THE LYTIC EFFECT OF POLYENE ANTIFUNGAL ANTIBIOTICS ON MAMMALIAN ERYTHROCYTES*

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Recent studies (Kinsky, 1962a) have suggested that the permeability alterations which polyene antifungal antibiotics induce in mycelial mats and protoplasts of Neurospora (Kinsky, 1961, 1962b) may be a consequence of antibiotic binding to the cell membrane. Gottlieb et al. (1958, 1961) have shown that sterols antagonize the inhibitory action of the antibiotics and, in confirmation of these results, evidence was presented indicating that ergosterol (or a related compound) may be required for nystatin binding to particles derived from the Neurospora membrane. Bacteria do not contain sterols (Asselineau and Lederer, 1960) and thus these investigations have provided a basis for the contention that the selective toxicity of the polyene antibiotics is due to a unique component in the membrane. Even stronger support for this argument would be provided by the converse demonstration that all cells, known to contain sterols in their membrane, manifest a permeability change in the presence of polyene antibiotics. This possibility was rendered probable by the recent observation of Dingle and Lucy (1962) that various mammalian erythrocytes were rapidly lysed by the most extensively investigated polyene compound, vitamin A.

METHODS

Blood, obtained from a single rat by cardiac puncture, was withdrawn with a syringe and immediately added to an equal volume of Alsever's solu-

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tion (Dingle and Lucy, 1962). After centrifugation for 10 minutes at 2000 x g, the resulting pellet was resuspended in 10 ml of isotonic saline (0.9 per cent NaCl) buffered with 0.02 M potassium phosphate, pH 6.7 (hereafter referred to as saline buffer). This procedure was repeated twice. The final pellet, representing approximately 1.5 ml of packed cells, was gently dispersed in 5 ml of saline buffer and maintained at 2° before use. Hemolysis was measured by addition of erythrocytes to saline buffer supplemented with the antibiotics as indicated below. After incubation for various time intervals at 37° the tubes were centrifuged and the extent of lysis was determined by measurement at 550 mμ (Zeiss spectrophotometer) of the hemoglobin present in the supernatant solution. An amount of erythrocytes was used (equivalent to approximately 0.06 ml of the final suspension) which, after total hemolysis with 3 ml of water, had an absorbancy at this wavelength of 0.450. Since the erythrocytes were isolated just prior to each experiment, standardization in this manner gave reproducible results with different blood samples.

The sources of the antibiotics and the preparation of stock solutions with dimethylformamide were described previously (Kinsky, 1962b). All dilutions of the antibiotics were made with saline buffer so that no change in tonicity resulted when the antibiotics were added. Lysis was not observed in control tubes containing dimethylformamide alone at concentrations (usually 0.5 per cent) equivalent to those in the experimental tubes.

RESULTS

The effect of increasing amphotericin B concentration on the extent of hemolysis is shown in Figure 1. Lysis was not obtained with 1.5 μg/ml but was nearly complete with 5 μg/ml. This sharp rise in the extent of lysis over a very narrow concentration range is similar to the results obtained by Dingle and Lucy with vitamin A and suggests that a critical threshold ratio of antibiotic per cell may be required. The time course of amphotericin B induced lysis is depicted in Figure 2. After an initial lag period, the rate increases rapidly and, after 15 minutes, is 87 per cent completed. Human erythrocytes, washed by the above procedure, were also rapidly lysed but the

