *et al.* (18), which is based on the stimulation of rabbit-muscle protein kinase by cAMP, we will show in this paper that these assumptions are correct.

MATERIALS AND METHODS

Cultures. The original culture of wild-type *Chlamydomonas reinhardtii*, (+) mating type, was the gift of Prof. R. P. Levine, who also provided the formulation of Tris-acetate-phosphate medium (TAP) that was used throughout this work. This formulation differs quantitatively from the one in the literature (19).

TAP medium is prepared by dissolving 2.42 g of tris(hydroxymethyl)aminomethane in a mixture of 1.0 ml of 1 M potassium phosphate buffer (pH 7.2), 50 ml of Beijerinck's solution, 1 ml of trace elements solution, and water. The pH is brought back to 7.2 by addition of about 1 ml of glacial acetic acid, and the volume is adjusted to 1 liter. Beijerinck's solution consists of 8 g of NH_4Cl , 1 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, dissolved in water to a final volume of 1 liter. To prepare the trace-elements solution, dissolve 50 g of disodium ethylenediaminetetraacetate (EDTA) in 250 ml of heated water. Then, dissolve 22.0 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 11.4 g of H_3BO_3 , 5.06 g of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 4.99 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.61 g of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 1.57 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 1.1 g of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ in 550 ml of H_2O at 70°. Add the EDTA solution, keep the temperature at or above 70°, and adjust the pH by use of pH paper to 6.5-6.8 with KOH solution, 20 g/100 ml of H_2O . Bring the volume to 1 liter. Allow to cool and stand until precipitate settles out. Remove the precipitate by filtration and discard.

The original culture was cloned shortly after receipt and all work reported here was done with this clone, which was maintained on 2% agar TAP slants at 28° under continuous illumination from cool-white fluorescent lamps. For cAMP extractions, cells were grown in 6-liter erlenmeyer flasks containing 3.5 liters of sterile TAP from an initial density of about 10^6 cells per ml to about 5×10^6 cells per ml, on a reciprocal shaker under the same temperature and light conditions described for agar cultures.

5-mM Solutions of aminophylline in TAP were prepared by dissolving the appropriate amount of aminophylline in the medium before the acetic acid had been added. After adjustment to pH 7.2, the medium was autoclaved.

Before cultures were harvested, they were examined under the phase-contrast microscope, and aliquots were plated on

Abbreviation: PKSA, protein kinase stimulating activity.

*Present address: Institute of Plant Physiology, Ruhr-University, D-463 Bochum, Germany.

Penassay Base Agar (20) to check for microbial contamination. In the rare cases when contamination was detected, the cultures were discarded.

Assay of Protein Kinase Stimulating Activity (PKSA). Protein kinase was prepared and assays were performed as described by Wastila *et al.* (18), except that ^{32}P -labeled casein was precipitated by addition of 5 ml of 10% Cl_3CCOOH , collected by vacuum filtration on Millipore glass-fiber pre-filter discs, and washed twice with 5 ml of 10% Cl_3CCOOH . The discs were transferred to glass scintillation vials and dried at 70° . 5 ml of counting solution [5 g of 2,5-diphenyloxazole (PPO) and 0.3 g of *p*-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) per liter of toluene] were added, and the radioactivity was determined in a Beckman LS-133 liquid scintillation counter. A standard curve with authentic cAMP was run for each set of assays.

Preparation of [γ - ^{32}P]ATP. The procedure described by Penefsky (21) was used. Labeled ATP was desalted by absorption to 1 mg/ml of Norit A (purified by washings with water, absolute diethyl ether, 2% NH_3 in 50% ethanol, water, 6 N HCl, and water, and dried) and desorption with 2% NH_3 in 50% ethanol. The eluate was lyophilized and redissolved in H_2O . The radiopurity of the ATP was checked by paper chromatography followed by radioautography. Radioactive ATP was diluted with unlabeled ATP to 5–10 Ci/mol for use in the protein kinase assays.

