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ACKNOWLEDGMENTS

Supported by a grant from Allergan Pharmaceuticals, Inc., Irvine, California, and by Grant EY-03816 from the National Eye Institute, Bethesda, Maryland.

Monolayer Studies of Insulin–Lipid Interactions

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Received December 28, 1981, from the *College of Pharmacy, University of Michigan, Ann Arbor, MI 48109.* Accepted for publication April 30, 1982.

Abstract □ The interactions between insulin and various lipids were studied by monolayer penetration experiments at constant surface area. The increase in surface pressure, $\Delta\Pi$, of a lipid film depended upon the particular lipid used and the concentration of insulin in the subphase. For all lipids studied, $\Delta\Pi$ was dependent on the initial surface pressure of the lipid film. Evidence of the interaction between insulin and the lipids was found in the ability of insulin to penetrate lipid films with initial pressures >16 dynes/cm, the maximum surface pressure obtained by insulin alone. For phospholipids, both the nonpolar and polar regions influenced the degree of interaction with insulin. Saturated chain lecithins exhibited less penetration than phospholipids with unsaturated hydrocarbon chains. The net charge of the lipid was not found to be an important determinant of penetration; however, the structure of the polar group can have a dramatic effect. Insulin penetration of mixed lipid films cannot be predicted by the penetration characteristics of the pure components. The possible role of these interactions in determining the geography of the insulin molecule within the liposome and its resultant effects on the stability is discussed.

Keyphrases □ Insulin—monolayer studies of interactions with lipids □ Monolayer studies—insulin–lipid interactions □ Lipids—monolayer studies of interactions with insulin

Monolayer interactions between proteins and lipids are of much interest in the study of cell membrane structure (1, 2), hormonal action (3, 4), and enzyme activity (5). Early studies (6) established the technique of injecting a protein into the subphase beneath a lipid film at constant surface area. Since then modifications of this procedure have been used in an effort to gain a more detailed understanding of the nature of these interactions.

Recent attempts to develop an oral dosage form of insulin have utilized liposomal entrapment in order to protect the hormone from proteolytic degradation in the GI tract. In theory, to achieve the necessary degree of protection, the entrapped insulin should not penetrate or decrease the stability of the lipid bilayer. The geography of the insulin molecule within the liposome should be dependent upon the degree and type of its interactions with the lipid components. This report investigates the interactions of insulin with cholesterol, stearylamine, and various phospholipids through the use of monolayer penetration studies.

BACKGROUND

The increase in surface pressure of a lipid film with the addition of protein has been termed penetration, although the exact nature of this phenomenon remains unclear. For example, as more protein is added to the subphase, the increase in surface pressure may be attributed to an increase in the number of protein molecules at the surface, enhanced interaction between the surface components, or protein–protein interactions just below the surface, which may alter molecular orientation at the surface. Although the various mechanisms cannot always be differentiated, the results of such penetration studies are still of value in determining the criteria that affect lipid–protein interactions.

The great variation in the behavior of lipid–protein films reported in the literature can be ascribed to the wide range of physical properties of the proteins used. The unique characteristics of each protein or polypeptide prevent accurate predictions of the nature and magnitude of its interactions with lipids. Also, relative protein penetration does not show consistent results based on the type of lipid involved. Therefore, it is necessary to individually assess the effect of insulin on films of pure lipids or lipid mixtures in order to better understand their interactions.

