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EFFECTS OF β-DIETHYLAMINOETHYL-DIPHENYLPROPYLACETATE (SKF 525-A) ON BIOLOGICAL MEMBRANES—II

SKF 525-A-INDUCED EFFECTS ON THE GROWTH AND UPTAKE OF L-AMINO ACIDS BY *ESCHERICHIA COLI* (DIPLOID MUTANT)*

INSU P. LEE†

Department of Pharmacology, School of Medicine, University of Washington, Seattle, Wash. 98105, U.S.A.

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Abstract—Previous work has indicated that β-diethylaminoethyl-diphenylpropylacetate (SKF 525-A), a well-known inhibitor of microsomal drug-metabolizing enzymes, lowers surface tension and has a biphasic hemolytic effect on the human red blood cell membrane. Present studies further investigate the effect of SKF 525-A on biological membranes and are concerned with the action of SKF 525-A on bacterial cells. A diploid mutant of *Escherichia coli* which has limited ability to synthesize pyrimidines due to the absence of orotidylate decarboxylase was used for these experiments. SKF 525-A at high concentrations (10^{-2} and 10^{-3} M) lysed *E. coli* (diploid mutant) cells either during the exponential or secondary growth phase. However, the rate of cell lysis was greater during the exponential than during the secondary growth phase. The magnitude of cell lysis in both growth phases correlated well with surface activities of SKF 525-A and was linear during the first 30 min. *E. coli* cells treated with SKF 525-A (10^{-5} M) for 4 hr prior to incubation with either uracil or L-isoleucine demonstrated decreased cellular accumulation of these substrates. The degrees of inhibition by SKF 525-A on the cellular uptake of these substrates were similar in spite of the dissimilarity of structures. The phenomena of cell lyses and impaired uptake of metabolic substrates suggest that the effect of SKF 525-A concerns an alteration of the cell wall and/or cell membrane rather than a specific effect on transport mechanisms.

β-DIETHYLAMINOETHYL-diphenylpropylacetate (SKF 525-A) is a well-known potent inhibitor of a wide variety of hepatic microsomal drug-metabolizing enzymes.[1] However, at the present time the mechanism by which SKF 525-A inhibits microsomal drug-metabolizing enzymes is not completely understood. Several mechanisms of action have been theorized to explain the inhibition of such diverse enzymatic reactions. Netter[2] proposed that, because the NADPH-dependent oxidase system is common to all oxidative reactions, SKF 525-A may act as uncoupling agent. However, SKF 525-A also inhibits non-NADPH metabolic reactions, for example, nitroreductase,[3] and glucuronyltransferase[4] reactions. Brodie[5] has suggested that SKF 525-A may have a physicochemical effect on the microsomal membrane to alter its permeability to drugs, and the mechanism of enzyme inhibition is for the most part noncompetitive.[2] On the other hand, Hollunger,[6] based on his microsomal amidase

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† Author's present address is: Pathologic Physiology Branch, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, N.C. 27709.

studies, reported that SKF 525-A competitively acts on the enzymes *per se* rather than on the microsomal membrane. In addition, Anders and Mannering[7] have suggested that microsomal demethylase is competitively inhibited by SKF 525-A. Recently Schenkman *et al.*[8] reported that SKF 525-A can be either a competitive or noncompetitive inhibitor for the liver microsomal mixed function oxidase system.

Previously, it was reported that SKF 525-A, at concentrations of 10^{-4} to 10^{-9} M, stabilized human red blood cell membrane against the effects of hypotonic solutions, while at higher concentrations SKF 525-A caused complete hemolysis.[10] It was further demonstrated that surface activity of SKF 525-A and degree of hemolysis showed good correlation.

The purpose of these experiments was to further investigate whether the membrane effects of SKF 525-A could be demonstrated in a bacterial cell wall and/or cell membrane that is structurally and chemically different from the mammalian cell membrane.

MATERIALS AND METHODS

Materials. Diploid mutant, *Escherichia coli*, obtained from Dr. Schachman, Molecular Biology and Virus Lab., University of California, was used in this study. This mutant has limited ability to synthesize pyrimidine, owing to mutational defects in orotidylate decarboxylase, and grows slowly in the absence of exogenous pyrimidines. The generation time is shortened by approx. 1 hr if the liquid medium is supplemented with 50 μg each of L-leucine and L-histidine/ml and 30 μg of uracil/ml.