Extraction and Purification of a Factor with PKSA from *C. reinhardtii*. Cells were harvested by centrifugation at $1000 \times g$ for 10 min in a swing-out rotor, frozen in liquid nitrogen, and stored at -80° until extracted. 15 g of frozen cells were added to 100 ml of cold 10% Cl_3CCOOH in a Potter-type homogenizer and extracted for 10 min. The homogenate was centrifuged at $15,000 \times g$ for 15 min at 0° . The supernatant was extracted with cold water-saturated diethylether until the pH was above 4. The aqueous phase was heated in a boiling-water bath until the smell of ether was no longer discernible. Material that precipitated during the ether extraction and the subsequent heating was removed by centrifugation at $15,000 \times g$ for 15 min. 2 mg/ml of Norit A were added to the supernatant and recovered by filtration through two layers of Whatman no. 1 filter paper. The charcoal was then treated on the filter with 100 ml of 2% NH_3 in 50% ethanol, and the eluate was lyophilized. 5 ml of H_2O were added to the lyophilized material and heated in a boiling-water bath. Insoluble material was removed by centrifugation in a clinical centrifuge. The volume of the supernatant was reduced to 0.5–1 ml in an air stream, and precipitated material was redissolved by heating. The concentrate was chromatographed on Whatman no. 52 paper (descending) in ethanol–1 M ammonium acetate (pH 7.5) (5:2). The region between $R_F = 0.3$ and $R_F = 0.5$ ($R_{F\text{cAMP}} = 0.4$) was eluted with H_2O . The eluate was lyophilized, redissolved in a small volume of hot H_2O , and chromatographed on previously coated silica-gel GF-254 thin-layer plates (Merck) in *n*-butanol-methanol-ethylacetate- NH_3 (7:3:4:4). The cAMP-region ($R_F = 0.43$) was eluted with hot 50% ethanol and lyophilized. The residue was dissolved in 50 or 100 μl of H_2O and assayed for PKSA. Recovery after this step {measured by the addition of about 5000 cpm of [^3H]cAMP (10^3 cpm/pmol) to the Cl_3CCOOH before addition of the frozen cells} was in the range of 40%.

Inactivation of PKSA from *C. reinhardtii* by Beef-Heart 3':5'-Cyclic Nucleotide Phosphodiesterase. 40 mM Tris·HCl (pH 7.5), 2 mM MgSO_4 , 100 pmol of cAMP-equivalents PKSA, and 0.001 units of 3':5'-cyclic nucleotide phosphodiesterase (Sigma) in a volume of 100 μl were incubated at 30° . A control was run with 100 pmol of authentic cAMP. 20- μl Samples were removed at intervals, kept in a boiling-water bath for 5 min, and subsequently stored at -20° until assayed for PKSA.

Extraction and Assay of a cAMP-Hydrolyzing Enzyme from *C. reinhardtii*. About 1 ml of packed cells ($1000 \times g$ pellet of a culture containing 5×10^6 cells per ml) was sonicated (Branson Sonifier, setting 5) for four 15-sec intervals in 3 ml of 40 mM Tris·HCl (pH 7.5), containing 2 mM MgSO_4 and 5 mM cysteine. The homogenate was centrifuged for 10 min at $1000 \times g$ and the pellet was discarded. The assay mixture contained 40 mM Tris·HCl (pH 7.5), 2 mM MgSO_4 , 5 mM cysteine, 0.9 mg of homogenate protein [determined by the procedure of Lowry *et al.* (22)], 500 pmol of [^3H]cAMP (130 cpm/pmol) and, for the inhibitor studies, 10 mM theophylline in a total volume of 0.6 ml. 0.1-ml Samples were removed at intervals and added to 1 ml of cold 10% Cl_3CCOOH . The precipitate was pelleted in a clinical centrifuge, and the supernatant was extracted five times with 10 ml of cold H_2O -saturated diethylether. The aqueous phase was then evaporated in an air stream to near dryness, and, after addition of authentic carrier cAMP, 5'-AMP, and adenosine, was chromatographed in the thin-layer system described above. cAMP, 5'-AMP, and adenosine were identified by their UV-absorption, scratched off the plate, transferred to scintillation vials, and powdered. 5 ml of toluene scintillation fluid (see above) were added, and the vials were left in the dark overnight to let gel phosphorescence subside before radioactivity was determined.

Identification of 5'-AMP as Product of cAMP Hydrolysis by the *C. reinhardtii* Extract. An assay of cAMP-hydrolyzing activity was run and chromatographed without 5'-AMP carrier. The AMP region was eluted with H_2O and rerun on silica gel in isopropanol-ammonia–0.1 M H_3BO_3 (7:1:2) after addition of carrier amounts of 3'-AMP and 5'-AMP. The nucleotides were identified on the plate by their UV-absorption and processed for determination of radioactivity as described above.

