Effect of High Concentrations of Nystatin upon Glycolysis and Cellular Permeability in Yeast.* (26882)

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The polyene antifungal agent nystatin inhibits the ability of fungi, especially yeasts, to grow and to utilize various substrates(1-3). Following treatment of yeast with nystatin, there is a leakage of acids in the tricarboxylic acid cycle(4,5), of inorganic phosphate (2,3) and of K^+(6). Since nystatin is equally inhibitory to glycolysis by intact yeast and by protoplasts, but has no effect on cell-free extracts(7), it was suggested that binding by the cell membrane may be the critical step in nystatin action(6). The resulting membrane damage would result in leakage of metabolites and death of the cell. The importance of permeability alterations in polyene action has also been indicated by Bradley and Jones (2), Drouhet, *et al.*(3), and Kinsky(8).

With low concentrations of nystatin, inhibition of glycolysis is apparently the result of K^+ leakage, since the inhibition at neutral pH can be prevented or reversed by exogenous K^+ or NH_4^+(6). Under these conditions the K^+ loss still occurs and the fungicidal action of the polyene is not prevented. Evidence is now presented that at higher concentrations of the antibiotic, a $K^+ - NH_4^+$ mixture will protect or restore glycolytic activity only in stationary phase cells and here only partially. For log phase cells, all the cofactors that are required in a cell-free glycolytic system must be added. The changes occurring in nystatin-treated cells and protoplasts are described, with particular emphasis on indications of altered membrane permeability.

Materials and methods. Saccharomyces cerevisiae strain LK2G12 was used for the present studies. Log phase and stationary phase cells and protoplasts were prepared as described by Marini *et al.*(6).

A highly purified preparation of nystatin (Lot HV 942, obtained from the Squibb Institute for Med. Research, New Brunswick, N. J.) was dissolved at 15 mg/ml in dimethyl sulfoxide and diluted in water. The citrate-phosphate buffers were those described by Gomori(9). Na^+ was replaced in certain experiments by K^+ or NH_4^+. For manometric measurements, Warburg flasks were flushed with nitrogen and equilibrated at 30°C. The yeast juice was prepared by freezing and thawing bakers yeast(10) and was heated for 5 min in a boiling water bath. The contents of the flasks are described in the text. Abbreviations used: ATP, adenosine triphosphate; AMP, adenosine-5'-phosphate; DPN, diphosphopyridine nucleotide; TPP, thiamine pyrophosphate; NY, nystatin; ($K^+ - NH_4^+$), a mixture of potassium and ammonium chlorides.

Results. Cofactor requirements of NY-treated cells. When 10 μg/ml or more of NY were added to log phase yeast cells in Na^+-buffer, pH 7.0, glycolysis was inhibited almost immediately (Fig. 1). No protection was afforded by 36 mM KCl and NH_4Cl, although one-fourth of this level prevented inhibition by 5 μg/ml of NY. In the presence of the cofactors required for glycolysis by soluble extracts, including small amounts of pyruvate as an oxidant primer, CO_2 production continued although at a rate only 30 to 40% of that in the absence of NY. With stationary phase cells, protection could be achieved by adding 36 mM ($K^+ - NH_4^+$) or, in other experiments, by 9 mM ($K^+ - NH_4^+$) and cofactors.

Similar results were obtained when the reversal of an existing inhibition by high NY concentrations was studied, although in this case both cell types reached only 30 to 40% of the uninhibited rate. High ($K^+ - NH_4^+$) levels were again effective only for stationary phase cells. The need for all the individual cofactors for reversal was somewhat more

* This study was supported in part by a research grant from Nat. Science Foundation.

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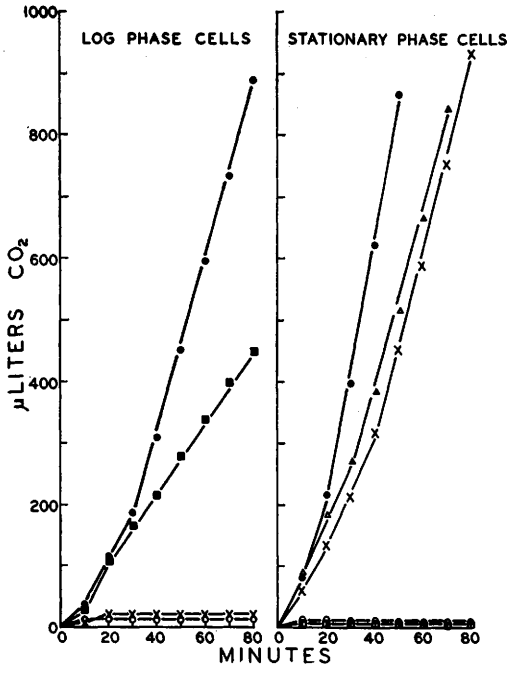

