Pharmaceutical Research, Vol. 5, No. 11, 1988

Report

The Reversibility of Absorption Promoter Interaction with Red Blood Cell Membranes Studied with Differential Scanning Calorimetry

Jurij Holinej,[1] Howard Y. Ando,[1,2] and Julian W. Snow[1]

Received January 19, 1988: accepted May 11, 1988

Absorption promoters, or adjuvants, are used to enhance the gastrointestinal absorption of poorly absorbed drugs such as macromolecules. In the present work, adjuvant–membrane interactions have been studied by differential scanning calorimetry (DSC) using red blood cell (RBC) membranes as model membrane. These interactions caused temperature shifts, amplitude changes, and broadening of the RBC transitions. Because more than one transition may be simultaneously affected by a given adjuvant, complex overlappings occur. Gaussian modeling and nonlinear regression analysis, therefore, were used to resolve these transitions. A correlation, which may serve as an indicator of adjuvant potency, was found between adjuvant concentration and induced transition temperature shifts. Further, these shifts recovered to baseline after successive washings with buffer (for most adjuvants). Sodium lauryl sulfate induced transition alterations, however, never recovered. Thus the DSC might be useful in monitoring reversible adjuvant–membrane interactions.

KEY WORDS: absorption promoters; adjuvants; differential scanning calorimetry (DSC); nonlinear regression analysis; red blood cells (RBC) membranes; thermal interactions.

INTRODUCTION

Many drugs are poorly absorbed orally due to a variety of factors, some of which are low lipophilicity, high molecular weight, and susceptibility to degradation by intestinal enzyme metabolism. The absorption of some of these drugs can be enhanced by the use of compounds known as absorption promoters or adjuvants (1–3). These adjuvants are generally believed to modify the absorption membrane; however, their actual mechanism of promotion remains speculative (4–6). In this research, reversible adjuvant–membrane interactions were examined by differential scanning calorimetry (DSC) using red blood cell (RBC) membranes. Alterations in the endothermic transitions of the RBC membrane due to varying adjuvant concentrations were analyzed by nonlinear regression.

Six adjuvants, which represented three different chemical classes, were chosen. The benzoic/salicylic acids are the largest class of agents which have been studied as gastrointestinal (GI) enhancers (1–3,5,6). Other agents include diethyl maleate (DEM) (5) and sodium lauryl sulfate (SLS) (7).

MATERIALS AND METHODS

RBC Membrane Preparation and Treatment. All adjuvants (Sigma Chemical Company, St. Louis, Mo.), with the exception of DEM, were used as the sodium salt. RBC membranes were prepared from recently outdated, packed human RBC (Philadelphia Chapter of the American Red Cross). Citrate phosphate dextrose with adenine (CPDA-1) was the anticoagulant.

Preparation of the RBC membranes was based on the method developed by Dodge *et al.* (8), with the exception that during the lysing procedure the membranes were washed a third time with 10 ideal milliosmolar (imOsm) phosphate buffer, pH 7.4, instead of 20 imOsm.

The adjuvant solutions were prepared by dissolving the appropriate amount of adjuvant in sodium phosphate buffer at 310 imOsm, pH 7.4. The RBC membranes were treated by mixing with the appropriate adjuvant solution. The membranes were then centrifuged and the supernatant was discarded. The membranes were stored overnight at 5°C to allow the adjuvant–membrane interaction to equilibrate prior to thermal analysis by DSC.

Experiments were also performed to determine whether or not the adjuvant interaction with the RBC membranes was reversible. In these experiments, the adjuvant-treated membranes were rewashed twice with adjuvant-free sodium phosphate buffer at 310 imOsm, pH 7.4. During the preparation and treatment of the RBC membranes, the membranes, buffer, and adjuvant solutions were maintained at 5°C.

Six different adjuvants were examined at three different concentrations in a randomized study. They were sodium benzoate (15, 30, 60 m*M*), sodium 3,5-dihydroxybenzoate (10, 20, 40 m*M*), sodium 5-methoxysalicylate (7.5, 15, 30

[1] Departments of Pharmaceutics and Chemistry, Philadelphia College of Pharmacy and Science, Philadelphia, Pennsylvania 19104.
[2] To whom correspondence should be addressed at Philadelphia College of Pharmacy and Science, 43rd Street and Woodland Avenue, Philadelphia, Pennsylvania 19104.

0724-8741/88/1100-0729$06.00/0

m*M*), sodium 3,5-diiodosalicylate (0.25, 0.5, 1 m*M*), diethyl maleate (5, 10, 20 m*M*), and sodium lauryl sulfate (0.06, 0.13, 0.25 m*M*).

