TUNICAMYCIN -- AN INHIBITOR OF YEAST GLYCOPROTEIN SYNTHESIS

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Received March 25, 1974

Summary: Tunicamycin, a glucosamine-containing antibiotic, halted synthesis of the external glycoproteins invertase, acid phosphatase and mannan by yeast protoplasts within 30 min; formation of two intracellular proteins, alpha-glucosidase and alkaline phosphatase, and of glucan continued at the control rate for at least 60 - 80 min. No accumulation of mannan-free acid phosphatase or invertase was evident in treated cells. Utilization of hexoses and incorporation of ^{14}C-amino acids into protein were not affected. Incorporation of ^{3}H-glucosamine into trichloroacetic acid-insoluble products was only partially reduced. In yeast tunicamycin acts primarily as an inhibitor of glycoprotein synthesis and not of general glucosamine metabolism.

TM[1], a glucosamine-containing antibiotic produced by Streptomyces lysosuperificus, is active against viruses, gram-positive bacteria, yeast and fungi (1). It preferentially inhibits the incorporation of glucosamine into acid-insoluble products by Bacillus subtilis and induces morphological changes in treated bacteria and yeast (2). From studies with B. subtilis and Newcastle disease virus (in chick embryo fibroblasts), Tamura and coworkers (3,4) suggested that TM interferes primarily with the biosynthesis of membrane glycoproteins.

We have investigated the effect of this antibiotic on yeast (Saccharomyces strain 1016) and found that growth was not extensively inhibited over a 2 to 3 hr period, but morphological changes -- spherical and enlarged cells -- were observed. These cells were not osmotically

[1] Abbreviations: TM, Tunicamycin; DE-52, Diethylaminoethyl cellulose 52.

sensitive, but became remarkably susceptible to lytic enzyme (contains mainly glucanase) from Arthrobacter luteus (5). Further studies with yeast protoplasts revealed that TM selectively blocks the synthesis and secretion of the wall mannan-protein complex and of mannan-containing enzymes.

Materials and Methods. Saccharomyces strain 1016 was grown in modified Vogel's medium N. Exponential phase cells were converted to protoplasts using snail gut enzyme in 0.6 M KCl medium, and then washed with 0.8 M sorbitol in the modified Vogel's medium or in that medium with the phosphate omitted (6, 7).

Conditions for enzyme formation and secretion by protoplasts were previously described (7). Suspensions of washed protoplasts (5×10^7 per ml) in 0.8 M sorbitol medium with the appropriate concentration of an energy source were incubated at 30 C with mild shaking (6). Samples were periodically removed and transferred to chilled tubes containing 4 volumes of ice-cold water to lyse the protoplasts. The resulting suspensions were assayed for invertase, alpha-glucosidase, and acid and alkaline phosphatases (6, 7).

To measure enzyme liberated into the medium, 1.0 ml of the incubation mixture was withdrawn at intervals and centrifuged at 1,000 x g; 0.2 ml of the supernatant fluid was pipetted into 0.8 ml of ice-cold water and assayed for the enzyme activity. To characterize the enzyme retained in the protoplasts, the protoplast pellets sedimented at 1000 x g were washed once with 0.8 M sorbitol medium and lysed in glass distilled water with 0.2% Triton X-100. The lysates were centrifuged at 10,000 x g for 10 min, and the supernatant fluids were applied to a column of DEAE-cellulose (DE-52) for separation of the different forms of invertase (8). Incorporation of

^{14}C-labelled amino acids and ^{3}H-glucosamine into CCl_3COOH-precipitable material and the uptake of ^{14}C-mannose into mannan and glucan according to Elorza and Sentandreu (9).

TM was supplied by Professor G. Tamura of the University of Tokyo, Japan. It was dissolved in a minimum amount of 1 M NaOH and diluted to a final concentration of 1 mg/ml with glass distilled water. These stock solutions were stored at - 20° C.

Results and Discussion. Yeast protoplasts suspended in an osmotically-supported medium with an appropriate energy source (usually hexose), synthesize many enzymes as well as the structural carbohydrates of the wall, i. e., glucan, mannan and chitin (10, 11). The glycoprotein enzymes invertase and acid phosphatase are synthesized and released into the medium, whereas alkaline phosphatase and alpha-glucosidase, which are not glycoproteins, are retained by the protoplasts (7, 12).

