tissue is heterogeneous with regard to cell type and contains a preponderance of cells inherently insensitive to the steroid; (v) after interaction of the receptor complex with chromatin some event at the translational level, such as new protein synthesis, does not occur; and (vi) there is more than one estrogen receptor in the cell, each with its own function.

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References and Notes

1. W. L. McGuire, P. P. Carbone, M. E. Sears, G. C. Eschert, in *Estrogen Receptors in Human Breast Cancer*, W. L. McGuire, P. P. Carbone, E. P. Vollmer, Eds. (Raven, New York, 1975), p. 1.
2. E. V. Jensen, S. Smith, E. R. DeSombre, *J. Steroid Biochem.* 7, 911 (1976).
3. W. L. McGuire, K. B. Horwitz, O. H. Pearson, A. Segaloff, *Cancer* **39**, 2934 (1977).
4. K. B. Horwitz, W. L. McGuire, O. H. Pearson, A. Segaloff, *Science* **189**, 726 (1975).
5. E. Milgrom, L. Thi, M. Atger, E.-E. Baulieu, *J. Biol. Chem.* **248**, 6366 (1973).
6. D. O. Toft and B. W. O'Malley, *Endocrinology* **90**, 1041 (1972).
7. J. Asselin, P. A. Kelly, M. G. Caron, F. Labrie, *ibid.* **101**, 666 (1977).
8. A. J. M. Koenders, A. Geurts-Moespot, S. J. Zolingen, T. J. Benraad, in *Progesterone Receptors in Normal and Neoplastic Tissues*, W. L. McGuire, J.-P. Raynaud, E.-E. Baulieu, Eds. (Raven, New York, 1977), p. 71.
9. L. S. Tsai and B. S. Katzenellenbogen, *Cancer Res.* **37**, 1537 (1977).
10. K. B. Horwitz and W. L. McGuire, *J. Biol. Chem.* **253**, 2223 (1978).
11. W. L. McGuire, K. B. Horwitz, D. T. Zava, R. E. Garola, G. C. Chamness, *Metabolism* **27**, 487 (1978).
12. U. Kim and M. J. Depowski, *Cancer Res.* **35**, 2068 (1975).
13. M. Ip, R. J. Milholland, F. Rosen, U. Kim, in preparation.
14. K. B. Horwitz and W. L. McGuire, *Cancer Res.* **37**, 1733 (1977).
15. G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).
16. D. W. Chan and W. R. Slaunwhite, *J. Clin. Endocrinol. Metab.* **44**, 983 (1977).
17. S. M. Shafie, S. L. Gibson, R. Hilf, *Cancer Res.* **37**, 4641 (1977).
18. P. A. Kelly, J. Asselin, M. G. Caron, F. Labrie, J.-P. Raynaud, *J. Natl. Cancer Inst.* **58**, 623 (1977).
19. F. Vignon and H. Rochefort, *Endocrinology* **98**, 722 (1976); G. Leclercq, J. A. Heuson-Stiennon, C. Nuyens, M. C. Deboel, J. C. Heuson, *Oncology* **34**, 168 (1977); J. S. Bodwin and Y. C. Cho-Chung, *Cancer Lett.* **3**, 289 (1977).
20. R. A. Hawkins, A. Hill, B. Freedman, E. Killen, P. Buchan, W. R. Miller, A. P. M. Forrest, *Eur. J. Cancer* **13**, 223 (1977).
21. B. W. O'Malley and A. R. Means, *Prog. Nucleic Acid Res. Mol. Biol.* **19**, 403 (1976).
22. W. L. McGuire, J. A. Julian, G. C. Chamness, *Endocrinology* **89**, 969 (1971).
23. M. M. Ip, R. J. Milholland, U. Kim, F. Rosen, *Cancer Res.* **38**, 2879 (1978).
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Chitin Synthetase Distribution on the Yeast Plasma Membrane

Abstract. *Purified, intact yeast plasma membranes were allowed to synthesize chitin, and the nascent chains of polysaccharide were observed either by the fluorescence produced with a brightener or by autoradiography. By both methods, it was concluded that the newly formed chitin emerged at many sites on each membrane. Thus, the synthetase that catalyzes chitin formation has a similar distribution. Since chitin synthetase is found mainly in a zymogen form, these results confirm the hypothesis that initiation of the chitinous primary septum of* Saccharomyces *occurs by localized activation of the uniformly distributed zymogen.*

The formation of the chitin primary septum of budding yeast is subject to precise temporal and spatial regulation (*1*). The onset of this event appears to be linked to the transformation of a zymogen, or latent form of chitin synthetase, into active enzyme (*2*). We have successfully solubilized the synthetase and have shown that the latency of the enzyme was preserved after solubilization and therefore was not due to shielding by a membranous structure (*3*).