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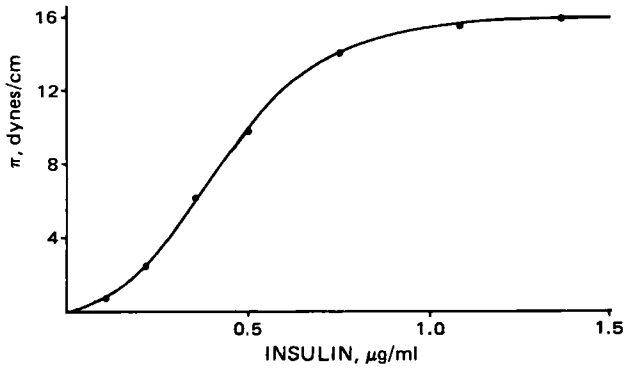

Figure 1—*The surface pressure–concentration profile of insulin upon injection into 0.05* M N-*2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid in 0.9% NaCl buffer, pH 7. The surface pressure was measured 30 min after each insulin addition. A limiting or collapse pressure for insulin of 16 dynes/cm was observed.*

EXPERIMENTAL

Phosphatidylcholine[1] (I) was purified on an activated alumina column. Cholesterol[2] was purified by recrystallizing three times from ethanol solution. All other lipids were used as supplied[2] and stored at 0°. TLC of all lipids revealed a single spot.

The subphase buffer was prepared from triple-distilled water, the second distillation was from alkaline permanganate. An all-glass distillation apparatus was used to prevent contamination. Sodium chloride, reagent grade, was heated to 600° to remove organic impurities. All other chemicals were reagent grade.

Research grade crystalline porcine insulin[3] with an activity of 28.5 units/mg and containing 0.6% zinc was used. The only impurity noted by polyacrylamide gel electrophoresis and high-performance liquid chromatographic (HPLC) analysis was 1–2% monodesamido insulin. Stock solutions of 4.0 mg/ml were prepared in 0.01 *N* HCl and stored at 4°. Further dilutions were prepared in 0.05 *M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid in 0.9% NaCl buffer, pH 7. The glass vials used for these dilutions were rinsed twice with the insulin solution to avoid loss of insulin due to adsorption.

The monolayer penetration studies were a modification of previous studies (6). The experiments were performed at 25 ± 1° on a surface tensiometer[4] having a sensitivity of 0.04 dynes/cm, using a sandblasted platinum Wilhelmy plate. The subphase consisted of 90 ml of 0.05 *M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid in 0.9% NaCl buffer, pH 7, contained in a circular polytetrafluoroethylene dish having an area of 46 cm^2. A polytetrafluoroethylene-coated magnetic stirrer was used for mixing of the subphase. The entire apparatus was enclosed to reduce airborne contamination.

All lipids were spread from solutions of hexane–ethanol (9:1) in a micrometer syringe[5] in amounts sufficient to produce the desired initial surface pressure. Each mixed lipid film was spread from a single solution in which the appropriate lipids were dissolved. The addition of the spreading solvent alone to a clean buffer surface produced no measurable change in surface tension. The dissolved lipids were stored below 0° under a dry nitrogen atmosphere for no longer than 5 days.

A stationary needle with a removable glass syringe was used to deliver concentrated insulin solutions beneath the surface into the subphase. Insulin was added in 1–2-ml increments, and an equal volume of the subphase was discarded prior to the additions. The needle and syringe were rinsed three times after each addition of insulin by withdrawing and reinjecting 1–2 ml of the subphase. A magnetic stirrer (10 rpm) mixed the subphase for 15 min, and the mixture was allowed to settle for 15 min for the monolayer to equilibrate before recording the surface tension. Surface pressure changes due to insulin addition were found to be virtually complete within 30 min.

Surface pressure (Π) was calculated as the difference in surface tension in the absence of the film (γ_0 = 72.3 dynes/cm) and that of the film-covered surface (γ). The change in surface pressure ($\Delta\Pi$) was calculated as the difference in pressure of the lipid film upon injection of insulin into the subphase (Π) and that of the film in the absence of insulin, *i.e.*, at its initial surface pressure (Π_0).

[1] Egg lecithin, Type IX-E, Sigma Chemical Co., St. Louis, Mo.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Lilly Research Laboratories, Indianapolis, Ind.
[4] Rosano Surface Tensiometer, VWR Scientific.
[5] Agla; Wellcome Reagents Ltd., Beckenham, U.K.

