J. Pharm. Pharmac., 1973, **25**, 401–407 *Received November* 11, 1972

The effect of pH and of temperature on the stability and bioactivity of nystatin and amphotericin B

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In the presence of phosphate-citrate buffers, nystatin and amphotericin B are optimally stable between pH 5 and 7. Loss of biological activity followed first-order kinetics, except under acid conditions. Apparent energies, enthalpies, free energies and entropies of activation of the two antibiotics have been calculated at pH 7, and the two sets of figures are similar. Variation in pH between 5 and 8 appeared to have little effect of the activity of amphotericin B against *Candida albicans*, while nystatin was more active at pH values between 6 and 8. Nystatin was more active at lower incubation temperatures (30–25°), while amphotericin B appeared to be more active at 41°.

The present work was undertaken to examine the effects of temperature and pH on the stability and the antimycotic activity of nystatin and amphotericin B, as there appears to be little information in the literature on these points.

METHODS AND MATERIALS

Buffer solutions. These were made basically as described in Documenta Geigy (1962), except that McIlvaine buffer was double-strength.

Antibiotics. Nystatin (4280 units mg^{-1}) and amphotericin B (84·4% pure) were kindly given by E. R. Squibb & Sons, Ltd., Twickenham. They were dissolved in dimethylsulphoxide to make stock solutions of 10 000 u ml^{-1}, and 2 mg ml^{-1}, respectively.

Organisms. Candida albicans 489 was used throughout; MIC for nystatin and amphotericin B, determined by the plate dilution technique, for this strain were 25 u ml^{-1} and 1 μg ml^{-1}, respectively. This and the other strains used had been isolated from clinical material.

Bioassay. The medium used routinely was double-strength Bacto Antibiotic Medium no. 2 ("Penassay agar"; 51 g $litre^{-1}$) containing 1% (w/v) glucose. One part of molten medium was mixed with one part of McIlvaine buffer pH 7, and 20 ml amounts poured into plastic Petri plates of 8·5 cm diameter, the surfaces of which were flooded with a 24 h culture of *C. albicans* 489 (37°, static in Sabouraud broth), excess culture sucked off and plates dried for 1 h at 37°. Six 7·5 mm wells were cut and filled with antibiotic solution, and the plates incubated at 30° overnight with their lids propped slightly open. One plate contained either 4 standards and 2 duplicate test samples, or 5 standards and 1 test (in which instance, two plates were

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used for each test sample). Nystatin standards were 50 to 400 u ml^{-1}, amphotericin B standards were 0·5 to 5 $\mu g\ ml^{-1}$; standard solutions were made up in buffer of the same composition as that used in the test samples. Dimethylsulphoxide at the highest concentration present during assay (4%) caused no inhibition of the indicator strain. Zones of inhibition were read with the aid of dark-ground illumination.

Stability of antibiotics at different pH values. 5 ml double-strength McIlvaine buffer (pH 3 to 8) + 4·5 ml water were warmed to 37°, and 0·5 ml of nystatin (8000 u ml^{-1}) or amphotericin B (100 $\mu g\ ml^{-1}$) solution was added. A sample was taken at once, and the tightly-capped bottle was incubated at 37°, further samples being taken at intervals.

Thermal stability of antibiotics. These experiments were carried out in a way similar to that described in the previous paragraph, except that the concentrated antibiotic solutions were added to buffer at pH 7, which had been prewarmed to temperatures between 37 and 100°. In the experiments at the higher temperatures, samples were deep-frozen (−20°) at once for convenience, then melted and bio-assayed not more than 6 h later.

Treatment of kinetic data. Plots were made of (log antibiotic concentration remaining) *vs* time, from which first-order constants (k_1) were calculated if the plots were linear. From the thermal stability data, an Arrhenius plot was made, from which were calculated the apparent activation energy E_a, the entropy (ΔS^*), the enthalpy (ΔH^*) and the free energy (ΔF^*) of activation (Glasstone, 1956).