Takatsuki et al. (2) reported that TM strongly inhibits incorporation of ^{14}C-glucosamine by B. subtilis but not the incorporation of ^{3}H-uridine, ^{3}H-thymidine and ^{14}C-amino acids into RNA, DNA and protein, respectively. We investigated the action of TM (5 - 10 μg/ml) on yeast protoplasts and found that formation of invertase ceased in 20 - 30 min (Fig. 1A). Incorporation of ^{3}H-glucosamine into acid insoluble products was partially inhibited (Fig. 1 C), but utilization of glucose and incorporation of ^{14}C-amino acids into the protein fraction were not affected during a 60 min incubation (Fig. 1A, B). A large proportion of the CCl_3COOH-insoluble radioactivity derived from ^{3}H-glucosamine was found to be present as chitin, whose synthesis was not sensitive to TM (data not present in detail).

To determine if TM primarily inhibits the formation of glycoproteins,

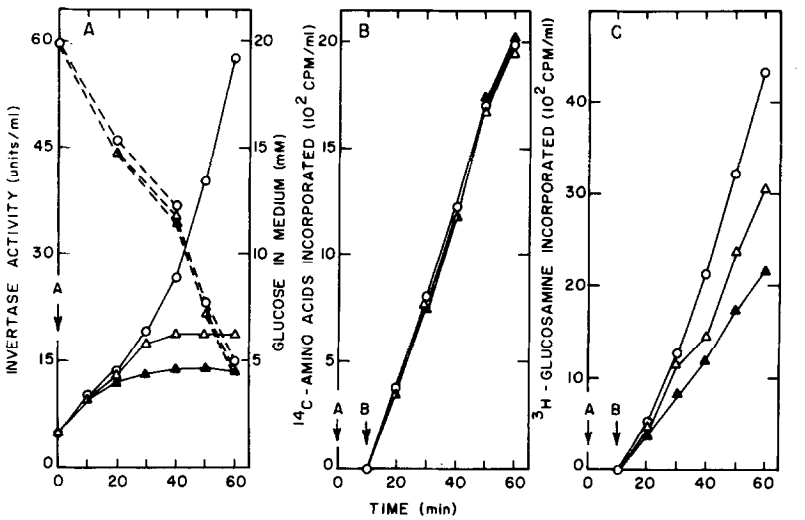

FIGURE 1. Glucose consumption, invertase formation and incorporation of ^{14}C-amino acids and ^{3}H-glucosamine in the presence of TM. Protoplasts from cells grown in 0.2 M glucose were suspended in modified Vogel's medium containing 0.8 M sorbitol and 20 mM glucose. After 60 min of incubation, the protoplasts were spun down and suspended in fresh medium with 20 mM glucose in the absence and presence of TM (Arrow A). ^{14}C-amino acids (0.1 μCi/ml) with 200 μg Casamino acid (Difco) as carrier or 1 mM ^{3}H-glucosamine (2 μCi/ml) was added at 10 min (Arrow B). The concentration of glucose in the supernatant fluids was measured with glucose oxidase (Miles Laboratories, Inc.). Radioactivity was determined in Aquasol (New England Nuclear Corp.) with a Packard Tri-Carb liquid scintillation counter. (O) No TM; (Δ) TM, 5 μg/ml; (▲) TM, 10 μg/ml.

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the induction and synthesis of alpha-glucosidase and alkaline phosphatase by protoplasts were examined since these are not glycoproteins. Alpha-glucosidase synthesis was induced by 20 mM maltose in the presence of an energy source (10 mM fructose) and an amino acid supplement. After 120 min of incubation, synthesis of alpha-glucosidase (and maltose utilization) had begun. During this period, invertase was formed and most of it was released into the medium. The protoplasts were spun down and resuspended in fresh medium with 20 mM maltose, and incubation continued in the presence and absence of TM. As in the experiment of Fig. 1, invertase production was severely inhibited (Fig. 2A), but production of alpha-