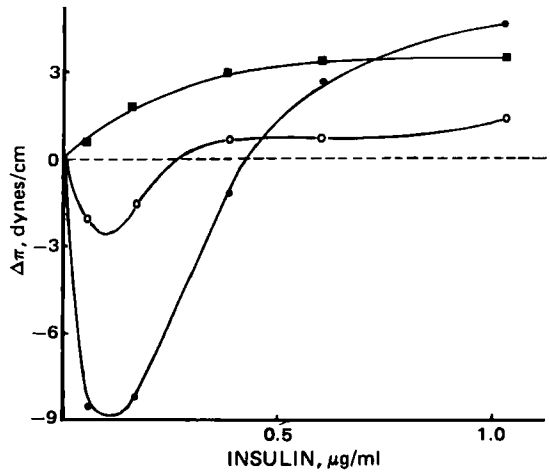

Figure 2—*The time-dependent changes in surface pressure of phosphatidic acid (II) monolayers upon the injection of insulin into the subphase. Each curve represents a film with an apparent initial surface pressure of ~13 dynes/cm. The delay times that followed the spreading of the films before the start of the experiments are as follows: (●) no delay; (○) 60 min; (■) 120 min. In control studies the surface pressure of films of II required 120 min to attain a stable value, which then remained constant for at least 3 hr.*

RESULTS

The insulin protein, although soluble in aqueous solutions, is a surface-active molecule denaturing at the air–water interface to give rise to surface pressures as high as 20 dynes/cm (7). Under the conditions of these experiments, the surface pressure–concentration (Π–*C*) curve of insulin approached a limiting or collapse pressure of 16 dynes/cm at pH 7, as seen in Fig. 1. This is in agreement with the collapse pressure obtained from the Π–*A* isotherms in a previous study (7) for insulin at pH 7.4.

The stability of the lipid films for the duration of the experiments (~3 hr) was confirmed by measuring the surface pressure as a function of time. With most of the lipids studied, a constant surface pressure was obtained within 15 min after spreading. However, with phosphatidic acid (II), phosphatidylserine (III), dipalmitoylphosphatidylcholine (VI), and distearoylphosphatidylcholine (VII) a slow decrease in surface pressure was observed for 1–2 hr, followed by constant values for at least 3 hr thereafter. Recognizing this time effect is important in obtaining accurate

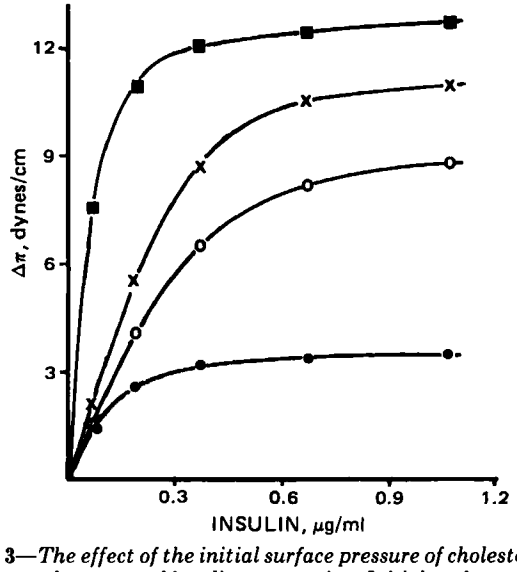

Figure 3—*The effect of the initial surface pressure of cholesterol monolayers on the extent of insulin penetration. Initial surface pressures (dynes/cm) are: (■) 4.8; (×) 11.1; (○) 15.8; (●) 19.5. This set of curves is qualitatively similar to those of all lipids studied in these experiments.*

