Kinetics of Association of Amphotericin B with Vesicles†

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ABSTRACT: Amphotericin B associates with vesicles prepared from phosphatidylcholines. The influence of lipid composition on the initial rate of amphotericin B association with vesicles was examined using stopped-flow kinetic measurements. A relationship was found between the tightness of packing of phosphatidylcholine molecules in the vesicles and the initial rate of amphotericin B association. Shortening of the fatty acyl chain length of saturated phosphatidylcholines and increasing the number of double bonds in the fatty acyl chains of unsaturated phosphatidylcholines enhance the initial rate, whereas addition of cholesterol to the bilayers reduces the rate. The initial rate of association with phosphatidylcholine–sterol vesicles follows the order, thiocholesterol > androst-5-en-3β-ol > epicholesterol > ergosterol > cholesterol and is thus inversely related to the order of phospholipid–sterol affinity, as revealed by permeability, surface area, and magnetic resonance measurements. These data suggest that the initial rate of amphotericin B uptake into vesicles depends on competition between lipid–lipid and amphotericin–lipid interactions.

Permeability changes produced by polyene antibiotics in model lipid bilayer systems such as liposomes (e.g., de Kruijff et al., 1974a,b; Singer, 1975) and planar bilayers (e.g., Andreoli, 1974; Holz, 1974), and in biological systems such as cells of acholeplasmas (de Kruijff et al., 1974a,b), mycoplasmas (Archer and Gale, 1975; Archer, 1976), fungi (Gale, 1974; Gale et al., 1975; Archer and Gale, 1975), and erythrocytes (Deuticke et al., 1973) have received considerable attention recently. It has been concluded that, in most membranes studied, sterol is required for large increases in permeability to ions and hydrophilic solutes, although occasional exceptions have been reported (Weissmann and Sessa, 1967; HsuChen and Feingold, 1973; Haupt et al., 1976). There is abundant evidence, originally circumstantial in nature (Lampen et al., 1962; Kinsky, 1963) and later direct (Weber and Kinsky, 1965; Feingold, 1965), that polyene antibiotics react only with cells whose membranes contain 3β-hydroxyl sterols. Complexation of amphotericin B with membrane-bound sterols has been shown (Bittman and Fischkoff, 1972; Norman et al., 1972; Bittman et al., 1974). In one model of amphotericin action, the permeability changes produced in membranes are attributed to aggregation of the initially formed polyene–sterol complexes, followed by formation of nonstatic transmembrane aqueous pores (Finkelstein and Holz, 1973; Andreoli, 1973; de Kruijff and Demel, 1974). An alternative explanation of the cholesterol requirement has been advanced in which no significance is assigned to the complexation of polyene antibiotics with membrane sterol. This model, which was proposed for the action of amphotericin B and the closely related antibiotic nystatin, visualizes cholesterol as a promoter of polyene–lipid interaction by virtue of its ability to increase the order of fluid membranes (HsuChen and Feingold, 1973; Abramson and Ockman, 1973). The increase in membrane rigidity may help align amphotericin B molecules parallel to the fatty acyl chains of the phospholipids, increasing the probability that on approach of amphotericin B molecules from opposite sides of the bilayer, two half-pores will be joined to form a transmembrane pore.

This paper is concerned with the role of sterols in the interaction between amphotericin B and phospholipid vesicles. In the preceding paper, the effects of phospholipid structure on the binding of another polyene antibiotic, filipin, to phospholipid–cholesterol vesicles are reported, and the differences between amphotericin B and filipin with respect to kinetics of association with vesicles are discussed (Blau and Bittman, 1977).