Radioactive istopes, [2-^{14}C]uracil (34·3 mCi/m-mole), L-[U-^{14}C]phenylalanine (366 mCi/m-mole) and L-[U-^{14}C]isoleucine (234 mCi/m-mole) were obtained from New England Nuclear Corp., Boston, Mass. SKF 525-A was a generous gift of Smith, Kline & French Lab., Philadelphia, Pa.

Culture media. The bacteria were grown in a medium containing the following chemicals in grams per liter of distilled water: Na_2HPO_4, 8·7; KH_2PO_4, 5·3; $(NH_4)_2SO_4$, 2·0; $MgSO_4.7H_2O$, 0·1; $Ca(NO_3)_2$, 0·005; $ZnSO_4.7H_2O$, 0·005; $FeSO_4.7H_2O$, 0·005; D-glucose, 4·0; L-leucine, 0·005; L-histidine, 0·005 and uracil, 0·004. The pH of the medium was 7·0. Solid medium was prepared by the addition of 15 g of Bacto-Agar (Difco). All the glassware and solutions were sterilized with a Castle Thermatic 50 autoclave.

Surface tension measurements of SKF 525-A. Surface activities of varying aqueous concentrations of SKF 525-A were determined at 23°, by means of the Wilhelmy plate method using a precision Rosano suface tensiometer (model ST 0500 MG).[10] Concentrations of SKF 525-A ranging from 10^{-2} to 10^{-8} M were dissolved in 300 mOsmolar NaCl (pH 5·7). A partition coefficient for SKF 525-A at 10^{-3} M (pH 5·70) in CH_3Cl/H_2O was 34.

To study the effects of SKF 525-A on the growth of *E. coli*, 10^6 cells were inoculated into media supplemented with L-amino acids and uracil as previously indicated and incubated at 37° in a Dubnoff metabolic shaker for 12 hr with several concentrations of SKF 525-A (10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} M). Bacterial cell numbers were estimated by reading optical densities (O.D.) of bacterial suspensions at 660 nm with a Beckman DU spectrophotometer.

To determine if the magnitude of drug effect varies with different growth phases, the

following experiments were carried out. *E. coli* was grown in a media supplemented with uracil and amino acids and during either exponential or secondary growth phase, cells were transferred to control flasks and flasks containing varying concentrations of SKF 525-A (10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} M). Optical densities of the final cell suspensions were adjusted to read 0·200 at 660 nm and then cell suspensions were incubated as described previously.

To study the effects of SKF 525-A on the cellular uptake of amino acids and pyrimidine, cells were grown in a medium supplemented with uracil and amino acids and grown for 4 hr with or without SKF 525-A (10^{-5} M). Subsequently, the cells were centrifuged at 200 *g* and washed three times with the nonsupplemented culture medium and the volume was adjusted to give an $O.D._{660nm}$ reading of 0·200. To block protein sysnthesis, chloramphenicol was added in the amount of 100 μg/ml to control or SKF 525-A treated cell suspensions. These cell suspensions were preincubated for 15 min at 37° in a Dubnoff metabolic shaker. Subsequently, either 0·125 μCi of [2-^{14}C]uracil, 0·05 μCi of L-[U-^{14}C]phenylalanine, or 0·05 μCi of L-[U-^{14}C]isoleucine was added to each flask and 20- and 40-min additional incubations were carried out. The final $O.D._{660nm}$ of cell suspension was 0·100 (approx. 10^8 cells). One-ml portions of cell suspension incubated with [^{14}C]-labeled uracil, L-phenylalanine or L-isoleucine were sampled at various times from the incubation flask and immediately filtered on a millipore filter (HAWP 04700; 0·45 μ) with model 3010 vacuum filtration equipment (Bradley Laboratory Corp.). The filters were then washed with 20 successive 1-ml aliquots of culture medium containing 10^{-5} M of cold uracil, L-phenylalanine, or L-isoleucine at 23°. The millipore filters were than placed in a radioactive counting vial and 0·5 ml of Hyamine hydroxide was added and incubated for 2 hr at 70°. After cooling, 10 ml of toluene containing 0·3% 2,5-diphenyloxazole and 0·05% 1,4-bis-2(5-diphenyloxazolyl)-benzene was added and radioactivity was determined with a Tricarb liquid scintillation spectrometer. Quenching was corrected by internal standards.