Chemicals. All nucleotides and commercially available enzymes used in this study, as well as aminophylline and theophylline, were obtained from Sigma Chemical Corp. [$8\text{-}^3\text{H}$]cAMP (2 Ci/mmol) and $^{32}\text{PO}_4^{3-}$ (carrier-free) were purchased from ICN Tracerlab (Irvine, Calif.). Casein (vitamin-free) was obtained from Nutritional Biochemicals.

RESULTS AND DISCUSSION

PKSA in Extracts of *C. reinhardtii*. Concentrated eluates from charcoal, to which Cl_3CCOOH extracts of *C. reinhardtii* had been absorbed, had little PKSA, and in fact inhibited the stimulation of the protein kinase reaction by authentic cAMP. However, when the charcoal eluates were subjected to paper chromatography, PKSA became detectable, appearing in the approximate region of the chromatogram where cAMP is located, and in no other region.

Ultraviolet absorbing material, which partially overlapped the PKSA region, was effectively separated from the PKSA

by thin-layer chromatography. The PKSA from paper chromatograms was less than proportional to concentration at high concentrations; after the thin-layer chromatography step, proportionality was maintained at higher concentrations. Therefore, the two-step chromatographic procedure was adopted for routine determination of PKSA. By this procedure, PKSA was detected in *C. reinhardtii* cells, and the activity was in the range of 20–30 pmol of cAMP equivalents per g dry weight of the alga, which had been grown to a density of about 5×10^6 cells per ml.

One serious consideration when dealing with picomole amounts of a compound is whether or not the compound was introduced into the system as a contaminant during the isolation procedure. To ensure that this was not the case, a quantity of Cl_3CCOOH , equal to that used to extract *C. reinhardtii*, was processed in parallel with the cell extract, and the resultant blank extract was used to estimate any PKSA due to contamination. No PKSA was detected after extraction of the Cl_3CCOOH blank.

Identity of PKSA of *C. reinhardtii* with cAMP. In order to determine if the endogenous PKSA was due to cAMP, we attempted a more detailed characterization of the active compound. Such work was made initially difficult because of the low PKSA in normal *C. reinhardtii* cells. However, it was found that after 1 hr in 5 mM aminophylline or after continuous growth in aminophylline, the alga contained at least ten times more PKSA (Table 1) [aminophylline does not affect the growth rate of *C. reinhardtii* (2)]. Extracts from *C. reinhardtii* cells treated with aminophylline were used in all experiments designed to characterize the PKSA. Again, we were concerned that PKSA might have been introduced as a contaminant, in this case due to aminophylline. In our hands, the limit of sensitivity of the protein kinase assay for cAMP is 1–2 pmol. Using this assay, we were able to exclude the presence of more than 2 pmol of cAMP equivalents in 2 μmol of aminophylline (that is, 0.0001 mol%); 2 μmol of aminophylline is the largest amount of the substance that could conveniently be introduced into the assay. Thin-layer chromatography of 20 μmol of aminophylline, followed by elution of the cAMP region and assay for PKSA in the eluate, gave equally negative results. However, since a contamination of the aminophylline with 0.0001 mol% PKSA would still introduce sufficient cAMP equivalents (i.e., 2500 pmol/500 ml, the volume used for the 1-hr incubations) into the medium, we tested whether *C. reinhardtii* could take up authentic cAMP from a 5-nM solution of the cyclic nucleotide in TAP medium. Cells (2 g dry weight) that had been incubated for 1 hr in 500 ml of TAP medium with 2500 pmol

TABLE 1. Influence of 5 mM aminophylline on PKSA of *C. reinhardtii* cells

Duration of treatment		cAMP pmol equivalents per g dry weight	
		5 mM aminophylline	Control
10 min		25	20
1 hr	Exp. 1	315	24
	Exp. 2	230	10
5 days	Exp. 1	322	25
	Exp. 2	262	23

FIG. 1. Effect of increasing cAMP (○—○) and *Chlamydomonas* factor (●—●) concentrations on the rate of rabbit-muscle protein kinase reaction.

of cAMP contained 60 pmol of cAMP equivalents per g dry weight, compared to 40 pmol/g dry weight of cells that had been incubated in TAP alone. This negative result, as well as the negative direct tests for PKSA in aminophylline, make it unlikely that the PKSA is introduced into the medium as a contaminant of aminophylline.