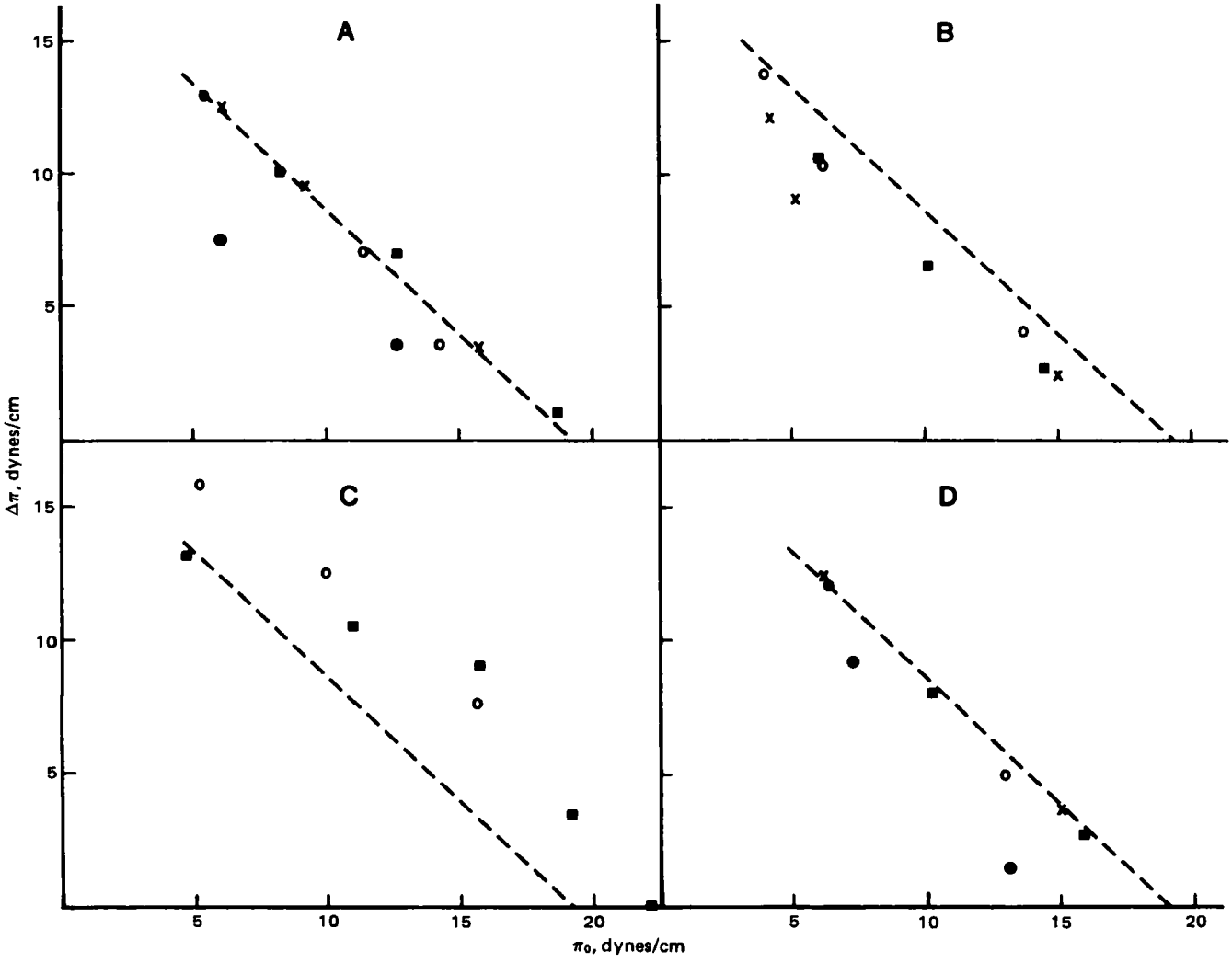

Figure 4—*Penetration of insulin (1.0 μg/ml) into monolayers of various lipids. Key: (A) unsaturated phospholipids: (■) phosphatidylcholine (I); (●) phosphatidic acid (II); (○) phosphatidylserine (III); (×) phosphatidylinositol (IV); (B) saturated lecithins: (■) dimyristoyl-(V); (×) dipalmitoyl-(VI); (○) distearoylphosphatidylcholine (VII); (C) other lipids: (■) cholesterol; (○) stearylamine; (D) mixed lipid films: (■) phosphatidylcholine–cholesterol (2:1) (VIII), (×) phosphatidylcholine–cholesterol–phosphatidic acid (4:2:1) (IX); (○) phosphatidylcholine–cholesterol–stearylamine (4:2:1) (×); (●) dipalmitoylphosphatidylcholine–cholesterol (2:1) (XI).*

results. The most dramatic pressure drop was observed with II. Figure 2 depicts three $\Delta\Pi$–C curves for films of II, each of which had an apparent initial surface pressure of ~13 dynes/cm. As can be seen, if the lipid film is not given sufficient time to equilibrate prior to addition of insulin to the subphase, both the magnitude and the shape of the curve is affected. For example, at 0- and 60-min delays, an initial condensation effect was observed (decrease in surface pressure), whereas with a 120-min delay, only an expansion effect (increase in surface pressure) was seen.

Placing additional pure spreading solvent on a film of II after its pressure had stabilized resulted in virtually no pressure change after 10 min, indicating that solvent retention in the lipid film was not a problem. Another study (8) also reported no evidence of solvent retention with a hexane–ethanol (9:1) solvent in lecithin and phosphatidylethanolamine films when $\Pi_0 > 2$ dynes/cm.

Two of the saturated lecithins, VI and VII, also showed a slow decrease in surface pressure during the first hour after spreading. With respect to the equilibrium-spreading pressure, Πe, which is defined as the pressure at which the monolayer is in equilibrium with the bulk lipid phase (9), films of both of these lipids may be considered thermodynamically unstable. For lecithins, it has been determined that spreading from the bulk phase does not occur below the gel–liquid crystal transition temperature (T_c), hence, $\Pi_e = 0$ at temperatures below T_c (10). The