According to a hypothesis explaining the localization in space of septum initiation (*2*), the chitin synthetase zymogen is uniformly distributed on the plasma membrane, and is activated only in a restricted area by a factor carried inside vesicles. The bulk of the chitin synthetase zymogen is indeed associated with purified, intact plasma membranes (*4*), but its distribution on the membrane remained unexplored. Although the most direct way of ascertaining the location of chitin synthetase would be through the use of a labeled antibody against the enzyme, this procedure is not available because the enzyme has not yet been purified to homogeneity. An indirect approach was suggested by the finding that the reaction product, chitin, remains associated with the particles used to catalyze its formation. It was concluded that the position of the enzyme could be inferred from that of the nascent chitin chains.

Saccharomyces cerevisiae X2180 (ATCC 26109) was grown, and plasma membranes were purified (*4*).

Purified plasma membranes were first activated with trypsin, and then incubated with UDP-*N*-[^{11}C]acetylglucosamine (UDP, uridine diphosphate) in the chitin synthetase reaction mixture; the mixture was finally divided into two portions: in one, the amount of radioactivity incorporated into chitin was determined (*5*). The other portion was subjected to centrifugation in a Renografin gradient identical to that previously used in the isolation of the membranes (*4*). Of the radioactivity incorporated into chitin, 73 percent was found in the reisolated membrane band, an indication that most of the chitin remained attached to the particles on which it was formed.

An attempt to visualize the polysaccharide synthesized by the particulate preparation was made. In order to observe the newly formed chitin, the fluorescence produced by interaction between the polysaccharide and a brightener, Calcofluor White MR2 New (*6*), was used. With this technique, membranes that had synthesized some chitin showed patches of fluorescent material all over their surface (Fig. 1, B and D). The spotty appearance of the fluorescence is more pronounced in Fig. 1D, where individual membranes are shown, than in Fig. 1B, where the membranes are in clumps. Under direct observation the spots appeared to be smaller and more numerous than in the photographs, which are somewhat blurred because of the impossibility of bringing each plane of the membrane into focus at the same time. Importantly, the fluorescent areas were restricted to the location of plasma membranes, thus indicating the absence of smaller particles with the ability to catalyze chitin synthesis. A control (Fig. 1, E and F) in which the chitin synthetase inhibitor polyoxin D was added to the reaction mixture, showed no fluorescence.

These observations were repeated with the electron microscope because of greater resolution. In this case UDP-*N*-[^{3}H]acetylglucosamine was used as substrate, and the product was located by autoradiography. Subsequent to the incubation, some manipulation of the membranes was required in order to eliminate the excess labeled substrate. It was found, however, that centrifugation of the membranes, followed by suspension and washing, resulted in the aggregation of chitin into large clumps, thereby destroying the intitial localization of nascent chains. In order to preserve the configuration of the system, and to immobilize membranes and nascent polysaccharide, the membranes were enrobed in small agar blocks. The agar allowed diffusion in and out of UDP-*N*-

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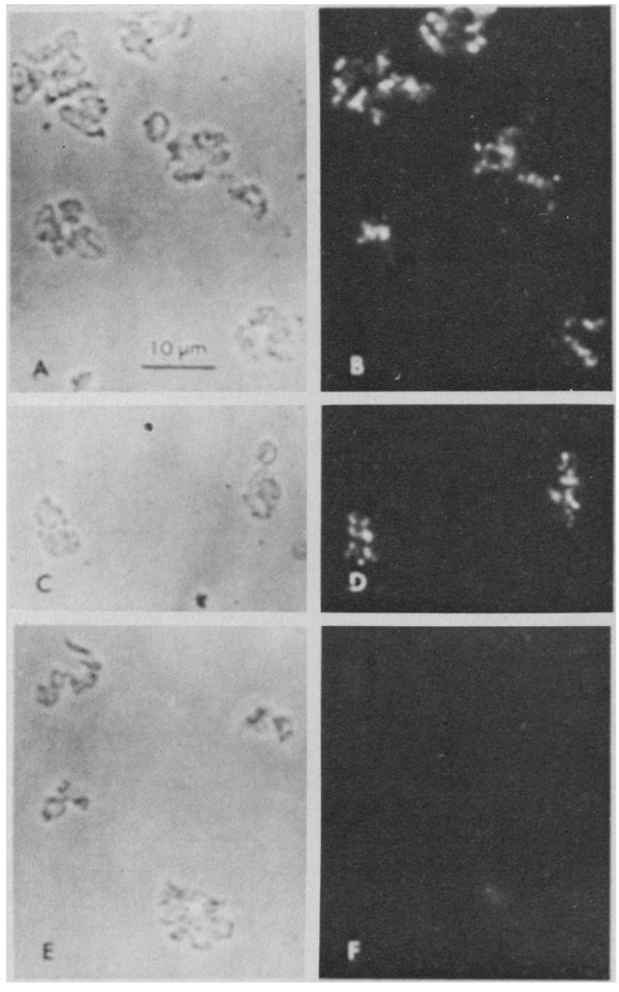

