Proc. Natl. Acad. Sci. USA
Vol. 78, No. 7, pp. 4354–4357, July 1981
Cell Biology

N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide, a calmodulin antagonist, inhibits cell proliferation

(Chinese hamster ovary K_1 cells/calmodulin function/cell cycle)

HIROYOSHI HIDAKA*, YASUHARU SASAKI*, TOSHIO TANAKA*, TOYOSHI ENDO*, SHINICHI OHNO†, YASUHISA FUJII†, AND TETSUJI NAGATA†

*Department of Pharmacology, Mie University School of Medicine, Tsu 514, Japan; and †Department of Anatomy, Shinshu University School of Medicine, Matsumoto 390, Japan

Communicated by Sidney Udenfriend, March 23, 1981

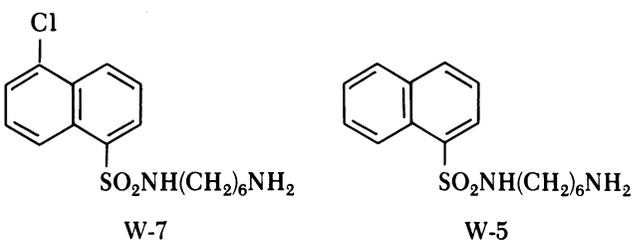
ABSTRACT ***N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and its derivatives are putative calmodulin antagonists that bind to calmodulin and inhibit Ca^{2+}/calmodulin-regulated enzyme activities. Autoradiographic studies using tritiated W-7 showed that this compound penetrates the cell membrane, is distributed mainly in the cytoplasm, and inhibits proliferation of Chinese hamster ovary K_1 (CHO-K_1) cells. Cytoplasmic [^{3}H]W-7 was excluded completely within 6 hr after removal of [^{3}H]W-7 from the culture medium. *N*-(6-aminohexyl)-1-haphthalenesulfonamide, an analogue of W-7 that interacts only weakly with calmodulin, proved to be a much weaker inhibitor of cell proliferation. CHO-K1 cells were synchronized by shaking during mitosis and then released into the cell cycle in the presence of 25 μM W-7 or 2.5 mM thymidine for 12 hr. Cell division was observed ≈6 hr later. The results suggest that the effect of W-7 on cell proliferation might be through selective inhibition of the G_1/S boundary phase, which is similar to the effect of excess thymidine. This pharmacological demonstration that cytoplasmic calmodulin is involved in cell proliferation is significant; W-7 and its derivatives may be useful tools for research on calmodulin and cell biology-related studies.**

Regulation of biological processes by calcium ion involves an interaction with high-affinity calcium-binding proteins. One such protein is calmodulin (1, 2), which has been referred to as an activator protein, a modulator protein, and a calcium-dependent regulatory protein. Calcium ion has been proposed to control a large number of functions, including cell mitosis (3), initiation of DNA synthesis (4, 5), phospholipid turnover (6), and stimulus–secretion coupling in endocrine and exocrine glands (7); however, the Ca^{2+} receptor has not been defined. All or some of these processes may be regulatory mechanisms involving calmodulin, and such potential involvement can be demonstrated by using pharmacological methods.

Calcium ion and calmodulin are considered to play important roles in regulating cell proliferation, being essential for the early DNA synthesis phase (early S phase) of the cell cycle (4, 5). We have examined the influence of a putative calmodulin antagonist (8–11), *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) on the cell division cycle of cultured cells and the intracellular distribution of this agent.