FIG. 1. Protection of glycolysis against high concentrations of NY. Each flask contained 1×10^8 cells, 0.1 M sodium citrate phosphate buffer, pH 7.0, 120 μmoles glucose, 180 μg NY, and supplements as indicated in 3.0 ml. Glucose, NY, and supplements added at 0 min. ●, Control; ○, NY; □, NY + 9 mM KCl and NH_4Cl; ×, NY + 36 mM KCl and NH_4Cl; ■, NY + 9 mM KCl and NH_4Cl + cofactors as in Table I; △, NY + 36 mM KCl and NH_4Cl + cofactors.

clearly defined than in protection experiments. Results with log phase cells are presented in Table I. In other tests, omission of ($K^+ - NH_4^+$) prevented reactivation. ATP could be replaced by equimolar AMP; as little as 0.1 μmole of either nucleotide produced about half-maximal reactivation. TPN could not be substituted for DPN. In several experiments, 0.3 to 2.0 ml of the boiled yeast juice was added per cup. Glycolysis by both NY-treated and intact yeast increased ca 20%, but the rate with the treated cells was still only one-third of that observed in absence of NY. Increased cofactor concentrations also failed to affect this relative rate.

TABLE I. Requirements for Glycolysis by Cells Treated with High Levels of NY.

NY, μg/ml	Supplement omitted	μl CO_2 0-25 min.	μl CO_2 25-95 min.
None		205	1100
60		74	470
60	ATP + DPN	63	74
60	Pyruvate	64	55
60	Mg^{++}	38	70
60	TPP	42	65
60	Glucose	4	20

The flasks contained 1×10^8 log phase cells in 3.0 ml of .05 M ammonium citrate phosphate pH 7.0 and the following supplements (μmoles), except where indicated: 120 glucose, 20 $MgCl_2$, 10 ATP, 5 DPN, 10 sodium pyruvate adjusted to pH 7.0, 5 TPP, and 27 μM each of NH_4Cl and KCl. Gas phase, N_2; temperature, 30°C. NY and glucose were added from a sidearm at 0 min. After 25 min. when metabolism had ceased, pyruvate was added.

Action of NY on protoplasts. The cofactor requirement at high NY levels was also determined for these altered cells. In this experiment (Fig. 2) all supplements except pyruvate were added at 0 min. Glycolysis ceased within 20 min in those vessels containing NY. At 40 min, pyruvate was added and CO_2 production measured. As previously noted with intact cells, the maximum rate attained by treated protoplasts was ca 30% of that in the absence of NY. Omission of any constituent resulted in a sharp reduction of glycolysis.

Lysates of protoplasts did not produce CO_2 from glucose unless the entire complement of cofactors was present (Fig. 2). Under these conditions, NY had no effect on glycolysis even at 90 μg/ml. The maximum glycolytic rate by lysates was 30-40% of that obtained with intact protoplasts.

NY-treated cells or protoplasts showed fermentation rates and cofactor responses essentially identical to those obtained with lysates. They cannot be considered as equivalent to lysates, however, since glycolytic activity was not released into the medium by the treated cells (Fig. 3). Suspensions of 1×10^8 protoplasts were incubated at 30°C for 20 min in 1.75 ml of pH 7.0 sodium citrate-phosphate buffer containing 0.82 *M* mannitol and either 0 or 30 μg of NY/ml. The treated proto-