Fig. 1. Fluorescence of plasma membranes after chitin synthesis. Purified plasma membranes were first treated with trypsin and then incubated with UDP - *N* - acetylglucosamine (unlabeled) (*5*), for 30 minutes at 30°C. A solution of Calcofluor White M2R New (provided by American Cyanamid) was added to yield a final concentration in brightener of 0.007 percent, and each sample was observed both in phase contrast (A, C, and E) and in the fluorescence microscope (B, D, and F), as reported (*6*). (E) and (F) represent a sample which contained polyoxin D (120 μg/ml) in the incubation mixture.

acetylglucosamine, which could be introduced before incubation and later washed out without disturbing the organization of the system. Fixation, embedding, sectioning, and subsequent autoradiography revealed that most of the grains were on or close to membrane profiles (Fig. 2, A and B). No grains were found in areas devoid of membranes. Thus the enrobing in agar seems to preserve the association between membranes and chitin, despite the large number of steps during the preparation. Since the sections were less than 0.1 μm thick and a yeast cell is about 6 μm in diameter, only a small portion of each membrane is represented by each section. Thus, many clusters of grains would be found in a complete membrane, in confirmation of the observations with the fluorescence microscope. Again, incubation in the presence of polyoxin D resulted in lack of visible grains (Fig. 2C).

The above described results confirm the previous conclusion that chitin synthetase is associated with the plasma membrane. Ruiz-Herrera and Bartnicki-Garcia have reported on a preparation of soluble chitin synthetase (*7*). It was later found that this "soluble" preparation contained chitin synthetase-bearing particles, which were termed "chitosomes" (*8*). No evidence was found of contamination of the membrane preparation