Experimental Section

Materials. Phosphatidylcholines (PC)[1] were obtained from the following sources: L-α-dimyristoyl-PC (DMPC) from Supelco, Inc.; L-α-dilauroyl-PC from Sigma Chemical Co.; and L-α-dioleoyl-PC, L-α-dilinoleoyl-PC, and L-α-diarachidonoyl-PC from Applied Science. The phospholipids were found by thin-layer analysis on silica gel G plates to be chromatographically pure, with the exception of dilinoleoyl-PC and diarachidonoyl-PC; the purities of the latter were estimated to be approximately 95%. The sources and the purification of the sterols were as reported previously (Bittman and Fischkoff, 1972). Dicetyl phosphoric acid was purchased from Sigma Chemical Co. Amphotericin B (subsequently referred to as amphotericin) was supplied by E. R. Squibb, Princeton, N.J. (batch no. 22-380-39568-001). Stock solutions of amphotericin were prepared in dimethyl sulfoxide and could be stored at −20 °C for ~3 days without loss of reproducibility in kinetic results. Aliquots of the stock solution were added to a well-agitated 2.5 or 5.0% aqueous dextrose solution. Fresh aqueous solutions were prepared daily. The concentration of amphotericin solutions was measured spectrophotometrically using an apparent molar absorbancy of 3.6×10^4 M^{-1} cm^{-1} at 385 nm in dilute (<4 μM) aqueous dextrose, or 9.0×10^4 M^{-1} cm^{-1} at 392 nm in dimethyl sulfoxide. The final concentration of amphotericin was 4.0 μM, except in experiments in which the concentration was varied. The final concentration of dimethyl sulfoxide, after mixing with vesicles, was 1.2% (v/v).

Preparation of Vesicles. Lipid suspensions were subjected to ultrasonic irradiation under nitrogen for six 30-s periods with a 20-kHz Branson sonifier (Model S-110) fitted with a solid tap horn and operated at power level 4, unless noted otherwise. Vesicles contained 4 mol % of dicetyl phosphoric acid, which was added to confer negative charge. The total lipid concen-

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[1] Abbreviations used: PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine.

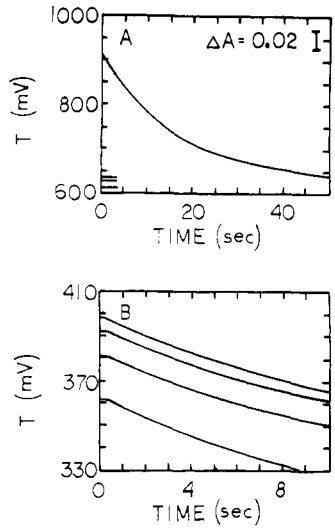

FIGURE 1: Stopped-flow oscilloscope traces showing a decrease in transmittance (increase in absorbance) on mixing of equal volumes of amphotericin and vesicles. (A) Vertical axis, 50 mV per division; horizontal axis, 5 s per division; initial signal, 1.6 V. Horizontal bars at lower left indicate the transmittance at (from top to bottom) 75, 105, and 170 s after mixing; the transmittance reached an end point at 170 s. The final concentration of DMPC was 0.096 mM. (B) Multiple traces of the initial portion of the reaction. Vertical axis (numbers in mV) refers to the top trace, 10 mV per division; horizontal axis, 1 s per division. The final concentration of DMPC was 0.192 mM.

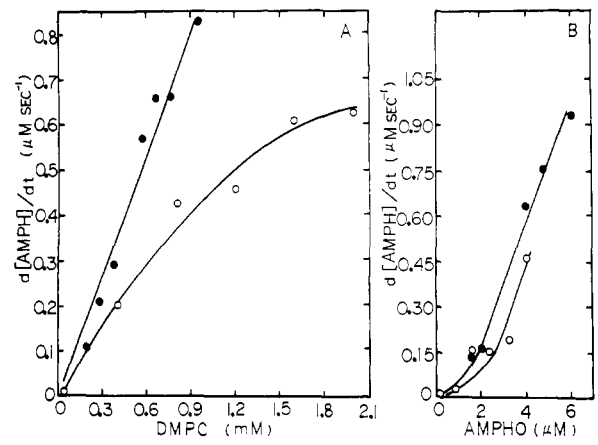

FIGURE 2: Initial rate of amphotericin association with vesicles as a function of DMPC and amphotericin concentrations. Vesicles were prepared from DMPC (●) and from DMPC and cholesterol at a molar ratio of 5 (○). (A) Plot of the initial rate as a function of DMPC concentration at constant amphotericin concentration (4.0 μM) and varying DMPC and cholesterol concentrations. (B) Plot of the initial rate vs. amphotericin concentration for association with DMPC vesicles (●) (total lipid concentration of 0.77 mM) and with DMPC–cholesterol vesicles (○) (total lipid concentration of 1.0 mM). The trapped aqueous volumes of DMPC vesicles and DMPC–cholesterol vesicles were 0.32 ± 0.04 and 0.42 ± 0.03 L per mol of lipid, respectively.