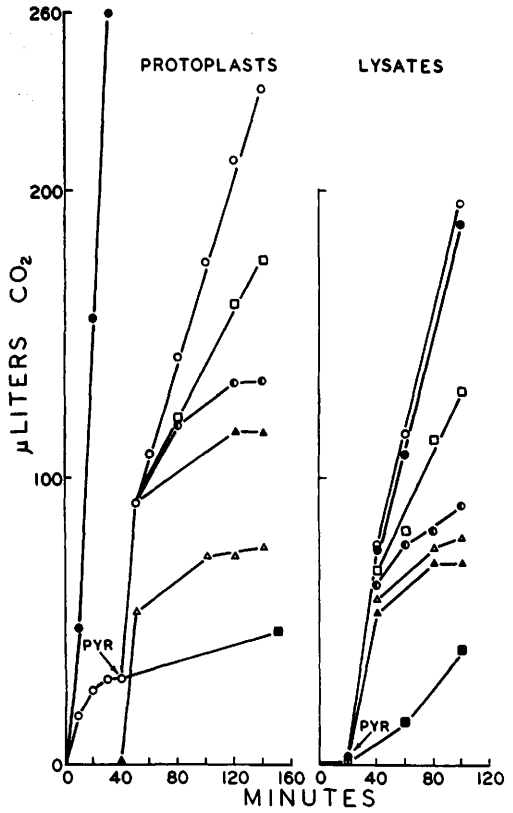

FIG. 2. Glycolysis requirements by NY-treated protoplasts and by lysates. Complete test system: 1.4×10^8 protoplasts in 0.04 M sodium citrate phosphate, pH 5.8, containing 0.82 M mannitol (or lysate prepared by suspending 1.4×10^8 protoplasts in buffer without mannitol), 2.4 μmoles $MgCl_2$, 7.5 μmoles NH_4Cl, 5 μmoles ATP, 2.5 μmoles DPN, 2.5 μmoles TPP, 30 μmoles glucose, and 5 μmoles pyruvate (pH 7.0) in 2.0 ml volume. ●, Complete system = A; ○, A + 30 μg NY/ml = B; △, B without glucose; ■, B without pyruvate; ▲, B without ATP and DPN; ◐, B without NH_4^+; □, B without TPP.

plasts were collected by centrifugation for 5 min at 1500 × g and resuspended in 1.75 ml of the buffered mannitol. Residual glycolytic activity was then determined in the presence of the following (μmoles): 30 glucose, 5 ATP, 5 DPN, 40 $MgCl_2$, 14 KCl, 14 NH_4Cl, 2.5 TPP, 5 pyruvate, and water to 2.0 ml. With the complete system, the rate of glycolysis by protoplasts preincubated with NY was ca one-fourth that exhibited by those preincubated in its absence—a relationship close to that existing before the washing procedure (Fig. 2). The treated and washed protoplasts were still completely dependent on ($K^+ - NH_4^+$) and pyruvate and partially dependent on one or more cofactors. No significant glycolytic activity was found in the supernatant fluid from the NY-treated protoplasts. Leakage of an incomplete complement of glycolytic enzymes has not, however, been excluded.

Of considerable interest is the fact that the treated cells still retain permeability barriers to sugar phosphates. Thus, glucose-6-phosphate and fructose diphosphate were not utilized by the treated cells even in the presence of the cofactors and primer required for CO_2 production from glucose. The same preparations were fermented readily by soluble extracts.

Alterations in cells. The relation between K^+ and NH_4^+ loss and the inhibition of glycolysis is illustrated by the data of Table II. At pH 5.8 in the presence of mannitol, gly-

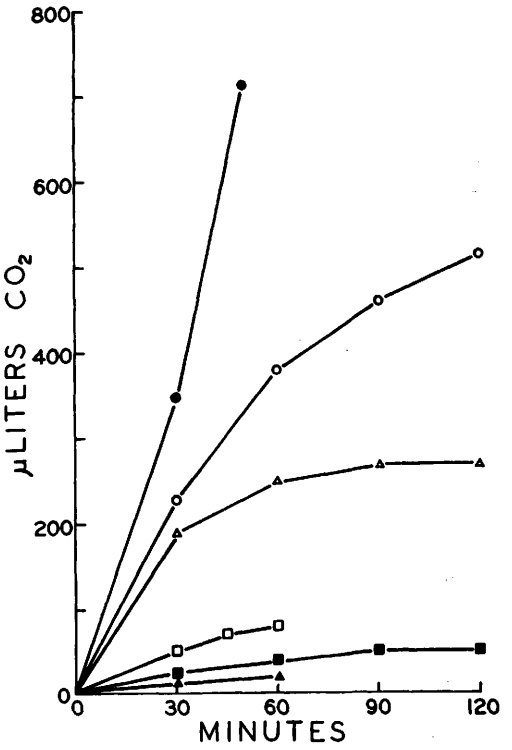

FIG. 3. Glycolysis of protoplasts pre-incubated with NY and then washed. See text for complete test system. NY-treated protoplasts: ○, complete supplement; △, no ATP, DPN, or TPP; □, no ($K^+ - NH_4^+$); ▲, no pyruvate. ●, no NY during pretreatment (complete supplement). ■, supernatant fluid (complete supplement).