MATERIALS AND METHODS

Chinese hamster ovary (CHO) cells, clone K_1 (CHO-K_1 cells) were cultured in Ham's F-12 medium (Flow Laboratories, McLean, VA)/10% fetal bovine serum (GIBCO)/penicillin (100 units/ml)/streptomycin (0.1 mg/ml) at 37°C. The cells were seeded in TD flasks (RKI, Tokyo) containing 9 × 50 mm glass coverslips for a few days. After the formation of monolayers, 0.2 ml of [^{3}H]W-7 (100 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) in sterile water was added to 1.8 ml of the old medium, and the cells were labeled for 1 hr. [^{3}H]W-7 (specific activity 59.7 Ci/mol) was synthesized by the method of Hidaka *et al.* (12). After labeling the cells with [^{3}H]W-7, the coverslips attached to them were rinsed in 0.1 M phosphate buffer, pH 7.4, at 4°C for 30 sec to remove unbound radioactivity and then blotted on a few sheets of absorbant paper to prevent the lateral movement of radioactive compounds. To eliminate all excess buffer, the coverslips were immediately dried in cold air, by using a hair dryer, and fixed with osmium tetroxide vapor for 30 min. On the other hand, the cells attached to the bottoms of the TD flasks were washed in the same buffer, treated with buffered 2.5% glutaraldehyde for 1 hr, detached from the flasks with lens paper, collected as pellets by centrifugation, and postfixed in buffered 1% osmium tetroxide for 1 hr. The preparations were then dehydrated in a series of ethanol mixtures and embedded in Epon. For light microscopic autoradiography, Sakura NR-M2 liquid emulsion (Konishiroku Photo Industry, Tokyo) was diluted with distilled water, and an equal volume of 0.04% sodium dioctyl sulfosuccinate at 45°C was added to the diluted emulsion. The coverslips were dry-mounted with this emulsion by means of a wire-loop method (13). For the other preparations, 2-μm thick sections cut from the Epon block with a Sorvall MT-2B ultramicrotome were dipped in liquid emulsion, exposed in dark boxes containing silica gel at 4°C for 1 month, and then developed in SDX-1 developer (Sakura) at 20°C for 5 min. These sections were fixed in acid hypofixer, washed in running water, stained with toluidine blue, and observed by bright-field or dark-field microscopy (Olympus Vanox).



W-7 W-5

W-7 and *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5) were synthesized by the method of Hidaka *et al.* (12).

Calmodulin-deficient, Ca^{2+}-dependent cyclic nucleotide phosphodiesterase was purified from bovine brain as described (14).

Myosin light chain kinase was purified from chicken gizzard,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; CHO, Chinese hamster ovary; W-5, *N*-(6-aminohexyl)-naphthalenesulfonamide.

and myosin light chain kinase activity was measured as described (15).

Calmodulin, purified to homogeneity from bovine brain (16), was assayed by measuring the extent of activation of a fixed amount of calmodulin-deficient phosphodiesterase under standard conditions (17). One unit of calmodulin was defined as the amount necessary to produce 50% maximum activation of the calmodulin-deficient phosphodiesterase and was equivalent to 10 ng of protein.

The displacement of [^{3}H]W-7 from purified calmodulin in the presence of calcium ion by W-7 or W-5 was investigated by the equilibrium binding technique of Hummel and Dreyer (18) on a Sephadex G-50 gel filtration column.

CHO-K_1 cells were grown in Ham's F-12 medium/5% fetal calf serum at 37°C in humidified 5% CO_2/95% air; 10^4 cells were plated in 35-mm dishes (Falcon) and cultured for 18 hr. The medium, containing an appropriate amount of the drug, was replaced and, after 2 days of additional culture, the cells were trypsinized and counted. The plating efficiency for CHO-K_1 cells was 96%. Cell number of the control (no drug) was 4.3 $\times$ 10^5 cells per dish. The viability of CHO-K_1 cells treated with various concentrations of W-7 or W-5 was determined by the method of colony formation. Cells 2 $\times$ 10^2 were plated in 35-mm dishes and cultivated for 18 hr. The cells in exponential growth were treated with various concentrations of W-7 for 2 days, and then the medium was gently replaced with fresh medium and the cells were cultured for another 5 days to form colonies. The cells were stained with crystal violet, and the colonies formed were counted. All cells subjected to the synchrony experiment were synchronized without the use of drugs by shaking during mitosis, as described by Terashima and Tolmach (19). The synchronous cells were plated in 35-mm dishes containing conditioned medium. Cell number was monitored at an appropriate period by using a Sysmex model CC-108. Generation time for CHO-K_1 cells in this experiment was $\approx$13 hr. These cultures have a G_1 phase of 6 hr, an S phase of 4 hr, a G_2 phase of 2 hr, and an M phase of 1 hr, as determined by the pulse-labeled mitotic procedure. The synchronous cells were plated at 6 $\times$ 10^4 cells per dish and treated with conditioned medium or medium containing either 25 μM W-7 or 2.5 mM thymidine for 12 hr. The treated cells were washed with conditioned medium and cultured in fresh conditoned medium or medium containing 10 μM cytidine. For the pulse labeling of DNA with [^{3}H]thymidine, cells that had been treated with W-7 and cultured in fresh conditioned medium were incubated for 20 min in 0.5 ml of medium containing [^{3}H]thymidine. Subsequently, the cells were washed and incubated in fresh medium for an additional 10 min. The resulting cells were washed with cold phosphate-buffered saline three times, and 1.0 ml of cold 6% trichloroacetate was added to obtain an acid-insoluble fraction.