trations given in the figure and table captions are those obtained after mixing with amphotericin. Suspensions of DMPC that were sonicated for 15 s were found by negative-staining electron microscopy to consist of approximately 70% multiconcentric structures and 30% single-shelled vesicles; after 30 s of sonication, approximately 70% of the preparation was in the form of single-shelled vesicles. Vesicles that were sonicated for six 30-s periods were predominantly (>80%) unilamellar, with an aqueous trapped volume of 0.32 ± 0.04 L/mol of PC. Trapped water volumes were measured as described in the accompanying paper (Blau and Bittman, 1977).

Kinetic Measurements. Reactions were performed in a Durrum-Gibson stopped-flow spectrophotometer. Amphotericin in 2.5 or 5.0% aqueous dextrose solution was mixed with vesicles suspended in the same concentration of dextrose. The temperature was 38 °C, unless otherwise noted. The changes in transmittance at 385 nm as a function of time and at equilibrium (within about 3 min of mixing) were recorded on a Tektronix storage oscilloscope. The initial rate of absorbance change, $(dA/dt)_0$, for the association of amphotericin with vesicles was computed from the slope of the initial transmittance change. The initial rate of change in amphotericin concentration, $(d[Amph]/dt)_0$, was computed using the equation:

$$(d[Amph]/dt)_0 = (1/\epsilon l)(dA/dt)_0$$

where ϵ and l are the molar absorbancy of amphotericin at 385 nm and the pathlength of the stopped-flow cuvette (2 cm), respectively. A monochromator slit width of 1 mm was used. Data reported for $(d[Amph]/dt)_0$ represent the mean of at least eight measurements of the reaction under study. No time-dependent transmittance changes were detected at 385 nm when amphotericin solutions (<6 μM final concentration) were mixed with an equal volume of aqueous dextrose solution (1.2% dimethyl sulfoxide, final concentration). In order to determine whether the rate processes observed for amphotericin–vesicle association depend on the size distribution of the vesicles, rate measurements were made with unfractionated and Sepharose 4B fractionated vesicles. The rates were found to be the same, within experimental error; therefore, measurements of the kinetics of association were made using unfractionated vesicles.

Results

The association of amphotericin with vesicles prepared from egg lecithin is accompanied by an increase in the absorbance of amphotericin at short wavelengths relative to the long wavelength band (Norman et al., 1972; Bittman et al., 1974). The absorbance change at 385 nm is convenient for monitoring the time course of amphotericin association with DMPC vesicles (Figure 1). A semilogarithmic plot of the reaction amplitude vs. time reveals that two rate processes can be resolved in the time range represented in Figure 1A (0–50 s). A slow reaction requiring approximately 3 min is also observed. Since the two relaxations are not well separated, the kinetics of amphotericin interaction with vesicles was investigated in terms of initial reaction rates. Initial rates were measured from the slope at zero time of the transmittance change with respect to time (Figure 1B); they were converted to changes in absorbance or in amphotericin concentration per second using the relationships shown in the experimental section.

Lipid Dependence of Kinetics of Association. The initial rate of amphotericin association with DMPC vesicles is faster than that with vesicles containing 16 mol % cholesterol (Figure 2A). The kinetic order of the association of amphotericin with DMPC vesicles was determined from the relationship between the logarithm of the initial rate and the logarithm of the lipid or amphotericin concentration. The reaction with DMPC vesicles is first order in DMPC. From the dependence of initial rate on amphotericin concentration (Figure 2B), it is found that, in the range of about 2.4 to 6.0 μM, the order with respect to amphotericin is approximately 1.5 in DMPC vesicles and approximately 2.0 in vesicles containing 16 mol % cholesterol.