Biological activities at different pH values. The hole-plate assay described was used, with a series of plates differing in the pH of the buffer used to dilute the double-strength agar (pH range 4·5 to 8). In a separate experiment, the pH of the resulting molten agar was measured in each case; values were in the range 5 to 8, the change in the pH of the buffer as the result of adding medium becoming progressively less marked as the pH increased. In these experiments, standard solutions (for nystatin 30 to 400 u ml^{-1}, for amphotericin B 0·5 to 8 $\mu g\ ml^{-1}$) were made up in water. Plots were made of log (antibiotic concentration) *vs* zone diameter for each pH value, and the slope and the intercept of each line were calculated.

Effect of temperature on activity of antibiotics. MIC determinations were made (Hamilton-Miller 1972), incubation being at 25, 30, 35 and 41°. Six *C. albicans* strains, including 489, were used.

The growth of *C. albicans* 489 in Sabouraud broth was followed nephelometrically; test-tubes containing a total of 10 ml, inoculated with 0·5 ml of an overnight shaken culture of *C. albicans* 489, were incubated at 25, 30, 35 and 41° until active growth started (60 to 90 min); at this point various amounts of nystatin or amphotericin B were added and tubes were read on an EEL nephelometer (filter OR2) at approximately 50 min intervals after homogenization on a vortex mixer.

The population structure of *C. albicans* 489 at different temperatures was analysed as follows: 0·1 ml volumes of serial decimal dilutions (down to 10^{-5}) of an overnight culture were inoculated on to four series of plates containing various concentrations of nystatin or amphotericin B. Each series was incubated for 40 h at 25, 30, 35 or 41° and the colonies on each plate were counted. Control counts were made on plain agar from the 10^{-5} dilution. From the results, the proportion of the organisms in the original culture able to grow at each temperature in the presence of the different concentrations of antibiotic were calculated. Plots were made of % original

inoculum killed *vs* antibiotic concentration for each set of conditions, and the concentration (ID99) that killed 99% of the original inoculum was read off by interpolation.

RESULTS

Effect of pH on stability. The results obtained at 37° in the presence of phosphate-citrate buffers of different pH values are shown in Fig. 1. The breakdown of nystatin

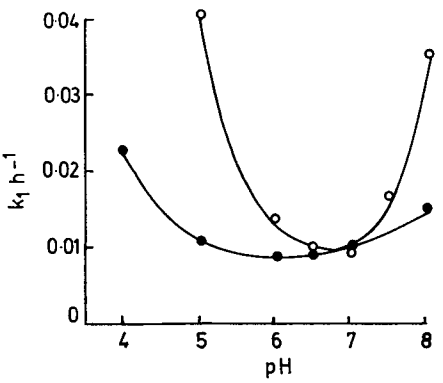

FIG. 1. Effect of pH on the stability of amphotericin B (●) and of nystatin (○).

at pH 3 and 4, and of amphotericin B at pH 3 was rapid (about 90% destruction in about 3, 6 and 17 h, respectively), and first-order kinetics were not followed.

In experiments during which nystatin solutions were incubated at pH 5, 6·5 and 7, samples were taken at intervals and their absorption spectra recorded. The loss of biological activity proceeded at a greater rate (4 to 8 times as fast) than did the loss of extinction at 321, 306 and 292 nm.

Effect of temperature on stability. First-order plots were obtained at all temperatures used; the thermodynamic data from the Arrhenius plots are given in Table 1.