RESULTS

Effects of SKF 525-A on the growth of bacteria. Figure 1 demonstrates complete inhibition of bacterial growth at 10^{-2} M of SKF 525-A, while at 10^{-3} M the lag period was significantly prolonged (200 per cent of control). This prolonged lag period was followed by a short exponential growth phase but no secondary growth phase occurred. At 10^{-4} M, the lag period was again significantly longer than the control lag period (160 per cent of control), and cell growth was significantly retarded both at the exponential growth phase and secondary growth phase. At 10^{-5} M, the lag period was not significantly different from the control but the growth rate at both the exponential and the seconary growth phase was retarded. A secondary lag period occurs after the uracil and amino acids in the medium are depleted, and the secondary growth phase occurs at a slower rate than the exponential growth phase.

Correlation between surface activity and the lytic effect of SKF 525-A. Surface tension measurements of varying concentrations of SKF 525-A indicated that the critical micellar concentration was 5×10^{-3} M (46 dynes/cm). A comparison of surface tensions and per cent of unlysed cells as a function of SKF 525-A concentration is shown in Fig. 2, which demonstrates that the lytic effects of drug correlated very well

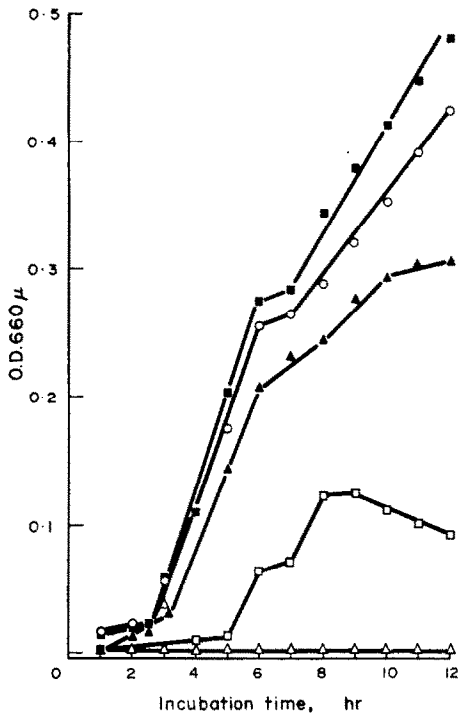

FIG. 1. Effects of SKF 525-A on *E. coli* (diploid mutant) growth. Control, ■——■; 10^{-5} M, ○——○; 10^{-4}, ▲——▲; 10^{-3}, □——□; and 10^{-2} M, △——△. 10^6 Bacterial cells were incubated at 37°.

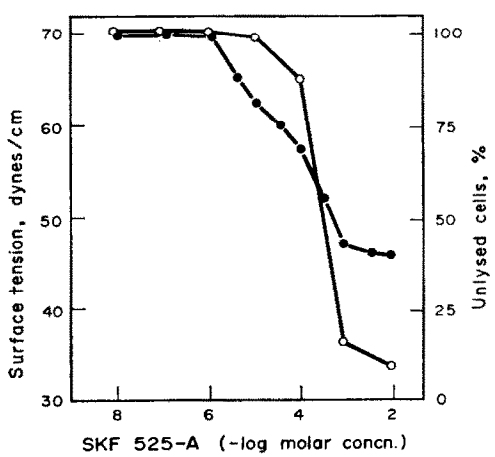

FIG. 2. Comparison of surface activities and lytic effect of SKF 525-A at varying concentrations. Surface tension, ●——●; cell lysis at 4-hr incubation, ○——○.

with surface activity. A concentration at which a significant cell lysis occurs corresponded exactly to a critical micellar concentration of drug. Cells lysed as per cent of control at 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} M were 95, 83, 9 and 0 of control, respectively, after a 4-hr incubation with drug.