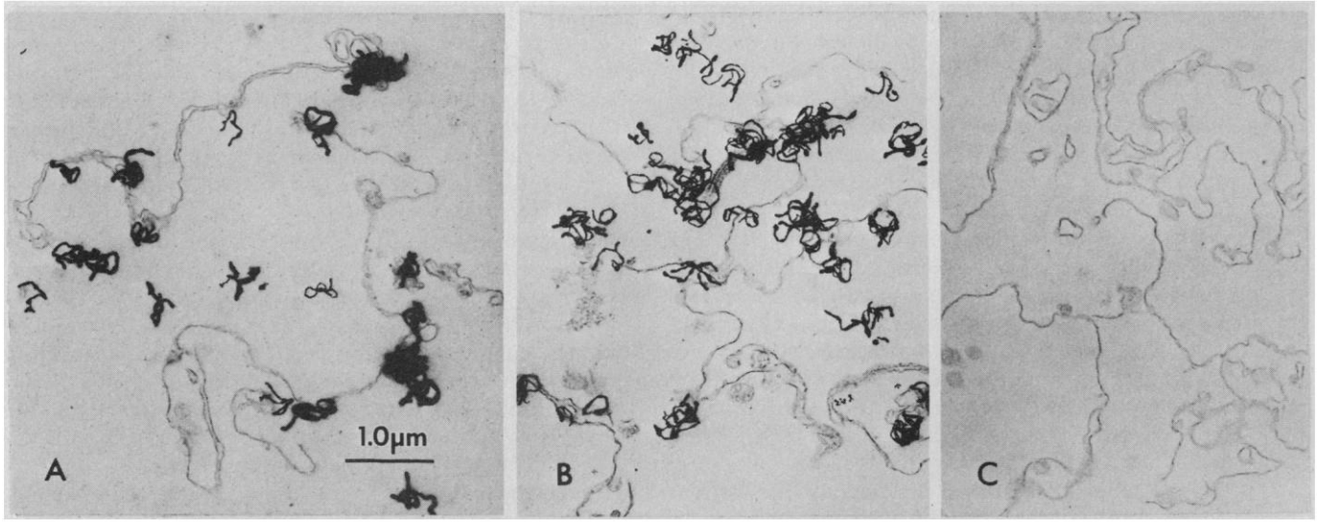

Fig. 2. Autoradiography of sectioned plasma membranes after chitin synthesis. To a suspension of purified membranes, activated with trypsin (*5*) and briefly warmed in bath at 44°C, an equal volume of a 3 percent solution of Noble agar at the same temperature was added and mixed well. The mixture was taken up in a long-stem Pasteur pipette fitted with a pipetting device and delivered slowly into ice-cold 0.05*M* tris (*p*H 7.5) with 2m*M* $MgSO_4$. As the agar solution emerged, it solidified into a long cylindrical "noodle," which was then cut into pieces 1 mm long with a razor blade. These blocks were incubated for 20 minutes at 30°C in a standard reaction mixture for chitin synthetase (*5*), with UDP-[6-^{3}H]*N*-acetylglucosamine (5×10^8 cpm/μmole) as substrate. After incubation, the liquid was withdrawn with a Pasteur pipette, the blocks were fixed with 3 percent glutaraldehyde, washed several times with the same solution, rinsed, and postfixed in 1 percent osmium tetroxide. The blocks were dehydrated in a graded series of ethanol and embedded in Epon 812 (*10*). Thin sections were coated with Ilford L-4 nuclear emulsion by the flat substrate method (*11*). The emulsion-coated sections were exposed for 3 weeks, then developed (Microdol-X) for 3 minutes. The sections were stained with uranyl acetate and lead citrate before microscopy. (A and B) Two representative sections after incubation with labeled substrate and autoradiography. (C) The incubation was carried out in the presence of polyoxin D (120 μl/ml).

with smaller chitin-synthesizing particles, such as chitosomes, as shown by the absence of fluorescence outside the membrane areas in Fig. 1. Both by fluorescence and autoradiography, it appears that the synthetase is present at many different locations on the membrane. Other evidence (*2, 3*) indicates that most of the enzyme is found in the zymogen state if proteolytic activation is prevented. Thus, the initiation of chitin septum at a specific site of the membrane most probably occurs by localized activation of the zymogen, as postulated in our scheme (*2*). The results are not in agreement with an alternative hypothesis, namely, that localization of septum synthesis is attained by delivery of chitin synthetase to specific sites. If this were the case, the enzyme would be found only in a very small zone of the membranes and most of it should already be in the active form, because it would be participating in septum synthesis.

Sloat and Pringle (*9*) have reported that a temperature-sensitive yeast mutant, which is defective in bud initiation, shows, at the nonpermissive temperature, delocalized chitin deposition over the whole cell wall. This behavior sharply contrasts with the precise localization of chitin at the mother cell-bud junction in normal cells. Our working hypothesis (*1, 6*) assumes that during budding initiation, vesicles containing an activator of the chitin synthetase zymogen is directed, by a still unknown apparatus, to a specific site. It seems possible that, when cells are arrested in a prebudding state for a relatively long time, the directional apparatus might become disorganized; the vesicles containing the activating factor would then collide with the plasma membrane at random: activation of chitin synthetase and chitin deposition would occur over the entire surface of the cell as observed by Sloat and Pringle.

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References and Notes

1. E. Cabib, R. Ulane, B. Bowers, *Curr. Top. Cell. Regul.* **8**, 1 (1974); E. Cabib, *Annu. Rev. Microbiol.* **29**, 191 (1975).
2. E. Cabib and V. Farkas, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2052 (1971).
3. A. Duran and E. Cabib, *J. Biol. Chem.* **253**, 4419 (1978).
4. A. Duran, B. Bowers, E. Cabib, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3952 (1975).
5. E. Cabib, *Methods Enzymol.* **28**, 572 (1972).
6. _____ and B. Bowers, *J. Bacteriol.* **124**, 1586 (1975).
7. J. Ruiz-Herrera and S. Bartnicki-Garcia, *Science* **186**, 357 (1974).
8. C. E. Bracker, J. Ruiz-Herrera, S. Bartnicki-Garcia, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 4570 (1976).
9. B. F. Sloat and J. R. Pringle, *Science* **200**, 1171 (1978).
10. J. H. Luft, *J. Biophys. Biochem. Cytol.* **9**, 409 (1961).
11. M. M. Salpeter and L. Bachmann, in *Principles and Techniques of Electron Microscopy*, M. A. Hyat, Ed. (Van Nostrand Reinhold, New York, 1972), p. 221
12. We thank Drs. G. Ashwell, W. B. Jakoby, J. Molano, I. Polacheck, L. Shematek, and M. Slater for a critical reading of the manuscript.