TABLE II. Alterations in NY-Treated Yeast.*

Buffer	NY, μg/ml	K^+ in cells, μg/mg	NH_3		Leakage of UV-absorbing material, O.D. (260 mμ) of medium‡	Pyruvate decarboxylation§	
			In cells, μg/mg	In medium, μg/mg cells†		Lag, min.	Rate, μl CO_2/30 min.
pH 5.8 NaC-P	0	15.7	2.1	.3	.100	—	4
+ .82 M	1‖	.06	1.3	.87	.100	25	66
mannitol	2		—			19	161
	5		.17			10	232
	10		—			6	297
	30		<.05	1.42	.156	2	327
pH 7.0 NaC-P	0	11.1	.90	.0	.015	—	20
	2	5.9	.45	.34	.080	90	38
	6‖	.05			.075	40	63
	10				—	22	95
	30		<.1	.75	.205	16	144

* Log phase cells used. K^+ and NH_3 and O.D. data represent values following incubation with NY for 30 min. NaC-P = 0.03 M sodium citrate-phosphate buffer.

† Corrected for NH_3 formed from NY during the analytical procedure (12.3 μg/mg—see text).

‡ Corrected for O.D. at 0 min. of mixtures without NY (.300 in pH 5.8 NaC-P + mannitol; .245 in pH 7.0 NaC-P).

§ NY added to 10^8 cells and 200 μmoles pyruvate in 3 ml of buffer. The lag period before decarboxylation and the subsequent constant rate of CO_2 evolution are presented.

‖ Rapid and complete inhibition of glycolysis occurred.

colysis of log phase cells is inhibited almost at once by 1 μg of NY per ml. At pH 7.0 without mannitol, cells are less sensitive, ca 5 μg per ml being required for rapid inhibition. This difference in sensitivity is attributable primarily to the high mannitol concentration (6), which may accelerate leakage of ions from a partially damaged cell membrane. Similarly, almost complete loss of K^+ occurred with 1 μg of NY at acid pH, whereas 6 μg per ml were required at pH 7.0. Cellular NH_4^+ was also lost following exposure to NY but the concentrations of the antibiotic required were generally higher than those needed to initiate K^+ leakage.

For measurement of ion loss, approximately 6 mg of log phase cells were incubated with the indicated levels of nystatin in 12 ml of buffer containing 0.04 *M* glucose. The suspensions were shaken 30 min at 28°C and then centrifuged. Certain supernatants were examined for leakage of ultra-violet absorbing material, or analyzed for NH_3 by the Markham technic(11). The treated cells were suspended in 5 ml water and placed in boiling water bath for 5 min. The cells were removed by centrifugation, and the extracts analyzed for K^+(6) or for NH_3(12). Levels of NH_3 in the original supernatants were too low to permit estimation by the specific Conway technic. Relatively good recoveries of the NH_3 lost from the cells were obtained by the Markham procedure when correction was made for NH_3 arising from NY during alkaline distillation.

Although the intact yeast cell is apparently impermeable to the pyruvate ion and does not decarboxylate pyruvate at neutral pH, damage of the membrane by freezing (10), drying(13), or by surfactants(14), will permit access of the ion to the decarboxylase. Yeast treated with NY will also decarboxylate pyruvate (Table II). Several points are of special interest: 1) Lower NY concentrations are required to initiate decarboxylation in pH 5.8 buffer containing mannitol than in pH 7.0 buffer, in parallel with the relative sensitivities of glycolysis to NY. 2) Decarboxylation of pyruvate occurs only after a lag and at concentrations of NY considerably higher than those required to produce K^+ and NH_4^+ loss and inhibit glycolysis. Thus, permeability to pyruvate appears to be a secondary result of the initial injury to the cell membrane. 3) Constant rates of CO_2 production were observed following the lags, and the rate was dependent on NY concentration. This suggests an all-or-none acquisition of

permeability to pyruvate by the yeast, as Scharff and Maupin(14) noted with benzalkonium chloride. 4) In pH 4.0 buffer, addition of 10-30 μg of NY per ml produced a rapid destruction of pyruvate decarboxylase comparable to that noted by Scholz, *et al.* (5) for other glycolytic enzymes. At pH values intermediate between 4 and 5.8, both an increased accessibility of the decarboxylase and gradual inactivation could be demonstrated.

In parallel tests, there was no pyruvate decarboxylation or K^+ leakage with the highest levels of solvent (dimethyl sulfoxide) used in any of these experiments. Also, the methyl ester and N-acetate of NY, which do not inhibit growth, did not initiate decarboxylation or leakage even at 30 μg per ml.

A detectable loss of ultra-violet-absorbing substances (acid-soluble) occurred even in the absence of NY (Table II) confirming the report by Higuchi and Uemura(15). This loss increased in the presence of NY, especially at those high concentrations which produced a deficiency of the nucleotide cofactors essential to glycolysis. At 30 μg NY per ml, some loss of protein from the cell was observed in several, but not all, experiments.