Effects of SKF 525-A on the exponential and secondary growth phase. The lytic effects of SKF 525-A on the cells at the exponential and secondary growth phase's were compared. Figure 3 demonstrates that the lytic effect of the drug on cells at two

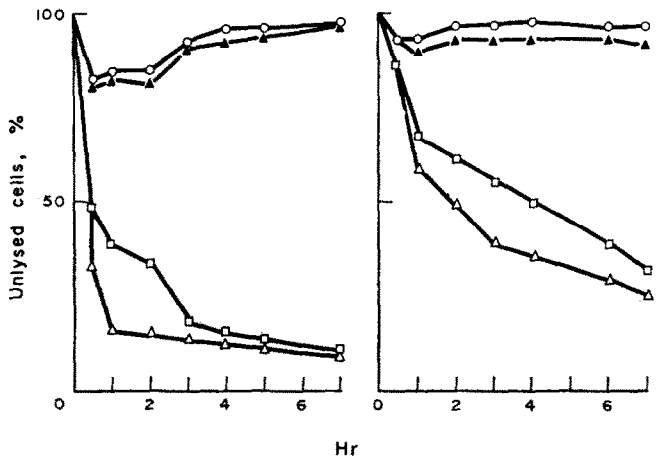

FIG. 3. Effects of SKF 525-A on the exponential and secondary growth phases. Left: exponential growth phase; right: secondary growth phase. 10^{-5} M, ○——○; 10^{-4} M, ▲——▲; 10^{-3} M, □——□; and 10^{-2} M, △——△. Per cent of unlysed cells was calculated from: per cent unlysed cells, $O.D._{660\,nm}\ t_{n\,hr}/t_{0\,hr} \times 10^2$.

different phases are quantitatively different. Significant cell lysis occurred with the first hr of incubation with all concentrations of SKF 525-A tested. During the first hour of incubation, the rate of cell lysis in both the exponential and secondary growth phases was linear (2·2 and 1·2 per cent cell lysis/min, respectively) at 10^{-3} and 10^{-2}M and, thereafter, cell numbers were further decreased in both growth phases, but quantitatively different. Effects of the drug at concentrations of 10^{-3} and 10^{-2} M on the exponential phase showed 62 and 85 per cent cell lysis, whereas in the same concentrations during the secondary growth phase exhibited 30 and 34 per cent cell lysis. Hence, not only the rate of cell lysis but also the total net cell lysis by SKF 525-A was greater in the exponential growth phase than during the secondary growth phase. It, therefore, indicated that the cells in the exponential growth phase are much more sensitive to SKF 525-A than the cells in the secondary growth phase. On the other hand, at lower concentrations of SKF 525-A (10^{-4} and 10^{-5} M), the rate of lysis for both the exponential and secondary growth phases in the first hour of incubation was 2·3 and 3·8 per cent cell lysis/minute respectively. However, its lytic effect at these lower concentrations is transient and cells were able to grow back to the initial cell number at the end of a 3-hr incubation.

Effects of varying concentrations of SKF 525-A on cell lysis. Figure 4 demonstrates the effects of varying concentrations of SKF 525-A on the cells in both growth phases. It indicates that during the first 30-min incubation, the degree of cell lysis in both

growth phases is a linear function of drug concentration but at different rates. Cells in the exponential growth phase were much more sensitive to lytic effects of SKF 525-A than cells in the secondary growth phase.

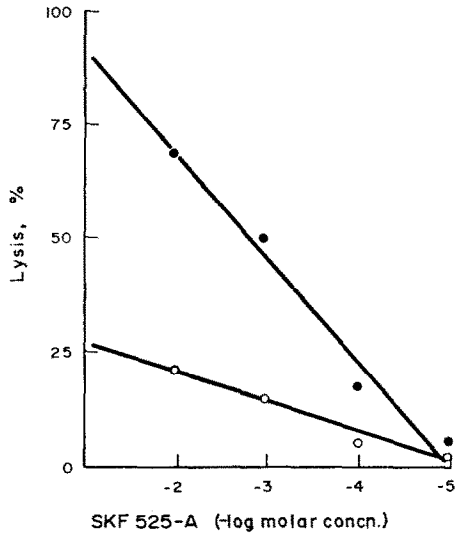

FIG. 4. Effects of varying concentrations of SKF 525-A on 4-hr old cells incubated for 30 min at 37°. Per cent of lysis was calculated from: per cent lysis = 100 − (O.D.$_{660\,nm}$ $t_{30\,min}/t_{0\,min}$ × 10^2). Cells in the exponential growth phase (●——●); cells in the secondary growth phase (○——○).

To determine if inhibition of cell growth by lower concentrations of SKF 525-A (10^{-5} M) was due to an alteration of uptake of uracil and amino acids, cells obtained during the exponential growth phase were investigated. The uptake of L-phenylalanine, L-isoleucine and uracil was increasingly inhibited by 10^{-5} M SKF 525-A as the incubation time was increased (Fig. 5). After 40 min of incubation at 37° under atmosphere, the uptake of L-phenylalanine, L-isoleucine and uracil was 33, 29 and 47 per cent of control, respectively.