values of T_c for VI and VII are 41 and 60°, respectively (11); therefore, their solvent-spread films at 25° are not at true thermodynamic equilibrium. The spread films, however, do maintain a constant surface pressure and thus can be considered metastable (8–10), since the attainment of equilibrium (collapsing of the monolayer to the gel state) is a slow process and does not occur within the time frame of the experiments.

For all lipids studied the degree of penetration or association of insulin into the monolayer, as measured by the magnitude of $\Delta\Pi$ (6), was very dependent on the initial surface pressure, Π_0, as is generally expected with insulin (12) and other proteins (6, 13–18). A greater degree of penetration was observed at lower Π_0 values as indicated both by the magnitude of $\Delta\Pi$ and the initial slope of the $\Delta\Pi$–C curves. Figure 3 shows a set of typical $\Delta\Pi$–C curves.

Plots of $\Delta\Pi$ *versus* Π_0 for the single component and mixed lipid films are shown in Fig. 4A–D. The data were obtained from $\Delta\Pi$–C curves for the various lipids at an insulin concentration of 1.0 μg/ml. For all lipids, the changes in $\Delta\Pi$ were virtually complete at this concentration of insulin. It was found that the lipids fell into three distinct groups based on the magnitude of $\Delta\Pi$. The first group (Fig. 4A, D), consisting of the unsaturated lipids I, III, and phosphatidylinositol (IV), and the mixed lipid films phosphatidylcholine–cholesterol (2:1) (VIII), phosphatidylcholine–cholesterol–phosphatidic acid (4:2:1) (IX), and phosphatidylcholine–cholesterol–stearylamine (4:2:1) (X), showed an intermediate degree of penetration by insulin. Their behaviors, as indicated by the dashed line, is included as a reference in these four plots. Cholesterol and stearylamine (Fig. 4C) form a second group of lipids characterized by enhanced penetration by insulin. The third group of lipids, those showing reduced or inhibited penetration, consists of the saturated lecithins dimyristoylphosphatidylcholine (V), VI, and VII (Fig. 4B), the unsaturated phospholipid II (Fig. 4A), and the mixed film of dipalmitoylphosphatidylcholine–cholesterol (2:1) (XI) (Fig. 4D). In all cases, $\Delta\Pi$ de-