Differential Scanning Calorimetric Analysis of RBC Membranes. Calorimetric scans were conducted on a Microcal Model MC-1 differential scanning calorimeter (MicroCal Inc., Amherst, Mass.) using matched 1-ml cells (9). The DSC data were recorded simultaneously on an $X-Y$ recorder Model 200 (Houston Instruments, Austin, Tex.) and, in digitized form, on a TRS-80 Model 4P microcomputer (Radio Shack, Fort Worth, Tex.) utilizing an analog-to-digital converter (D & A Research, Satellite Beach, Fla.). A heating rate of 1°C/min was used. The weight of each sample was determined to +0.02 mg for specific heat capacities computations.

The digitized DSC data were converted into MS-DOS format (Microsoft Corp., Bellevue, Wash.) by transferring the data through a commercially available program, Supercross/XT (Powersoft Products, Dallas, Tex.).

Nonlinear Regression Analysis of Calorimetric Data. A function composed of seven Gaussian terms with a linear baseline was used to model the DSC scans of the RBC membranes (10). Each Gaussian term was composed of parameters controlling its amplitude, width, and transition temperature.

The nonlinear regression program PCNONLIN (Statistical Consultants, Inc., Lexington, Ky.) was used to fit the digitized DSC scans with this mathematical model on an IBM-PC microcomputer.

RESULTS

The peaks observed in a DSC scan of RBC membranes are due to localized endothermic (structural) transitions involving membrane protein and/or lipid. The area under each peak is proportional to the enthalpy absorbed during the transition. Both transition temperatures and enthalpies associated with several of these transitions were affected differently by the various adjuvants used in this study. The concentration ranges over which these changes took place also differed from one adjuvant to the next.

Figure 1 illustrates the nonlinear regression fit of the calorimetric data to the mathematical model for untreated RBC membranes. The points on the graph are the experimental data and the curve drawn through them is the mathematically fitted regression. The curves and straight line below the calorimetric scan are the resolved transitions and rising baseline, respectively. The transitions labeled A, B_1, B_2, C, and D follow the initial nomenclature by Brandts *et al.* (11,12). The A_o and H transitions are new to this study (see Discussion). Figures 2 and 3 show the mean fitted curves for the highest concentration of each adjuvant used in this study.

Treatment of the RBC membranes with BNZ shows little change from the control. Slight downward temperature shifts in the A, B_1, and B_2 transitions are observed, with minor upward shifts in the C transition. Both DHB and MSA follow the same trend as BNZ but with a slightly more pronounced effect. The A, B_1, and B_2 transitions shift downward as the C transition shifts upward.

For DEM- and DIS-treated RBC membranes, the A and C transition temperatures shifted noticeably downward. The C transition shifted by as much as four degrees.

SLS produced the most drastic changes. With the exception of the A_o and A transitions, all the transition temperatures were shifted downward. The most noticeable shift was the 6°C decrease in the C transition.

Comparisons among adjuvants were made with respect to the concentration that was needed to shift the transition temperature 1°C (Table I). For the A and B_1 transitions, the potency of the adjuvant increased in the following manner: BNZ < DHB < MSA < DEM < DIS < SLS. A similar relationship also held for the B_2 and C transitions: (BNZ, DHB, MSA) < DEM < DIS < SLS. Concentrations of the adjuvants, BNZ, DHB, and MSA, were poorly correlated with transition temperature.

Additional experiments were carried out in which the reversibility of the calorimetric effect produced by the adju-

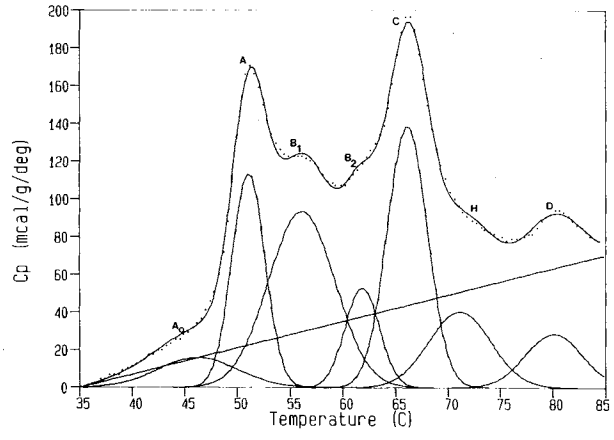

Fig. 1. DSC scan of untreated RBC membranes at 310 imOsm sodium phosphate, pH 7.4 (single control scan). The points represent the experimental data. The line drawn through the points is the nonlinear regressional fit of the data to the equation. The resolved transitions and linear baseline are shown below the experimental and calculated data. The letters identify each transition.