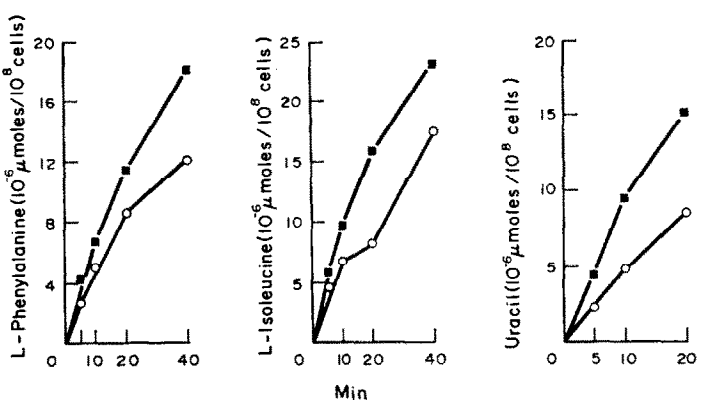

FIG. 5. Effects of SKF 525-A (10^{-5} M) on the uptake of L-amino acids and uracil. Left: the uptake of L-phenylalanine; middle: the uptake of L-isoleucine; right: the uptake of uracil. The control uptake of all substrates was ■——■; 10^{-5} M SKF 525-A, ○——○.

DISCUSSION

It has been reported previously that human red blood cells were either lysed (10^{-3} M) or stabilized by SKF 525-A (10^{-5} M) in a range of hypotonic solutions.[10] In addition, the stabilization of biological membranes by pharmacologically active tranquilizers, antihistamines, barbiturates, steroids and local anesthetics is well known.[9,11] All of these drugs exert a biphasic effect on biological membranes which is drug concentration dependent. A similar observation has been reported wherein chlorpromazine has not only a lytic action on bacteria but also has altered premeability of *Lactobacillus plantarum*.[12] The most common physicochemical properties of phenothiazine derivatives are associated with their surface activity and high lipid solubilities at physiological pH.

It has been reported that phenothiazine derivatives accumulate at the biological membrane.[13,14] The degree of accumulation of a drug at the membrane depends on its surface activity. The size of micelles has been postulated as the mechanism in pharmacological action.[15]

The present data indicate that SKF 525-A (10^{-3} M) at high concentrations lysed *E. coli* mutant bacterial cells within 30 min, either during the exponential growth phase (with uracil and L-leucine and L-histidine supplemented medium) or the secondary growth phase in which uracil as well as the amino acids was the limiting factor for its rate of growth. On the other hand, SKF 525-A at lower concentrations lysed only a small fraction of cells and subsequently slowed cell growth rate during both growth phases.

It was also demonstrated that at all SKF 525-A concentrations, the cells in exponential growth phase were much more sensitive to the lytic effect of SKF 525-A than the cells in the secondary growth phase. The mechanism of the growth phase dependent effect of SKF 525-A has not been elucidated in the present studies. However, similar growth phase dependent effects of other surface active drugs have been reported previously.[16,17] It has been reported that, during the period of profuse synthesis of DNA, the cell wall has been found to be highly vulnerable to chlorpromazine.[18] From the results of [^{14}C]-labeled uracil and amino acid uptake studies, it appears that the slow rate of growth of bacteria cells previously exposed to a low concentration of SKF 525-A (10^{-5} M) could be attributed to an inhibition of uptake of these substrates. SKF 525-A is capable of inhibiting the cellular uptake of two unrelated L-amino acids and also pyrimidine, and that there was no apparent specificity in the inhibition of uptake of any substrate used in the present study. It is suggestive that at low concentrations of the drug, either bacterial cell wall and/or cell membrane may have altered to prevent adequate transport of L-amino acids and pyrimidine across these cells.

It is compatible with the data that cell lysis at higher concentrations of SKF 525-A (10^{-3} and 10^{-2} M) appears to be a direct effect whereas at a lower concentration, SKF 525-A (10^{-4} and 10^{-5} M) appears to inhibit the transport of substrates for cell growth. Therefore, the action of SKF 525-A on bacterial cell wall and/or cell membrane appears to be nonspecific and correlates well with its surface activity as well as its high partition coefficients (chloroform/H_2O).

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