creases with increasing values of Π_0 until at a critical Π_0 value, no pressure change is observed. This value of Π_0, where $\Delta\Pi = 0$ is termed the limiting Π (18). For the lipids in the second group a limiting Π of 19.2 dynes/cm was in close agreement with the value obtained previously (12) of 19 dynes/cm for phosphatidylcholine, stearyl alcohol, and stearic acid monolayers. For cholesterol, however, our limiting Π value of ~22 dynes/cm is lower than that of a previously derived value of 27 dynes/cm (12). Repeated trials with cholesterol films at pressures between 22 and 24 dynes/cm failed to show any response to insulin under our conditions.

Insulin, which is soluble in the subphase, is able to penetrate monolayers with an initial surface pressure greater than the collapse pressure of insulin alone (16 dynes/cm). This indicates that insulin is interacting in some manner with the lipids, and that the observed behavior is not merely competition for the surface by two different surface-active molecules.

The combined data for the three saturated lecithins (Fig. 4B) indicate a limiting Π value of 17.7 dynes/cm. The absence of double bonds in the acyl chains in these lecithins results in closer packing of their molecules at the air–water interface (15, 19) as compared with unsaturated lecithin (I). As a result, insulin penetration is reduced. The temperature at which the experiments were performed (25°) is above the gel–liquid crystal transition temperature of V (T_c = 23°) (11), but is below that of VI and VII as previously mentioned. The influence of this parameter alone was not found to be of great importance in determining the degree of insulin penetration in this monolayer system.

Of the three lipids (II, III, and IV) with a net negative charge, only II showed decreased penetration (Fig. 4A). Since all three lipids, like I, are unsaturated, it is likely that the structure of the polar group, not the charge, is a more important determinant of penetration. The positively charged stearylamine films enhanced penetration by insulin, which has a net negative charge of pH 7; this effect may be due to electrostatic interactions. However, direct comparisons between stearylamine and lecithin films are inappropriate due to gross structural differences.

The mixed lipid films showed some interesting behavior and, as expected, were unique with respect to their individual components. Although cholesterol is strongly interactive with both I and VI as shown by monolayer studies (19, 20), the addition of cholesterol to films of I did not change the degree of insulin penetration, whereas incorporation of cholesterol into films of VI significantly reduced the penetration of insulin into the mixed monolayer. In both negatively (IX) and positively (X) charged mixed films, the individual behavior of the charged lipid was not apparent and the films demonstrated penetration similar to that of films of VIII or I.

DISCUSSION

Previously published results on the penetration or association of various proteins with lipid monolayers have yielded few consistent patterns of behavior based on the type of lipid involved. Since proteins encompass a wide range of physical properties including net charge, structure, and hydrophobicity, it is reasonable to expect that their interactions with lipids will be dependent on the structure and orientation of the lipid. The present results, which indicate decreased penetration by insulin into films of saturated lecithins as compared with I were also observed for α-lactalbumin (14) and delipidated high-density lipoprotein (21). However, a previous study (22) reported that I and fully hydrogenated I showed identical penetration by rabbit IgG. With β-casein (17, 21) and a fraction of delipidated lipoprotein (17), more penetration was observed into saturated lecithins than into I. Cholesterol films have consistantly allowed greater penetration than films of I (12, 13, 22), which is also apparent from the results of the present study.

It is widely recognized that properties of the subphase can affect the interactions of proteins with lipid films. Variations in pH (6, 14, 16, 18), temperature (13), ionic strength (18, 23), and the method of introducing the lipid and protein at the surface (13) all determine, in part, the changes in the interfacial region.