Table 1. *Enthalpy, Gibb's free energy and entropy of activation for loss of biological activity of nystatin and amphotericin B.*

	E_a k cal mol^{-1} (k J mol^{-1})	ΔH^* k cal mol^{-1} (k J mol^{-1})	ΔF^* k cal mol^{-1} (k J mol^{-1})	ΔS^* cal mol^{-1} deg^{-1} (J mol^{-1} deg^{-1})
Nystatin	18.6 (78)	18.1 (76)	24.8 (104)	−21.9 (−92)
Amphotericin B	16.4 (69)	15.9 (67)	24.8 (104)	−28.8 (−120)

Effect of pH on biological activity. In the plots made of log dose *vs* response for nystatin and amphotericin B, each slope represents the sensitivity of the assay, and each intercept represents the maximum concentration of antibiotic that just fails to produce a zone of inhibition, and thus gives a measure of the intrinsic activity. The latter has been referred to previously (Hamilton-Miller, 1971) as the "maximum non-inhibitory concentration", or MNIC. Over the range studied (pH 5 to 8), pH had virtually no effect on the sensitivity of assay for either antibiotic; the effect on the

intrinsic activity of amphotericin B was also negligible, while nystatin showed decreased activity (of the order of 50% of maximal) at pH values of 6 and below.

Effect of temperature on biological activity. Plate diffusion experiments gave highly misleading results because both antibiotics diffuse poorly through agar, and zone sizes could be increased (in size but not clarity) by preincubation of the prepared assay plates at 4°. Also, as the lag phase of the indicator organism increases with a lowering of incubation temperature, plates incubated at lower temperatures showed a preincubation effect (i.e. increased zone size) superimposed upon any intrinsic effect of temperature that might exist. The obvious solution—preincubation of all plates for an experimentally-determined optimal period before incubation at the desired growth temperature—could not be adopted because zone edges became progressively more indistinct as the prediffusion period was prolonged. No clear-cut results were

Table 2. *Concentration (ID99) of antibiotics required to kill 99% of inoculum of* C. albicans *489 at different temperatures; see text for full details.* Figures in brackets are relative to values at 41°.

Incubation temperature	ID99 amphotericin B μg ml^{-1}	ID99 nystatin u ml^{-1}
25°	0.87 (1.47)	5.1 (0.32)
30°	0.93 (1.58)	8.5 (0.53)
35°	0.85 (1.44)	11 (0.69)
41°	0.59 (1)	16 (1)

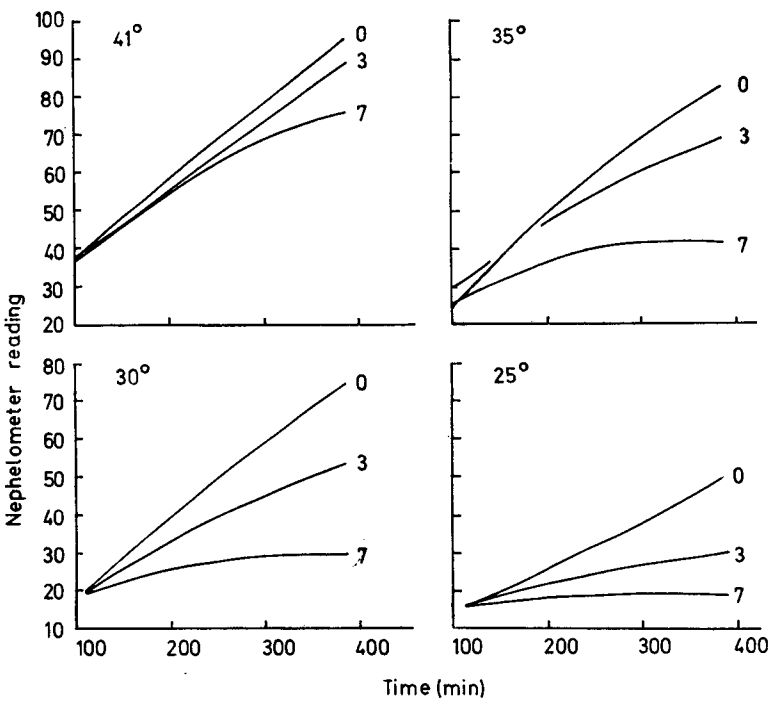

FIG. 2. Effect of temperature on growth inhibitory activity of nystatin. Figures against each urve show the concentration of the antibiotic (u ml^{-1}).

obtained from MIC determinations using six *C. albicans* strains at different temperatures, but population structure experiments (Table 2) suggested that nystatin was progressively more active as temperature is decreased; amphotericin B, on the other hand, appeared to be of higher activity at 41°. These impressions were strengthened by the results of growth curve experiments, which show (Fig. 2) that nystatin (3 u ml^{-1}) was virtually inactive at 41° but became more and more active as the incubation temperature was decreased. The results with amphotericin B (Fig. 3) were not so clear-cut.