RESULTS

Dry-mounted autoradiograms of whole CHO-K_1 cells on glass coverslips were first observed unstained by conventional bright-field microscopy (Fig. 1*A*). The different focus levels of silver grains localized on the surface of the flat cells were not clearly visible. Dark-field microscopic observations showed that the generalized distributions of silver grains were more numerous over the cytoplasm than over the nuclei and, to a lesser extent, in the extracellular space (Fig. 1*B*). In the wet-mounted radioautograms from 2-μm thick Epon sections stained with toluidine blue and observed by bright-field microscopy, almost all of the silver grains were clearly localized in the cytoplasm of the CHO-K_1 cells, with a few in the nuclei (Fig. 1*C*). The vacuoles and lipid droplets were not labeled. It was also obvious that the silver grains were distributed densely in the cytoplasm and sparsely in the nuclei. Cytoplasmic [^{3}H]W-7 was thus demonstrated, as the silver grains decreased exponentially after removal of [^{3}H]W-7 from the culture medium and disappeared within 6 hr.

As shown in Fig. 2, W-7 proved to be a potent inhibitor of

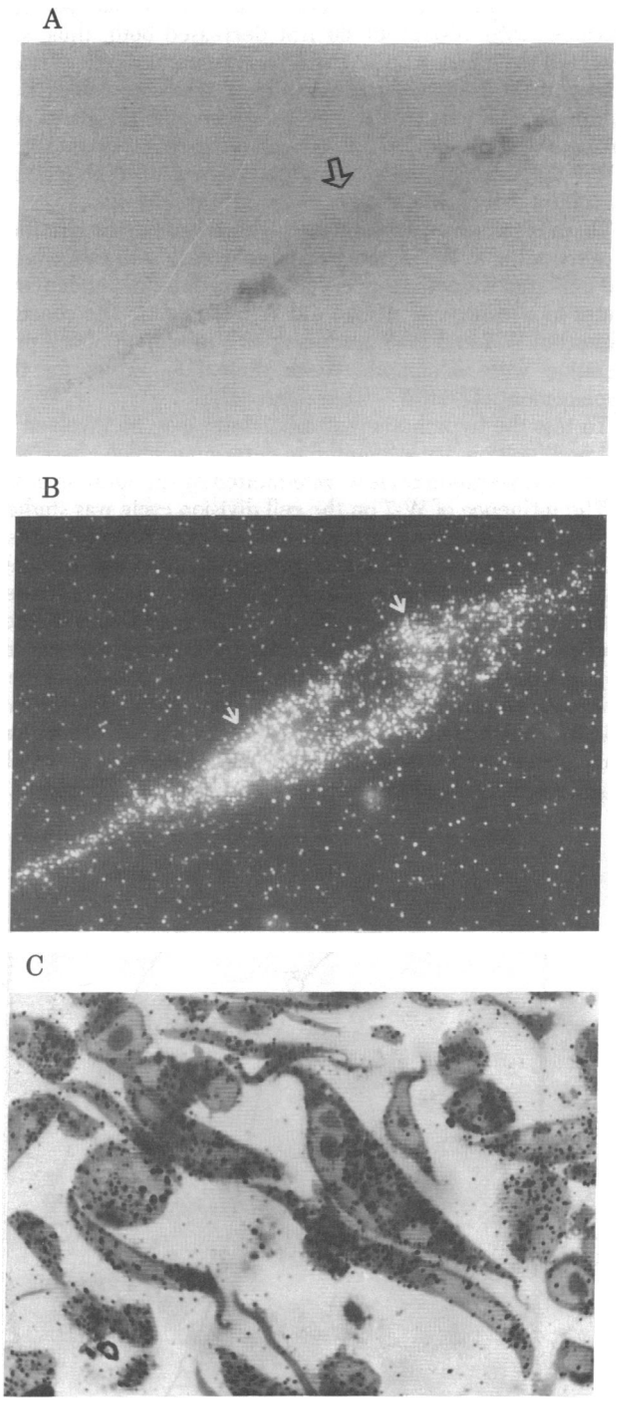


FIG. 1. (*A*) Bright-field photomicrograph of unstained dry-mounted whole cells. Note the nucleus (large arrow) at the center. (×950.) (*B*) Dark-field photomicrograph of unstained dry-mounted whole cells. Many silver grains were observed in the cytoplasm (white arrows) whereas the silver grains in the nucleus were few and the background level of the grain density in the adjacent area was lower. (×1100.) (*C*) Bright-field photomicrograph of stained cells in 2-μm-thick wet-mounted Epon sections. Note the silver grains in the cytoplasmic matrix. Few silver grains can be seen in the nucleus. (×530.)