The mechanism of protein penetration and interaction with lipid films remains a topic of current research. The structure of proteins in lipid films and bilayers is also receiving more attention as new and more powerful analytic methods become available. Early work (24, 25) suggested that various proteins, including insulin, formed two distinct layers under a lipid film. The first layer was said to consist of fully denatured protein interacting by an ionic mechanism with the choline residue of lecithins, with penetration into the film by hydrophobic side chains of the protein producing a change in surface pressure. Beneath this was adsorbed a second layer of native protein. A monolayer study of insulin (12) stressed that hydrophobic interactions control penetration. However, results here indicate that this is not the only factor.

Several mechanisms of monolayer and membrane interactions have been proposed (1, 13, 22, 26) with proteins dependent upon the nature of both the lipid and protein. In these studies the role of many factors, including protein hydrophobicity and flexibility, lipid compressibility, electrostatic interactions, and water structuring about the lipid polar groups, are discussed. Other reports (16–18, 21, 27) also conclude that lipid–protein interactions cannot be explained on the basis of hydrophobic or electrostatic mechanisms alone. The importance of lipid compressibility (6) has also been discussed in reports of film penetration experiments with β-casein and apoprotein (17). However, the contention that decreased compressibility due to the incorporation of cholesterol in a lecithin film (2, 15) should lead to decreased penetration was not apparent in several of the mixed films which were studied. Measurements of the surface concentration of proteins indicate that $\Delta\Pi$ is not necessarily a measure of the amount of penetrating protein (22), and protein continues to adsorb beneath a film after a maximum in $\Delta\Pi$ has been attained (18, 27).

Monolayer penetration experiments are a valuable tool for exploring the interactions between lipids and proteins and can, with limitations, provide insight into the interactions occurring in liposomal systems (2, 8, 16, 17). We can conclude from our data that insulin is capable of some degree of penetration into lipid films, which can be controlled by the choice of lipid. Such penetration into the bilayer of a liposome may render the hormone susceptible to attack by proteases present in the external medium and also change the bilayer integrity. Saturated lecithins alone or in combination with cholesterol, which exhibit inhibited penetration, would be good candidates for liposomal delivery systems of insulin. These lipids would be better able to confine insulin within the aqueous compartments of the liposome than lipids that allow greater penetration.

Adsorption of insulin on the external and internal faces of the bilayer is also variable and can greatly affect the degree of entrapment. The incubation of liposomes in solutions of free insulin can result in the irreversible binding of the protein on the vesicle surface (28). Many reports of oral administration (29, 30) include externally bound insulin when calculating entrapped insulin and the administered dose. Upon oral administration, such insulin would be expected to undergo rapid destruction.

A better understanding of the underlying insulin–lipid interactions may aid in the design of an effective and reliable oral insulin dosage form.

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ACKNOWLEDGMENTS

This research was supported by the Michigan Diabetes Research Training Center, sponsored by the NIH National Institute of Arthritis, Diabetes, and Digestive and Kidney Disease, Grant No. 5 P60 AM20572.

Dr. David L. Schwinke wishes to thank the American Foundation for Pharmaceutical Education for their gracious support of his graduate studies. The authors also wish to thank Dr. George Zografi for his valuable suggestions during the preparation of this manuscript.

Bilirubin-Displacing Effect of Ampicillin, Indomethacin, Chlorpromazine, Gentamicin, and Parabens *In Vitro* and in Newborn Infants

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Received November 23, 1981, from the *Department of Biochemistry, University of Aarhus, DK-8000 Aarhus C, and the ‡Department of Neonatology, Rigshospitalet, DK-2100 Copenhagen φ, Denmark. Accepted for publication April 30, 1982.