Let us now consider the evidence that the PKSA that accumulates in *C. reinhardtii* during aminophylline treatment is in fact cAMP. This evidence is based on a comparison of PKSA and authentic cAMP according to the following criteria: (a) their effect on rabbit skeletal-muscle protein kinase, (b) their chromatographic and electrophoretic behavior, and

FIG. 2. Chromatography of the *Chlamydomonas* factor on silica gel in *n*-butanol-methanol-ethylacetate- NH_3 (7:3:4:4). (a) The entire chromatogram was divided into 10 regions, which were eluted and assayed for PKSA. (b) The main fraction of (a) was rechromatographed on silica gel in the presence of a trace of $[^3\text{H}]\text{cAMP}$ with negligible PKSA (4% of the total PKSA). 6 Regions between $R_F = 0.25$ and $R_F = 0.55$ were eluted with H_2O and assayed for PKSA and radioactivity.

FIG. 3. Paper electrophoresis of the *Chlamydomonas* factor. A trace of [³H]cAMP with negligible PKSA was mixed with the *Chlamydomonas* extract and applied to the origin (arrow) on a 44-cm strip. Electrophoresis was performed for 5 hr at pH 3.5 in 0.05 M ammonium acetate buffer at 11.5 V/cm and 2 mA. 20 2.2-cm long segments were eluted with H₂O and assayed for PKSA and radioactivity.

(c) their inactivation by beef-heart cyclic nucleotide phosphodiesterase.

(a) The activity of rabbit-muscle protein kinase depends on the concentration of the *C. reinhardtii* factor in the same way as it depends on the concentration of authentic cAMP (Fig. 1).

(b) When the chromatographic and electrophoretic behavior of PKSA from *C. reinhardtii* was compared with that of authentic cAMP, by addition of trace amounts of [³H]cAMP to the PKSA-containing preparations, PKSA behaved exactly like authentic cAMP (Figs. 2 and 3). Furthermore, the chromatograms and electrophorograms contained only one region with PKSA.

(c) Authentic cAMP as well as PKSA are completely inactivated by beef-heart cyclic nucleotide phosphodiesterase (Fig. 4). However, the rate of inactivation with PKSA obtained after the thin-layer step was significantly lower than for authentic cAMP. At least two alternative explanations for this difference in the inactivation rates of cAMP and the PKSA have to be considered: (i) PKSA is a cyclic nucleotide that resembles cAMP and has similar activity in the protein kinase assay but is not identical with cAMP. (ii) The PKSA preparations from *C. reinhardtii* contain an inhibitor of cyclic nucleotide phosphodiesterase.

FIG. 4. Time-course of inactivation of PKSA of authentic cAMP (O—O) and *Chlamydomonas* factor (●—●) by beef-heart 3':5'-cyclic nucleotide phosphodiesterase.

FIG. 5. Hydrolysis of cAMP by a crude extract of *C. reinhardtii*. Time-course of disappearance of cAMP (O—O) and appearance of AMP (Δ—Δ) and adenosine (□—□) in the reaction mixture. The reaction rate of cAMP hydrolysis in the presence (●—●) of 10 mM theophylline is inhibited to about 80%. [Beef-heart 3':5'-cyclic nucleotide phosphodiesterase is inhibited to about 90% by 10 mM theophylline (23)].

None of the 3':5'-cyclic monophosphate derivatives of cytidine, guanosine, inosine, or uridine cochromatographed with PKSA in the chromatographic systems used. 2':3'-Cyclic AMP, which does cochromatograph with cAMP in these systems, has no PKSA even at high concentrations. Thus, we were not able to find any evidence indicating a difference between cAMP and PKSA. We did, however, find evidence for an inhibitor, which would seem to explain the discrepancies in the inactivation rates. The existence of this inhibitor became apparent from experiments in which authentic cAMP was subjected to hydrolysis by cyclic nucleotide phosphodiesterase in either the presence or absence of *C. reinhardtii* extracts that had been processed for PKSA. Presence of these extracts in the reaction mixture reduced the reaction rate to as little as 20% of the control rate. The inhibitor is unlikely to be aminophylline or theophylline because PKSA and these compounds are very effectively separated in the paper and thin-layer chromatographic steps.