Effect of Sonication. To determine whether surface area influences the reaction rate, the effect of sonication time on the initial rate was examined. Table I shows the marked rate

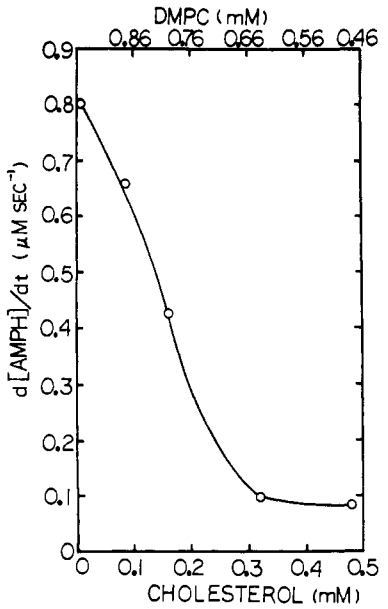

FIGURE 3: Dependence of the initial rate of amphotericin association with vesicles on the cholesterol content of the bilayer. Vesicles were prepared with a constant total lipid concentration (1.0 mM) and various concentrations of cholesterol and DMPC. The cholesterol to DMPC molar ratio varied from 0 to 1.

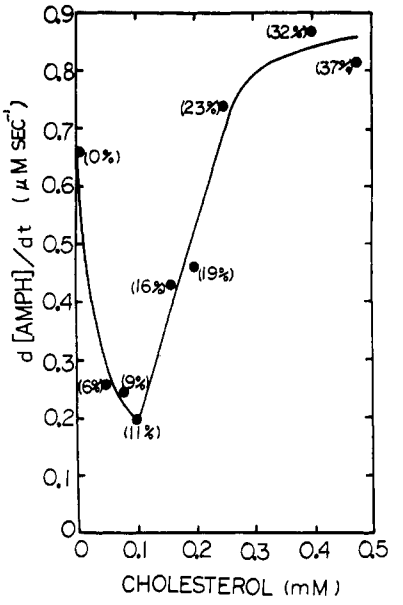

FIGURE 4: Influence of cholesterol on the initial rate of reaction of amphotericin with vesicles prepared with a constant concentration of DMPC (0.80 mM) and various cholesterol and total lipid concentrations. The numbers in parentheses refer to the mole percent of cholesterol in the bilayer.

TABLE I: Effect of Sonication Time on the Initial Rate of Amphotericin Association with DMPC Vesicles.[a]

Time (s)	d[Amph]/dt (μM s^{-1})
0	0.013 ± 0.002
10	0.029 ± 0.009
20	0.022 ± 0.002
30	0.042 ± 0.003
45	0.053 ± 0.003
60	0.238 ± 0.013
90	0.867 ± 0.042
120	0.833 ± 0.026
150	0.785 ± 0.085

[a] The total lipid concentration was 1.0 mM. The results represent the mean ± average error of the mean.

enhancement for amphotericin association with unilamellar vesicles relative to multilamellar bilayers.

Effects of Cholesterol Incorporation. With vesicles prepared with 16 mol % cholesterol and various total lipid concentrations, the initial rate of amphotericin association is slower than that with vesicles prepared from DMPC alone (Figure 2A). Figure 3 shows that the initial rate of association with vesicles prepared at a constant total lipid concentration of 1 mM decreases as the cholesterol content increases. The rate approaches a limiting value at a phospholipid to cholesterol molar ratio of approximately 2. In order to investigate whether the decrease in rate results from the decrease in DMPC concentration that accompanies the increase in cholesterol concentration of the bilayer, vesicles were made with a constant concentration of DMPC (0.80 mM) and various concentrations of cholesterol. The initial rate of amphotericin association decreases when cholesterol is incorporated to 11 mol %, despite the increase in total lipid concentrations (Figure 4). Additional increases in cholesterol and total lipid concentrations lead to increases in the rate. In the region of rapidly increasing reaction rate (between 0.94 and 1.1 mM total lipid), the order with respect to cholesterol is approximately 1.0.