the cell proliferation of CHO-K_1 cells in culture; 32 μM W-7 suppressed 50% of cell proliferation, while 200 μM W-5 was required for 50% inhibition of cell proliferation. This inhibitory effect of W-7 at up to 50 μM was found to be reversible by the method of colony formation. The viability of cells treated with W-7 or W-5 was determined by using the colony-formation technique. Treatment of the cells with W-7 at up to 30 μM did not decrease either the number or the sizes of the colonies, but treatment with W-7 at 40–60 μM decreased both; thus, we examined the effect of 25 μM W-7 on synchronous cells. Treatment with W-7 at >70 μM completely inhibited colony formation. In addition, >60 μM W-7 detached the cells from the surface of the plastic dish. W-5, a chlorine-deficient analogue of W-7 that interacts with calmodulin only weakly (Table 1) did not inhibit cell proliferation at up to 50 μM (Fig. 2).

These results are explained on the basis of different affinities to calmodulin, as W-5 interacted more weakly with calmodulin and inhibited the activation of Ca^{2+}/calmodulin-dependent enzymes to a lesser extent than did W-7 (Table 1). The concentrations of W-7 and W-5 producing 50% inhibition of cell proliferation were comparable with their IC_{50} values for the displacement of [^{3}H]W-7 from calmodulin.

To test the hypothesis that calmodulin may be involved in cell proliferation, we attempted to determine which phase(s) of the cell proliferation cycle were inhibited by the addition of W-7. The influence of W-7 on the cell division cycle was studied by using synchronous cells prepared by shaking during mitosis (19); this procedure resulted in starting populations of cells having mitotic indices >90%. The synchronous cells were treated with 25 μM W-7 or 2.5 mM thymidine for 12 hr and then washed with conditioned medium and cultured, resulting in cell populations having mitotic indices of 81% and 94%, respectively. As shown in Fig. 3, after the exclusion of W-7 or thymidine from the culture medium, the cell division of these partially synchronized cells was observed at ≈6 hr. The results suggest that W-7 and excess thymidine block similar phases of the cell cycle. Moreover, a sharp increase in DNA synthesis, determined by [^{3}H]thymidine incorporation, was observed after the cells were incubated in fresh medium without W-7.