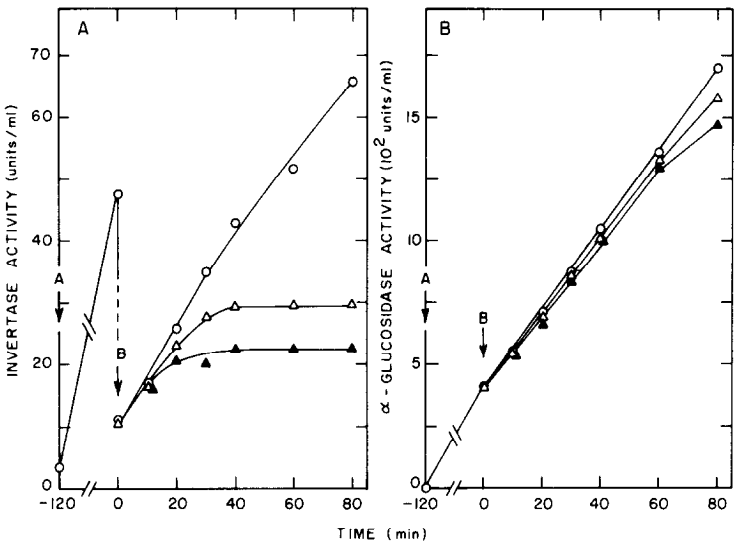

FIGURE 2. Synthesis of invertase and alpha-glucosidase by TM-treated protoplasts. Protoplasts from cells grown in 0.2 M glucose medium were suspended in modified Vogel's medium containing 0.8 M sorbitol, 10 mM fructose, 0.5% Casamino acids (Difco), and 20 mM maltose as inducer (Arrow A). After 120 min of incubation (Arrow B), when synthesis of alpha-glucosidase had begun, the protoplasts were centrifuged at 1,000 x g and the pellets were suspended in fresh medium containing 20 mM maltose and 0.5% Casamino acids (Difco) and incubation continued. (O) No TM; (△) TM, 5 μg/ml; (▲) TM, 10 μg/ml.

glucosidase did not decrease during 80 min (Fig. 2B).

We have established (7) that the synthesis of acid and alkaline phosphatases by protoplasts of Saccharomyces 1016 is readily derepressed in phosphate deficient medium if a high level of hexose (100 mM fructose) is present. When TM was added to these derepressed protoplasts (Fig. 3; arrow), further formation of acid phosphatase was severely curtailed after 20 min (Fig. 3A). Synthesis of alkaline phosphatase by the same protoplasts was not affected (Fig. 3B) nor did this enzyme leak out into the medium.

It was important to determine if TM causes a selective blocking of the synthesis of mannan, since this polysaccharide contains 4 - 8% protein

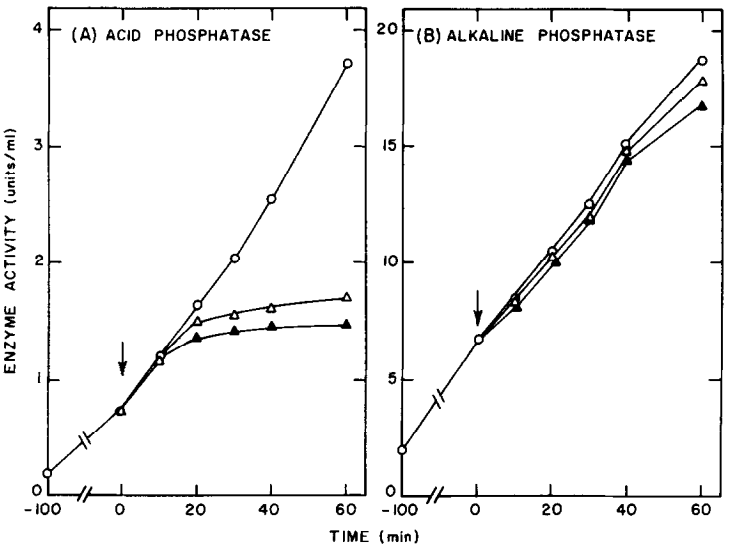

FIGURE 3. Effect of TM on the concurrent synthesis of acid and alkaline phosphatases by protoplasts. Protoplasts from cells grown in 0.05 M maltose were suspended in modified Vogel's medium (phosphate omitted) containing 0.8 M sorbitol and 100 mM fructose, and preincubated for 100 min before tunicamycin was added (Arrow). (O) No TM; (Δ) TM, 5 μg/ml; (▲) TM, 10 μg/ml.

(13) and its synthesis is strongly prevented by cycloheximide (9, 14). TM (5 μg/ml) was added to a suspension of protoplasts actively taking up ^{14}C-mannose and incubation was continued for another 60 min. TM did not affect ^{14}C-mannose uptake by the protoplasts or incorporation of the sugar into glucan (Table 1). In contrast, the incorporation of ^{14}C-mannose into mannan was strongly inhibited.

We have examined the possibility that TM might cause the accumulation of carbohydrate-free forms of acid phosphatase and invertase, since general protein synthesis and formation of two non-glycoprotein enzymes were unimpaired (Figs. 1, 2, and 3). In the presence of TM both invertase and acid phosphatase continue to be secreted into the external medium (Table 2), but the quantity appearing is reduced. Thus TM does not appear

TABLE 1. Effect of TM on ^{14}C-mannose uptake by protoplasts and on incorporation into glucan and mannan [a]

Time (min)	Radioactivity (cpm/ml)					
	^{14}C-mannose uptake by protoplasts		Incorporation			
			Glucan		Mannan	
	-TM	+TM	-TM	+TM	-TM	+TM
0	1506	-	581	-	450	-
20	2602	2716	841	808	810	763
40	3786	3460	1208	1040	1031	850
60	4508	4280	1404	1404	1299	858