Effects of Other Sterols. The decrease in reaction rate observed on increasing the cholesterol content of the bilayer at constant total lipid concentration (Figure 3) and at total lipid concentrations between 0.84 and 0.94 mM (Figure 4) may result from the decrease in motion of the phospholipid fatty acyl chains and/or from the increase in thickness of the hydrocarbon region. To examine the possibility that a decrease in membrane fluidity affects the initial rate of association, the influence of other sterols on the reaction rate was studied. The initial rates of amphotericin association with DMPC–sterol (2:1 molar ratio, 1.0 mM total lipid concentration) vesicles, d[Amph]/dt, were as follows: no sterol, 0.78 μM s^{-1}; thiocholesterol, 0.48; androst-5-en-3β-ol, 0.42; epicholesterol, 0.34; ergosterol, 0.13; cholesterol, 0.084. Thus the decrease in the initial rate of amphotericin association with sterol-containing vesicles follows the order thiocholesterol < androsten-3β-ol < epicholesterol < ergosterol < cholesterol. This parallels the order of phospholipid–sterol affinity as revealed by permeability (Demel et al., 1972a; Bittman and Blau, 1972), surface pressure (Demel et al., 1972b), and electron spin resonance (Butler et al., 1970; Schreier-Muccillo et al., 1973; Suckling and Boyd, 1976) measurements. Thus at a constant total lipid concentration and 33 mol % of cholesterol, the initial rate of amphotericin association with vesicles appears to be sensitive to the extent of membrane rigidity. The observation that addition of benzyl alcohol, which decreases the packing of polar head groups, increases the mobility of the fatty acyl chains, and depresses the phospholipid transition temperature (Hubbell et al., 1971; Haynes, 1972; Colley and Metcalfe, 1972), causes a 28% increase in rate of amphotericin association with DMPC vesicles at an alcohol concentration of 37 mM is consistent with the hypothesis that lipid mobility influences the kinetics of amphotericin association with bilayers.

Effect of PC Chain Length and Extent of Unsaturation. In order to assess the effects of bilayer rigidity on the initial rate of amphotericin association, vesicles were prepared from saturated lecithins having different chain lengths and from unsaturated lecithins differing in the extent of unsaturation. It is known from permeability measurements that shortening of

fatty acyl chain length or introduction of unsaturation into the paraffin chains increases membrane fluidity (de Gier et al., 1968; Demel et al., 1972a,c; de Kruijff et al., 1973; McElhaney et al., 1973). The initial rate at 30 °C, which is above the phase transition temperature of all the phospholipids, is enhanced with the more fluid vesicles. The initial rates of amphotericin association with vesicles (0.5 mM total lipid concentration) formed from dilauroyl-PC and from DMPC were 0.51 and 0.068 $\mu M\ s^{-1}$, respectively.[2] Thus the initial rate decreases as the interaction between phospholipid hydrocarbon chains within the vesicle becomes stronger. The lower stability and poorer permeability barrier properties of vesicles prepared from dilauroyl-PC relative to longer chain phosphatidylcholines (Hauser and Barrat, 1973) may also contribute to the large difference in the initial rate of amphotericin association. With vesicles made from unsaturated lecithins, the initial rate displays a dependence on the number of double bonds in the acyl chains: dioleoyl-PC, 0.10 $\mu M\ s^{-1}$; dilinoleoyl-PC, 0.14; diarachidonoyl-PC, 0.19. (Initial rates were measured at 30 °C, and vesicles of 0.5 mM total lipid concentration were prepared using a Branson Model S-75 sonifier fitted with a microtip.) The extent of amphotericin association, as measured by ΔA_{total}, was approximately the same for all of the vesicles except those prepared from dilauroyl-PC; ΔA_{total} was markedly higher in vesicles prepared from the latter phospholipid.

Discussion

The association of amphotericin with vesicles does not require the presence of sterols. The initial rate of amphotericin association with vesicles decreases with increasing cholesterol incorporation, increasing chain length of saturated lecithins, and decreasing extent of unsaturation in unsaturated lecithins, suggesting that increases in mobility within the membrane interior facilitate amphotericin uptake. These results are not consistent with the reports that amphotericin penetration into monolayers was enhanced by an increase in membrane order (Ockman, 1974) or that the amount of bound nystatin was higher in aqueous dispersions of DMPC below the lipid phase transition temperature than above it (Abramson and Ockman, 1973).