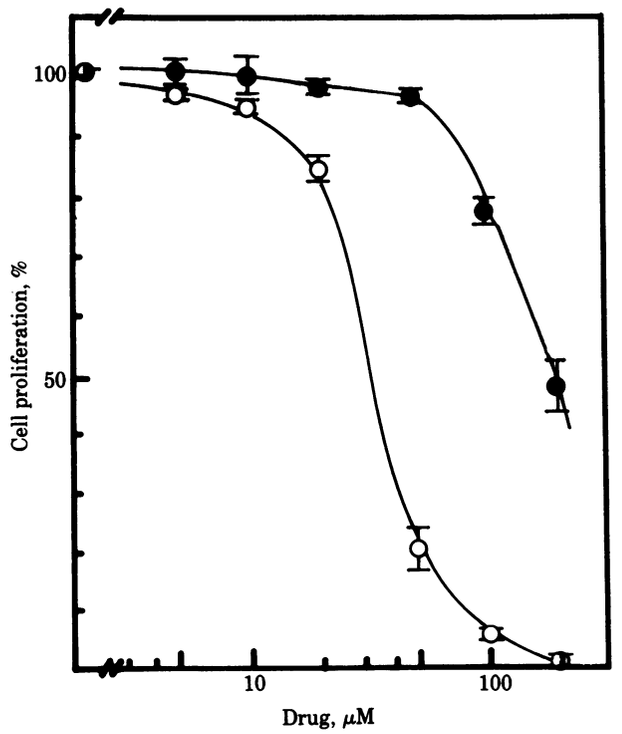


FIG. 2. Effects of W-7 (○) and W-5 (●) at various concentrations on proliferation of CHO-K_1 cells. Experiments were carried out as described in *Materials and Methods*.

DISCUSSION

The importance of calcium in the regulation of cellular processes such as motility, secretion, division, cell shape, and metabolic activity is well established. However, although a growing body of evidence suggests that calmodulin may be an intracellular receptor for calcium ion *in vitro*, the nature or existence of calcium control mechanisms through calmodulin has not been clarified *in vivo*.

A monospecific calmodulin antibody and the indirect immunofluorescence technique are effective tools for the study of calmodulin, as related to cell biology (20–22). Means and associates have shown by the indirect immunofluorescence method that calmodulin is present throughout the cytoplasm but excluded from the nucleus. Our results suggest that the subcellular localization of W-7 is similar to that of calmodulin and that this agent penetrates the cell membrane and probably interacts selectively with calmodulin *in vivo*. Other calmodulin antagonists such as neuroleptic drugs may not be suitable for use in cell biology because they are highly toxic in intact cells and more soluble in the membranous than in the aqueous phase (23). Therefore, W-7 is probably more appropriate for *in vivo* studies as a calmodulin antagonist.

The present results suggest that calmodulin antagonists such as W-7 may selectively block the phase of the cell cycle (G_1/S boundary phase) in a manner similar to that found with excess thymidine treatment. It is well established that high concentrations of thymidine inhibit cell proliferation through inhibition of DNA synthesis (24).

We have shown that 25 μM W-7 arrests the growth of the cells at the G_1/S boundary phase of the cell cycle. Moreover, our results suggest that the initiation of DNA synthesis requires not only Ca^{2+} but also calmodulin and support the finding that the initiation of DNA synthesis requires Ca^{2+} or calmodulin or both (4, 5). Recently, Boynton *et al.* (25) also reported the im-

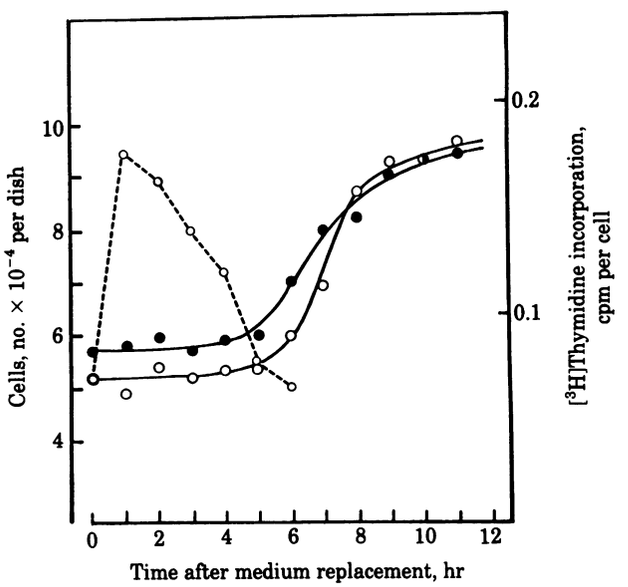


FIG. 3. CHO-K_1 cells synchronized by shaking during mitosis were incubated in the presence of 25 μM (●) W-7 or 2.5 mM (○) thymidine for 12 hr. The treated cells were washed with and incubated in fresh medium. At the indicated time, the incorporation of [^{3}H]thymidine into the trichloroacetic acid-precipitable fraction was determined (○---○).

portance of Ca^{2+}–calmodulin complex in DNA synthesis of T518 rat liver cells. These observations support the hypothesis that Ca^{2+}–calmodulin complex may be important, particularly in the G_1/S transition.