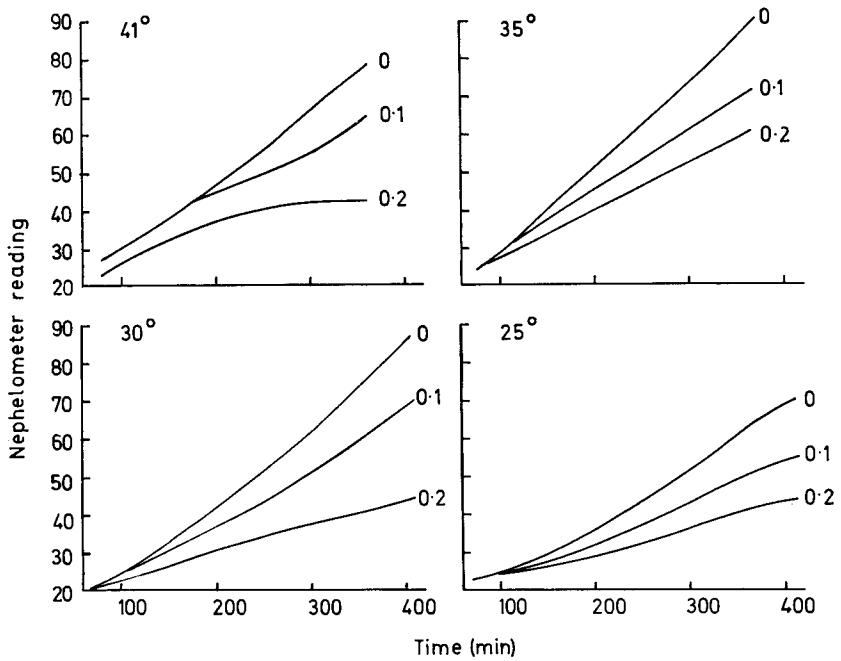

FIG. 3. Effect of temperature on growth inhibitory activity of amphotericin B. Figures against each curve show the concentration of the antibiotic (μg ml^{-1}).

DISCUSSION

There have been few systematic studies on the stability of specific polyene antibiotics under physiological conditions. There are several general statements in the literature (e.g. Waksman, Lechevalier & Schaffner, 1965; Dutcher, 1968, Rickards, Smith & Golding, 1970), describing the polyenes as a group as unstable compounds, decomposed at extremes of pH and light, heat and air. Reports on specific compounds have often been vague quantitatively; however, pimaricin, in aqueous conditions, has been reported to be fairly heat-stable (Struyk, Hoette & others, 1958), but is easily oxidized or photolysed (Dekker & Ark, 1959).

The findings summarized in Table 1 require comment. The observed values of E_a, a practical (experimentally determined) parameter, are consistent with a chemical rather than a physical mechanism for the heat-mediated destruction of these two compounds. This precludes the possibility that the changes in the biological activity of polyenes with temperature are due to alterations in their micellar nature. Under physiological conditions (as indicated in Table 1), the value of E_a is virtually identical

to that parameter representing the energy that each molecule must acquire before it is capable of undergoing reaction, ΔH^*. A high negative entropy term (ΔS^*) is to be expected, in terms of the collision theory, for a reaction involving large and complex molecules like those of the polyenes; in practical terms, the values of ΔS^* found here show that the thermal decomposition of polyenes is a "slow" reaction (by chemical standards). The precise rate of reaction at any one temperature, measured in the laboratory by the reaction constant k_1, is actually governed by ΔF^*. Thus, from Table 1 it can be deduced that the mechanism of breakdown of nystatin and of amphotericin B is a chemical process, similar in both cases, and that the rate of breakdown is increased by a factor (Q_{10}) of approximately 3 for each 10° increment of temperature. At 4°, for instance, the half-life of aqueous solutions of nystatin and of amphotericin B may be calculated, using the data in Table 1, to be about 41 and 34 days respectively.