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Lithium: A Modulator of Cyclic AMP–Dependent Events in Lymphocytes?

Abstract. *Theophylline, salbutamol, isoproterenol, and dibutyryl cyclic AMP inhibited E-rosette formation by human T lymphocytes and immunoglobulin M secretion from human plaque-forming B cells and augmented T-suppressor cell activity in three patients with agammaglobulinemia. Lithium chloride increased mitogen-induced lymphocyte proliferation and inhibited suppressor cell activity. In the presence of lithium, the effects of all the drugs except dibutyryl cyclic AMP could be prevented. The data suggest a role for lithium in the modulation of cyclic AMP-dependent events in lymphocytes. Its potential role as an inhibitor of suppressor cell activity warrants further attention.*

The regulatory role of cyclic nucleotides on cell function has been demonstrated in a number of experimental systems. Although much of the work has been carried out with nonlymphoid tissues, there is now evidence that these nucleotides are important mediators of hormonal action on a variety of specific lymphocyte functions [reviewed in (*1*)]. Thus, adenosine 3′,5′-monophosphate (cyclic AMP) has been shown to regulate the formation of E rosettes, a marker of T cells, as well as the activation, proliferation, differentiation, and effector function of T lymphocytes. In addition, there is considerable evidence that cyclic AMP regulates the synthesis and release of immunoglobulin from antibody-producing cells. Membrane adenylate cyclase, through the production of cyclic AMP, plays a crucial role in the transduction of regulatory signals in cells following recognition and triggering (*2*). Lithium, probably through its ability to interfere with adenylate cyclase activation, can affect a variety of cyclic nucleotide–dependent events in the kidney, thyroid, and adrenal glands (*3*). We have examined the role of drugs known to involve cyclic nucleotide synthesis or degradation, in modulating the expression of different human lymphocyte functions, and have shown that these drug-induced effects were effectively countered by lithium chloride.

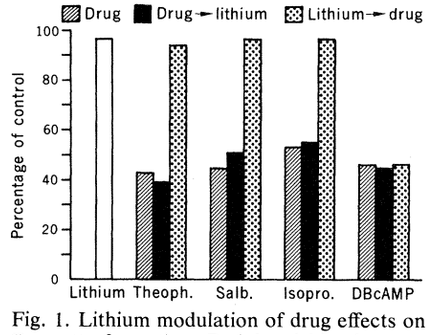

Fig. 1. Lithium modulation of drug effects on E-rosette formation. Peripheral blood mononuclear cells, obtained by Hypaque-Ficoll gradient centrifugation, were incubated with the drugs for 60 minutes at 37°C. In the study shown, lithium was added either 5 minutes before or 60 minutes after the drugs, and the mixtures were incubated for a further 60 minutes at 37°C. Sheep red blood cells were then added, the mixtures were centrifuged (200*g*) for 5 minutes at room temperature, and the pellet was maintained at 4°C for 2 hours. The pellet was then gently resuspended and the number of rosetting cells counted and expressed as a percentage of mononuclear cells. Controls, in the absence of any treatment, ranged from 50 to 62 percent. The means of three experiments, carried out in duplicate, are shown, in which an optimal concentration of lithium ($5 \times 10^{-3}M$) was used; values are expressed as a percentage of the control. Molar concentrations are: lithium, 5×10^{-3}; theophylline, 5×10^{-3}; salbutamol, 1×10^{-4}; isoproterenol, 1×10^{-4}; and dibutyryl cyclic AMP, 1×10^{-4}. Abbreviations: *Theoph.*, theophylline; *Salb.*, salbutamol; *Isopro.*, isoproterenol; and *DBcAMP*, dibutyryl cyclic AMP.

Formation of E rosettes by human T lymphocytes is inhibited by drugs that increase the intracellular levels of cyclic AMP via different mechanisms (*4*). Theophylline, salbutamol, isoproterenol, and dibutyryl cyclic AMP lead to a dose-dependent inhibition of E-rosette formation. As shown in Fig. 1, incubation with $5 \times 10^{-3}M$ lithium chloride, for as little as 5 minutes prior to the addition of the drugs, could abrogate drug-related inhibition of rosette formation in all instances except for inhibition induced by dibutyryl cyclic AMP. Moreover, lith-

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