Abstract □ Displacement of bilirubin bound to human serum albumin by ampicillin, indomethacin, chlorpromazine, gentamicin, methylparaben, and propylparaben was investigated quantitatively. Two methods were used *in vitro*: measurement of bilirubin displacement by studying the rate of bilirubin oxidation with hydrogen peroxide and peroxidase and determination of the albumin reserve for binding of bilirubin by observation of the dialysis rate of an added trace amount of a deputy ligand monoacetyldapsone (*p*-acetamido-*p'*-aminodiphenyl sulfone). The latter method was also used for the determination of the albumin reserve in sera from treated newborn infants. The following doses were given: ampicillin, 100 mg/kg iv; indomethacin, 0.2 mg/kg iv; chlorpromazine hydrochloride, 0.7 mg/kg im; gentamicin sulfate, 2.5 mg/kg im. The parabens were present in injectable preparations of chlorpromazine and gentamicin and were therefore given in the following doses: methylparaben, 0.35 mg/kg, and propylparaben, 0.05 mg/kg. All drugs were given in a single dose. A few additional additives and metabolites were studied *in vitro*. Ampicillin, given to 19 infants, produced a small, significant decrease in plasma albumin reserve, to 82% of the pretreatment level and, thus, had a slight bilirubin-displacing effect, quantitatively consistent with a weak displacing effect measured *in vitro*. None of the other substances showed any measurable displacement *in vivo*, likewise in agreement with the results from *in vitro* studies.

Keyphrases □ Bilirubin—displacing effect of ampicillin, indomethacin, chloropromazine, gentamicin, and parabens, *in vitro* and in newborn infants □ Ampicillin—bilirubin-displacing effects of indomethacin, chlorpromazine, gentamicin, and parabens, *in vitro* and in newborn infants □ Indomethacin—bilirubin-displacing effects of ampicillin, chlorpromazine, gentamicin, and parabens, *in vitro* and in newborn infants □ Chlorpromazine—bilirubin-displacing effects of ampicillin, indomethacin, gentamicin, and parabens, *in vitro* and in newborn infants □ Gentamicin—bilirubin-displacing effects of ampicillin, indomethacin, chlorpromazine, and parabens, *in vitro* and in newborn infants □ Parabens—bilirubin-displacing effects of ampicillin, indomethacin, chlorpromazine, and gentamicin, *in vitro* and in newborn infants

A few drugs, notably sulfonamides, are capable of occupying the bilirubin-binding capacity of albumin in plasma, thereby increasing the risk of kernicterus in icteric human neonates (1, 2) as well as in experimental animals (3). Studies of such binding interactions *in vitro* have indicated displacing effects of ampicillin (4), indomethacin (4), some antimicrobial additives (parabens and sodium benzoate) (4–6) and chlorpromazine (7). Gentamicin in itself does not interfere with binding of bilirubin to albumin but is marketed with displacing additives (8). Measurement of the rate of dialysis of an added trace amount of monoacetyldapsone (I) into an otherwise identical plasma sample without this additive has recently been introduced as a technique for quantitative studies of occupation of albumin by drugs (9). Compound I serves as a deputy ligand for bilirubin, since one molecule of I competes selectively with binding of one molecule of bilirubin to human albumin (10). Determinations in undiluted sera at 37° are possible with this technique. Drug effects can thus be studied quantitatively *in vivo* as well as *in vitro*.

Previous work (11, 12) has shown that the concentration of free bilirubin in plasma may return quickly to the pretreatment level after administration of a bilirubin-displacing drug. Since there are no suitable methods for measuring the free bilirubin concentration in undiluted samples of infant serum (often hemolytic) at body temperature, it was decided to base the present *in vivo* studies on a combination of three measurements: determinations of albumin, bilirubin (bound), and albumin reserve in serum samples obtained before giving a single dose of the drug and at one point of time thereafter. The theoretical basis for this principle will be discussed, and the drugs mentioned will be tested accordingly.

EXPERIMENTAL

Human serum albumin[1] was obtained in the lyophilized state with its natural content of fatty acids and was used as a standard in albumin and

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0022-3549/83/0300-0248$01.00/0