Presence of a Cyclic Nucleotide Phosphodiesterase in C. reinhardtii. The 10-fold and greater increase in PKSA within 1 hr of aminophylline treatment of *C. reinhardtii* strongly suggested that the organism contained a cyclic nucleotide phosphodiesterase that could be inhibited by methylxanthines. We were able to demonstrate the presence of such an enzyme in crude extracts of *C. reinhardtii*. Time-course experiments were performed in which [³H]cAMP was used as substrate. Reactants and products were separated chromatographically and counted. Adenosine monophosphate was found to be the first product of the reaction; it was then converted to adenosine (Fig. 5). Chromatography of the first reaction product in a system that separates 3'-AMP from 5'-AMP showed that more than 95% of the reaction product was 5'-AMP. Based on the initial rates of degradation of cAMP, at a concentration of 0.83 μM in the reaction mixture, 10 mM theophylline inhibited the reaction by 80% (Fig. 5).

CONCLUSION

We cannot yet present a rigorous chemical proof that the structure of the PKSA factor of *C. reinhardtii* is identical with that of cAMP. Efforts in this area are obviously hampered by the technical problem of preparing sufficient amounts

of the compound. However, the biochemical evidence that we were able to gather leads to the conclusion that cAMP occurs in *C. reinhardtii* and that the endogenous concentration is governed, at least in part, by a cyclic nucleotide phosphodiesterase that is subject to inhibition by theophylline.

This research was conducted under U.S. Atomic Energy Commission Contract AT(11-1)-1338. N. A. held a fellowship from the Deutsche Forschungsgemeinschaft. We are indebted to Mr. Jon Kosteris for his technical assistance.

1. Rubin, R. W., Amrhein, N. & Filner, P. (1971) "Abstracts," 11th Annual Meeting of the American Society for Cell Biology, p. 254.
2. Rubin, R. W. & Filner, P. (1973) *J. Cell. Biol.*, in press.
3. Robison, G. A., Butcher, R. W. & Sutherland, E. W. (1971) *Cyclic AMP* (Academic Press, New York).
4. Narayanan, A., Vermeersch, J. & Pradet, A. (1970) *C. R. H. Acad. Sci.* **271**, 2404-2407.
5. Wood, H. N., Lin, M. C. & Braun, A. C. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 403-406.
6. Duffus, C. M. & Duffus, J. H. (1969) *Experientia* **25**, 581.
7. Galsky, A. G. & Lippincott, J. A. (1969) *Plant Cell Physiol.* **10**, 607-620.
8. Kamisaka, S. & Masuda, Y. (1970) *Naturwissenschaften* **57**, 546.
9. Pollard, C. J. (1971) *Biochim. Biophys. Acta* **252**, 553-560.
10. Salomon, D. & Mascarenhas, J. P. (1971) *Z. Pflanzenphysiol.* **65**, 385-388.
11. Salomon, D. & Mascarenhas, J. P. (1972) *Biochem. Biophys. Res. Commun.* **47**, 134-141.
12. Pollard, C. J. (1970) *Biochim. Biophys. Acta* **201**, 511-512.
13. Salomon, D. & Mascarenhas, J. P. (1971) *Life Sci.* **10**, 879-885.
14. Azhar, S. & Krishna Murti, C. R. (1971) *Biochem. Biophys. Res. Commun.* **43**, 58-64.
15. Shimoyama, M., Kawai, M., Tanigawa, Y., Ueda, I., Sakamoto, M., Hagiwara, K., Yamashita, Y. & Sakakibara, E. (1972) *Biochem. Biophys. Res. Commun.* **47**, 59-65.
16. Vandepute, J. & Huffaker, R. C. (1971) *Plant Physiol.* **47**, suppl., 33.
17. Alvarez, R. (1971) *Diss. Abstr. B* **770**.
18. Wastila, W. B., Stull, J. T., Mayer, S. E. & Walsh, D. A. (1971) *J. Biol. Chem.* **246**, 1996-2003.
19. Gorman, D. S. & Levine, R. P. (1965) *Proc. Nat. Acad. Sci. USA* **54**, 1665-1669.
20. *Difco Manual* (1953) (Difco Laboratories, Detroit), p. 204.
21. Penefsky, H. S. (1967) in *Methods in Enzymology*, eds. Estabrook, R. W. & Pullman, M. E. (Academic Press, New York), Vol. X, pp. 703-704.
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275.
23. Butcher, R. W. & Sutherland, E. W. (1962) *J. Biol. Chem.* **237**, 1244-1250.