Discussion. A graded series of effects upon yeast permeability can be obtained by addition of increasing concentrations of nystatin. The pH range of 5.8 to 7.0 was used to avoid secondary autolytic changes believed to occur at acid pH(1). At the lowest levels of nystatin, the only alteration observed thus far is the rapid loss of K^+ ions(6), which correlates well with inhibition of glycolysis. NH_4^+ ions, which can also restore glycolytic activity when added to the system, are not eliminated from the cell unless higher nystatin levels are used. However, the concentration of ammonia in the cell is low, and values obtained even with the comparatively mild Conway procedure may still represent substances other than ammonium ion available for glycolytic activity. The results are consistent, therefore, with the idea that K^+ depletion is the critical factor in the inhibition. Although in the test system used by Marini *et al.*(6) no effect of phosphate ion could be shown, in pH 7 sodium acetate buffer under the general conditions of Bradley and Farber(16), both phosphate ions and K^+ or NH_4^+ were required for reversal of nystatin inhibition of glycolysis. Thus, phosphate leakage must also take place at relatively low nystatin concentrations. Increasing the level of nystatin will initiate leakage of UV-absorbing nucleotides and cause sufficient change in the membrane that decarboxylation of the pyruvate ion is possible. Leakage of pyruvate, malate and other carboxylic acids and subsequently of sugar phosphates occurs under approximately the same conditions(4,5). This leakage, and consequent dilution of cofactors and of intermediates, especially pyruvate, is probably the reason glycolysis halts under these conditions. At the highest nystatin levels, there is some loss of protein, but the bulk of the glycolytic enzymes must remain with the cell.

At least partial reactivation of CO_2 production can be attained by addition of external glycolytic cofactors. It should be noted that this response to external cofactors can be demonstrated only with very high nystatin levels and with the most sensitive cell types, that is, log phase cells or protoplasts. The reversal experiments presented here emphasize the role of pyruvate in initiation of glycolytic activity but, as seen from the experiments on protection, the other cofactors are also required under comparable conditions. A requirement of external ATP for glycolysis has also been reported recently by Fox and Stevenson(17) for streptococci whose permeability had been altered by treatment with trypsin.

Even at the highest nystatin levels the yeast membrane does not appear to be open to the entrance of all ions by simple diffusion. For instance, the cell retains the barrier to glucose-6-phosphate and fructose diphosphate characteristic of yeast. The cellular alterations at lower concentrations of the antibiotic are probably even more specific, particularly since there is significant loss of only certain cell constituents.

Summary. Permeability alterations induced in yeast by high levels of the polyene antifungal antibiotic nystatin were examined. Glycolytic activity could be protected

against nystatin either by adding ca 0.04 *M* K^+ and NH_4^+ salts (stationary phase cells) or by 0.01 *M* K^+ and NH_4^+ salts and the cofactors essential for a cell-free glycolytic system (log phase cells or protoplasts). Under these conditions there was leakage of K^+, NH_4^+, nucleotides and some protein, although glycolytic activity remained with the treated cells or protoplasts even after repeated washing. Pyruvate decarboxylase in NY-treated cells became accessible to pyruvate ions, but glucose-6-phosphate and fructose-1,6-diphosphate were still not fermented. The rate of CO_2 production by NY-treated cells or osmotically-lysed protoplasts did not exceed 25 to 30% of that obtained with the original preparations. The nature of the membrane alterations is discussed.

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Received July 7, 1961. P.S.E.B.M., 1961, v108.

Apparatus for Change of Medium in Extracellular Maintenance *in vitro* of an Intracellular Parasite (Malaria). (26883)

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Methods have been described whereby the avian malarial parasite, *Plasmodium lophurae,* can be removed from its host erythrocytes and maintained extracellularly *in vitro* for up to 4 days at 40°C(1,2). These methods have made possible a study of some of the nutritional requirements of an intracellular parasite itself, as distinguished from the host cell-parasite complex.

To get the best survival and development it was necessary to change the culture fluid after the first 18 hours and at 12 hour intervals thereafter. This was done as follows: Each 50 ml Erlenmeyer culture flask received 0.25 ml of duckling plasma (obtained without use of an anticoagulant). To this was added 0.02 ml of a chick embryo extract (1 10-day embryo ground in 4 ml of glucose-salt solution, mixture centrifuged, supernatant used). The flask was then rotated rapidly in such a way that the plasma clotted as a thin layer along the lower part of the wall of the flask, with the excess sometimes forming a larger clot at the bottom. Each flask then received the culture medium and finally the inoculum of hemolyzed red cells and free parasites prepared by the treatment of blood from an infected duckling with guinea pig