There is a considerable difference in the stability to H^+ of the two antibiotics; as Fig. 1 shows, although their stabilities are virtually identical at pH 6·5 and 7, nystatin (a tetraene) is much less stable at more extreme pH values than is amphotericin B (a heptaene).

The fact that, for nystatin, loss of the tetraenic chromophore was much slower than loss of biological activity suggests that the mechanism of inactivation under these conditions is not an epoxidation of the type established by Rickards & others (1970) to account for the aerial oxidation of methanolic solutions of filipin and lagosin. Dekker & Ark (1959) have suggested that, for aqueous preparations of rimocidin and pimaricin, ultraviolet photolysis is brought about by a *trans—cis* isomerization, and light-independent degradation by an oxidative provess. Gerke & Madigan (1961) and Horvath & Koczka (1964) have also observed that spectrophotometric and biological assays of polyene antibiotics do not always correlate; on the other hand, Tingstad & Garrett (1960) reported parallel decrease in bioactivity and extinction during the thermal degradation of filipin. On the whole, it would seem prudent not to assume direct proportionality between extinction and bioactivity for partially degraded polyene preparations, bearing in mind that the probable micellar nature of "solutions" of these compounds (Lampen, Morgan & others, 1959) may lead to deviations from Beer's Law.

The findings here in respect to the effect of pH on biological activity are in general consistent with those made by others; Lechevalier (1960) reported that nystatin was more active at pH7 than at 4·5 against *C. albicans* in solid media, but that against *Saccharomyces cerevisiae* in liquid medium this effect was much less marked. Three other groups have also found that the activities of nystatin and amphotericin B are relatively independent of pH—Lampen, Morgan & Slocum (1957) using *S. cerevisiae*, Kinsky (1961) using *Neurospora crassa* and Gerke & Madigan (1961) using *C. tropicalis*.

The action of nystatin against *C. albicans* seems to be favoured by lower temperatures, both in solid and in liquid media; with amphotericin B the picture is not quite as clear, but it appears that a higher temperature may enhance its activity, at least in solid media. These findings thus confirm those of Gerke & Madigan (1961) for *C. tropicalis*.

An analysis of the effect of temperature and pH on the anti-yeast activity of nystatin and amphotericin B must of necessity take into account numerous factors. What is germane to the present discussion is the extent to which the stability of the polyenes

is concerned in this matter. Polyenes are first absorbed to sterols in the yeast cell membrane; this causes alterations in membrane function, leading to leakage of essential metabolites, inhibition of glycolysis and cell death (Lampen, 1969). It is easy to vizualise alterations in pH and temperature affecting any of these cellular processes (e.g. sterol content is affected by growth conditions—Hunter & Rose, 1972). Thus, both polyene binding and overall cell death were increased on raising the temperature or on lowering the pH (Lechevalier, 1960, Lampen & others, 1959; Lampen, Arnow & others 1962; Lampen, 1966). However, the crucial question is the time scale involved in cell death; Lampen & others (1957) found that glucose oxidation in yeast was inhibited after 20 min, and cell death was about 95% complete after 30 min, following contact with nystatin. Lampen & others (1959) showed that binding sites had been saturated after approximately 1 h. These figures tend to suggest that any part polyene instability has to play in the overall effect of pH and temperature variations on the biological activity of these antibiotics is minimal, especially under physiological conditions.

Acknowledgements

I thank Professor H. I. Winner for his encouragement, and Miss P. Thorne for assistance. This work was carried out during the tenure of a Squibb Research Fellowship, which I gratefully acknowledge.

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