While these results agree with the experiments by HsuChen and Feingold (1973) indicating that the nature of the phospholipid is important, the conclusion that the association rate increases with increasing fluidity may lead one to predict that a direct correlation exists between membrane fluidity and polyene-induced permeability change; however, these workers concluded that the membrane must be in an ordered state for amphotericin to induce permeability changes. Although the association of an amphipathic agent such as amphotericin with phospholipids certainly does not imply that this process is relevant to the activity or selectivity of the antibiotic toward membranes, it is of interest to note that membrane fluidity has recently been implicated in the mechanism of amphotericin action. For example, under specified conditions, sterol-free egg lecithin vesicles and *Mycoplasma mycoides* var. *capri* membranes became sensitive to the polyenes amphotericin methyl ester and filipin, and increases in the cholesterol content did not increase the extent of permeability change (Archer, 1976). Moreover, ion leakage across amphotericin-treated bilayers was maximal at 20 to 30 mol % cholesterol and declined at higher sterol content (Singer, 1975; Archer, 1976). The model of polyene antibiotic action that emphasizes complexation of polyene with membrane sterol assigns an important role to the movement of the initially formed complexes within the plane of the membrane, resulting in aggregate formation. The results we report here showing the role of membrane fluidity in the association process are consistent with a model in which diffusion of polyene–sterol complexes is a necessary condition for polyene sensitivity, but not with a model in which a highly ordered membrane is a prerequisite to antibiotic action.

The possibility that the reaction we monitor arises from a lipid-mediated change in amphotericin–amphotericin interaction seems unlikely. The fact that slopes of log–log plots of the initial rate of amphotericin association with DMPC and DMPC–cholesterol vesicles vs. amphotericin concentration are approximately 1.5–2 indicates a lack of extensive changes in cooperativity among amphotericin molecules during association with these vesicles. A process involving association with vesicles rather than one involving dissociation of polyene self-aggregates is also suggested by the reaction order with respect to DMPC (1.0) in DMPC vesicles.

The increase in conductance and in ion and nonelectrolyte permeability induced by amphotericin in thin lipid membranes depends on antibiotic concentration to a high power (4–12, varying with membrane composition) (e.g., references in McLaughlin and Eisenberg, 1975). This power dependence is thought to arise because channel formation involves a large number of amphotericin molecules; models of pore structure postulate a multimolecular array of amphotericin–cholesterol complexes (Finkelstein and Holz, 1973; Andreoli, 1973; de Kruijff and Demel, 1974). The dependence of the initial rate on amphotericin concentration below 4 μM in DMPC vesicles is similar to that in DMPC–cholesterol vesicles (Figure 2B), and extensive cooperativity is not observed under these conditions. Therefore, we conclude that the absorbance changes on the time scale reported here arise from adsorption of amphotericin monomers onto the vesicles, and not from amphotericin molecules in a channel configuration. An appreciable time lag has been reported for the full appearance of the amphotericin-induced increase in the permeability of biological and artificial membranes (Cass et al., 1970; de Kruijff et al., 1974b; Gent and Prestegard, 1976; Chen et al., 1977). Transmembrane aqueous channels are apparently formed relatively slowly, with the magnitude of the time delay for full polyene activity dependent on the temperature, lipid, sterol concentration, antibiotic concentration, and mode of addition and availability (to one side or to both sides of the membrane) of the antibiotic.

The origin of the initial decrease, subsequent increase, and later apparent leveling off in initial rate on increasing cholesterol concentration in vesicles prepared with a constant DMPC concentration (Figure 4) is unclear at present. Cholesterol incorporation causes an increase in bilayer thickness as a result of the increase in phospholipid hydrocarbon chain extension (Lecuyer and Dervichian, 1969). The rate decrease at low cholesterol incorporation may arise from the increased bilayer rigidity. The increase in initial rate with cholesterol incorporation above 11 mol % may represent binding to cholesterol in these thickened bilayers. If phase separation into phases of pure PC and cholesterol–PC, with a cholesterol content of 33 mol %, occurs (Engelman and Rothman, 1972), rate enhancement for amphotericin association may result when the maximum interface area at the phase boundary becomes available. The rate may level off when higher proportions of cholesterol are incorporated because of the absence of new interfaces or because cholesterol–cholesterol interactions (which would compete with cholesterol–amphotericin and PC–cholesterol–

[2] Dilauroyl-PC and DMPC were sonicated using a Branson Model S-75 sonifier fitted with a microtip. The slower rate of association with DMPC vesicles relative to the results shown elsewhere in this paper may be due to the lower power used to prepare these vesicles and the lower temperature (30 °C) at which the rates were measured.

amphotericin interactions) may become significant when insufficient PC is available to surround each cholesterol molecule (Engelman and Rothman, 1972).

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