Cell proliferation is a complex phenomenon involving several systems. It is likely that the entire range of pharmacological effects of calmodulin antagonists on CHO-K_1 cell proliferation results from a combination of several types of molecular interactions and cannot simply be explained by a single mechanism. For example, we have shown the interaction between calmodulin and a hydrophobic probe, 2-*p*-toluidinylnaphthalene-6-sulfonate, in the presence of Ca^{2+} (26). Although the possibility that W-7 binds to other hydrophobic proteins or lipids and inhibits CHO-K_1 cell proliferation cannot be excluded completely, our findings show that putative calmodulin antagonists such as W-7 and its derivatives are useful tools for cell biology research.

We thank M. Ohara for critical reading of the manuscript.

1. Kakiuchi, S., Yamazaki, R. & Nakajima, H. (1970) *Proc. Jpn. Acad.* **46**, 587–592.
2. Cheung, W. Y. (1970) *Biochem. Biophys. Res. Commun.* **38**, 533–538.
3. Lestourgeon, W. M., Forer, A., Yang, Y., Bertram, J. S. & Rusch, H. P. (1975) *Biochim. Biophys. Acta* **379**, 529–552.
4. MacManus, J. P., Whitfield, J. F., Boynton, A. L. & Rixon, R. H. (1975) *Adv. Cyclic Nucleotide Res.* **5**, 719–734.
5. MacManus, J. P., Boynton, A. L. & Whitfield, J. F. (1978) *Adv. Cyclic Nucleotide Res.* **9**, 485–491.
6. Allan, D. & Michell, R. H. (1977) *Biochem. J.* **164**, 389–397.
7. Douglas, W. W. (1968) *Br. J. Pharmacol.* **34**, 451–474.
8. Hidaka, H., Yamaki, T., Asano, M. & Totsuka, T. (1978) *Blood Vessels* **15**, 55–64.
9. Kobayashi, R., Tawata, M. & Hidaka, H. (1979) *Biochem. Biophys. Res. Commun.* **88**, 1037–1045.
10. Hidaka, H., Naka, M. & Yamaki, T. (1979) *Biochem. Biophys. Res. Commun.* **90**, 694–699.
11. Hidaka, H., Yamaki, T., Naka, M., Tanaka, T., Hayashi, H. & Kobayashi, R. (1980) *Mol. Pharmacol.* **17**, 66–72.
12. Hidaka, H., Asano, M., Iwadare, S., Matsumoto, I., Totsuka, T. & Aoki, N. (1978) *J. Pharmacol. Exp. Ther.* **207**, 8–15.
13. Nagata, T. & Nawa, T. (1966) *Histochemie* **7**, 370–371.
14. Hidaka, H., Yamaki, T. & Yamabe, H. (1978) *Arch. Biochem. Biophys.* **187**, 315–321.
15. Tanaka, T., Naka, M. & Hidaka, H. (1980) *Biochem. Biophys. Res. Commun.* **92**, 313–318.
16. Hidaka, H., Yamaki, T., Totsuka, T. & Asano, M. (1979) *Mol. Pharmacol.* **15**, 49–59.
17. Hidaka, H., Asano, T. (1976) *J. Biol. Chem.* **251**, 7508–7516.
18. Hummel, J. P. & Dreyer, W. J. (1962) *Biochim. Biophys. Acta* **63**, 530–532.
19. Terashima, T. & Tolmach, L. J. (1963) *Exp. Cell Res.* **30**, 344–362.
20. Welsh, M. J., Dedman, J. R., Brinkley, B. R. & Means, A. R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1867–1871.
21. Welsh, M. J., Dedman, J. R., Brinkley, B. R. & Means, A. R. (1979) *J. Cell Biol.* **81**, 624–634.
22. Dedman, J. R., Welsh, M. J. & Means, A. R. (1978) *J. Biol. Chem.* **253**, 7515–7521.
23. Seeman, P., Staiman, A. & Chau-wong, M. (1974) *J. Pharmacol. Exp. Ther.* **190**, 123–130.
24. Xeros, N. (1962) *Nature (London)* **194**, 682–683.
25. Boynton, A. L., Whitfield, J. F. & MacManus, J. P. (1980) *Biochem. Biophys. Res. Commun.* **95**, 745–749.
26. Tanaka, T. & Hidaka, H. (1980) *J. Biol. Chem.* **255**, 11078–11080.