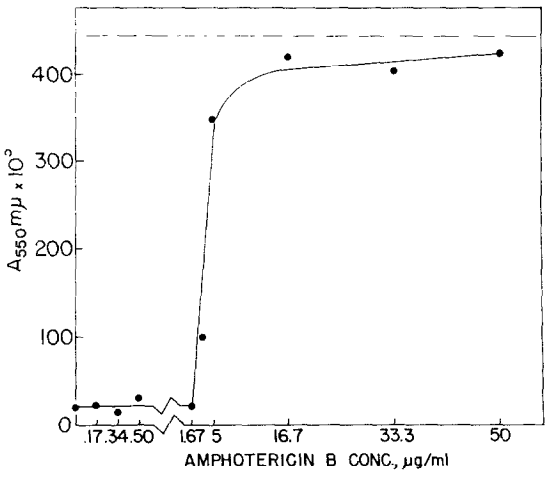

Figure 1

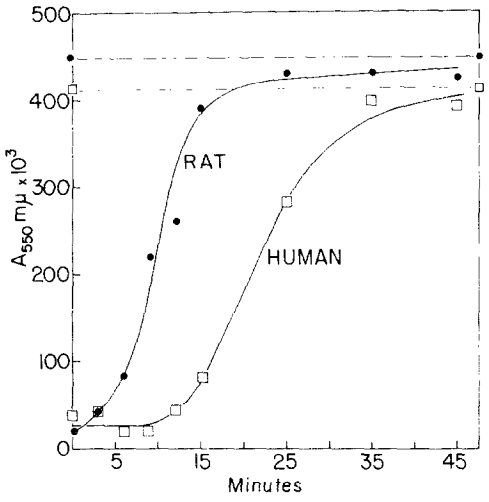

Figure 2

Figure 1. Effect of Amphotericin B Concentration on Extent of Hemolysis. Rat erythrocytes were added to saline buffer, containing amphotericin B at the concentrations shown on the abscissa, to give a final volume of 3 ml. Incubation time: 15 minutes. The dashed line indicates the absorbancy of the supernatant at 550 mμ after complete lysis in 3 ml of water.

Figure 2. Time Course of Amphotericin B Induced Hemolysis. Conditions were similar to those described in Figure 1 except that each tube contained amphotericin B at a final concentration of 5 µg/ml and the incubation time was varied as indicated on the abscissa.

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lag phase was longer. The significance, if any, of this lag phase must still be determined. Although all polyene antifungal antibiotics thus far tested have been shown to induce lysis, important quantitative differences have been observed (Table 1). The order of effectiveness was filipin > amphotericin B > nystatin. These results are particularly interesting in view of the earlier evidence that filipin causes more extensive permeability changes and is more tightly bound to Neurospora membrane particles than either amphotericin B or nystatin (Kinsky, 1962a,b).

DISCUSSION

Apart from bacteria, sterols are found in all living organisms. Cholesterol is known to be a constituent of erythrocyte membranes (references cited in Cook (1958)) and the experiments reported here support the contention

that the presence of sterol is a prerequisite for sensitivity towards polyene antibiotics. Evidence demonstrating marked effects of polyene antibiotics on yeasts (Gottlieb et al., 1961; Marini et al., 1961), algae (Hunter and McVeigh, 1961; Lampen and Arnow, 1961), protozoa (Ghosh and Chatterjee, 1962), flatworms (Johnson et al., 1962), and snails (Seneca and Bergendahl, 1955) is also consistent with this conclusion.[1]

Table 1. Relative Effectiveness of Nystatin, Amphotericin B, and Filipin. Conditions were similar to those described in Figure 1 except that different polyene antibiotics were used at the final concentrations cited in the table.

Antibiotic Added	Minutes Required for Complete Hemolysis: Antibiotic Concentration, μg/ml		
	1	10	50
Filipin	>180[a]	<1	<1
Amphotericin B	>180[c]	21	17
Nystatin	>180[c]	>180[c]	>180[b]

[a] 37 per cent lysis observed after 3 hours.
[b] 32 per cent lysis observed after 3 hours.
[c] No lysis observed after 3 hours.

The present experiments are also significant for the following reasons: (1) They provide an adequate explanation for the hemolytic anemia often observed in patients undergoing amphotericin B therapy (Newcomer et al., 1960). Many of the undesirable effects of the polyene antibiotics (e.g. renal damage) may be a reflection of antibiotic interaction with sterols in mammalian membranes. (2) These results suggest that erythrocytes might be appropriate objects for the study of the mechanism of polyene antifungal antibiotic action.

[1] Rothblat and Smith (1961) have demonstrated the presence of sterol in the membrane of various strains of pleuropneumonia-like organisms (PPLO); the intriguing possibility, from a phylogenetic view, that these organisms might also be inhibited by polyene antifungal antibiotics has not yet been determined

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