[a] Protoplasts were preincubated in 20 mM ^{14}C-mannose (specific activity 0.2 mCi/mmol) for 60 min and incubation then continued with or without TM (5 µg/ml). At various intervals, samples were removed and uptake by the protoplasts and incorporation of radioactivity into the polymers measured as described in Materials and Methods. Most of the mannan formed is secreted by protoplasts and thus is not included in the values for ^{14}C-mannose uptake by the protoplasts.

to inhibit the release of the glycoproteins from the protoplast, but rather their formation. There are (at least) two forms of invertase in yeast cells; a large (L) mannan-protein, mainly external, and a small (S) carbohydrate-free internal enzyme (10). The two can readily be separated by chromatography on DEAE-cellulose or by gel filtration on Sephadex G-200 (6, 8, 15). As shown in Table 2, there was no significant change in the level of the small form after treatment of protoplasts with TM. We could not detect any acid phosphatase (pH 4.0 optimum) smaller than the external glycoprotein enzyme either in the medium or in lysates of treated protoplasts (Table 2).

TABLE 2. Formation and distribution of invertase and acid phosphatase activity in external medium and protoplasts in the presence of TM

Enzyme	Time incubated	Enzyme activity (units/ml)		
		Medium	Protoplasts	
		(L-form)[b]	L-form[b]	S-form[b]
Invertase[a]	60 min (A)	57	3.4	4.2
	A + 40 min (-TM)	150	3.5	3.9
	A + 40 min (+TM)	91	2.2	3.7
acid phosphatase[a]	120 min (A)	1.0	0.25	-
	A + 40 min (-TM)	3.1	0.35	-
	A + 40 min (+TM)	1.8	0.15	-

[a] Protoplasts were incubated in 20 mM and 100 mM fructose for invertase and acid phosphatase experiments, respectively. Phosphate was omitted from the medium for the experiment on phosphatase production. Enzyme activities in the external medium and retained in protoplasts were measured as described in Materials and Methods.

[b] L and S-forms represent large (glycoprotein) and small (non-glycoprotein) enzymes, respectively.

We conclude that TM acts primarily as an inhibitor of glycoprotein synthesis. It is not a general inhibitor of glucosamine utilization, since chitin formation is not affected, but may inhibit a transfer reaction specific to glycoprotein synthesis.

The precise role of the glycosylation process in regulating the formation of glycoprotein enzymes is not clear, but the findings reported

here, like those obtained using 2-deoxy-D-glucose (7), indicate that addition of the sugar moiety is a prerequisite for continued production and secretion of the protein portion of the molecule. TM should be a valuable agent for investigation of the biosynthesis of glycoproteins and their cellular role in yeast.

Acknowledgments. We thank Professor G. Tamura, University of Tokyo, Japan, for the gift of tunicamycin. This research was supported by Public Health Service grant AI-04572 from the National Institute of Allergy and Infectious Diseases.

References.

1. Takatsuki, A., K. Arima and G. Tamura, J. Antibiotics 24: 215-233 (1971).
2. Takatsuki, A., K. Shimizu and G. Tamura, J. Antibiotics 25: 75 - 85 (1972).
3. Takatsuki, A., and G. Tamura, J. Antibiotics 24: 785 - 794 (1971).
4. Takatsuki, A., and G. Tamura, J. Antibiotics 24: 232 - 238 (1971).
5. Kitamura, K., T. Kaneko and Y. Yamamoto, J. Gen. Appl. Microbiol. 18: 57 - 71 (1972).
6. Kuo, S.-C., and J. O. Lampen, J. Bacteriol. 106: 183 - 191 (1971).
7. Kuo, S.-C., and J. O. Lampen, J. Bacteriol. 111: 419 - 429 (1972).
8. Lampen, J. O., S.-C. Kuo, F. R. Cano, and J. S. Tkacz, Ferment. Tech. Today (G. Terui, ed.). Proc. Intl. Ferment. Symp. (IV), pp. 819 - 824 (1972).
9. Elorza, M. V., and R. Sentandreu, Biochem. Biophys. Res. Commun. 36: 741 - 747 (1969).
10. Lampen, J. O., Antonie van Leewenhoek, J. Microbiol. Serol. 34: 1 - 18 (1968).
11. Mendoza, C. G., and M. N. Ledieu, Nature 220: 1035 (1968).
12. Halvorson, H., and L. Ellias, Biochim. Biophys. Acta 30: 28 - 40 (1958).
13. Eddy, A. A., Proc. Roy Soc. B. 149: 425 - 440 (1958).
14. Sentandreu, R., and J. O. Lampen, FEBS Letters 11: 95 - 99 (1970).
15. Gascon, S., and J. O. Lampen, J. Biol. Chem. 243: 1567 - 1572 (1968).