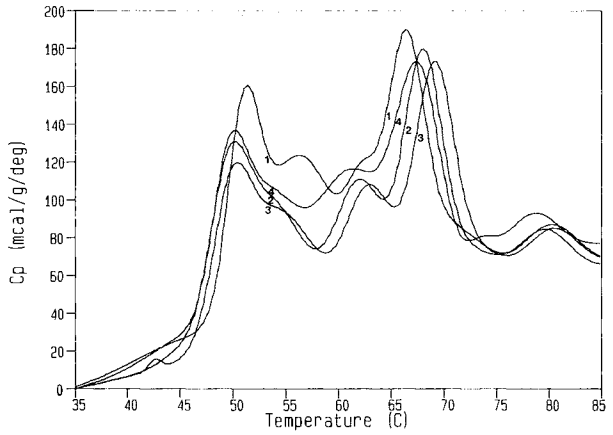

Fig. 2. DSC scans of adjuvant-treated RBC membranes at 310 imOsm sodium phosphate, pH 7.4 (average of three experiments). The key to the scans is as follows: (1) control (untreated); (2) sodium benzoate at 60 m*M*; (3) sodium 3,5-dihydroxybenzoate at 40 m*M*; and (4) sodium 5-methoxysalicylate at 30 mM.

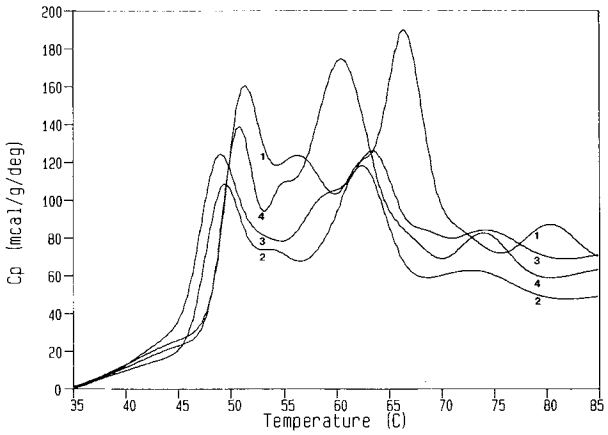

Fig. 3. DSC scans of adjuvant-treated RBC membranes at 310 imOsm sodium phosphate, pH 7.4 (average of three experiments). The key to the scans is as follows: (1) control (untreated); (2) diethyl maleate at 20 m*M*; (3) sodium 3,5-diiodosalicylate at 1 m*M*; and (4) sodium lauryl sulfate at 0.25 m*M*.

vants was determined. After treating membranes with the highest adjuvant concentration used in these studies and after repeated washings with adjuvant-free buffer, DSC scans were run. A reversible adjuvant effect was determined by comparing the DSC scan of these membranes with that of a control (untreated) membrane sample. Figure 4 shows that, with the exception of sodium laurel sulfate, all of the adjuvant treatments return their adjuvant-induced shifts back to baseline.

DISCUSSION

Modeling of RBC Membrane Calorimetric Data. At the present time, there are no theoretical models for the heat capacity curve of irreversible RBC membrane transitions. Statistical thermodynamic models are valid only for reversible transitions (13,14).

The choice of the widely used Gaussian function for our model was based on the observation that calorimetric transitions of RBC membranes appear symmetric if transitions are separated by more than 5°C. The assumption was made that any asymmetries that occurred were the result of overlapping transitions. The work of Orlov *et al.* (15) supports this assumption, and Gaussian resolution of asymmetries in this study is consistent with this assumption.

Seven Gaussian terms were required to fit the RBC calorimetric data to the mathematical model. Five of these correspond to those originally described by Brandts *et al.* (11,12). The addition of two more transitions, the A and H, were found to improve the goodness of fit significantly as measured by the sum of squared residuals and standard deviations of fitted parameters. The existance of the A transition near 42°C was postulated by Brandts *et al.* (11) and others (15,16). The H transition near 70°C appears to be due to residual binding of hemoglobin to the membrane.

In the model, a rising linear baseline term was added to correct for differences in baseline heat capacities between native and denatured species. When multiple, overlapping transitions occur, the heat capacity was assumed to change in a linear fashion between initial native species and final denatured species.

Adjuvant Effects on the A Transition. A negative linear correlation was found between the transition tempearature of the A transition and the concentration of adjuvant required to shift the A transition, as shown in Table I. The adjuvant-induced shifts in the A transition also showed rank

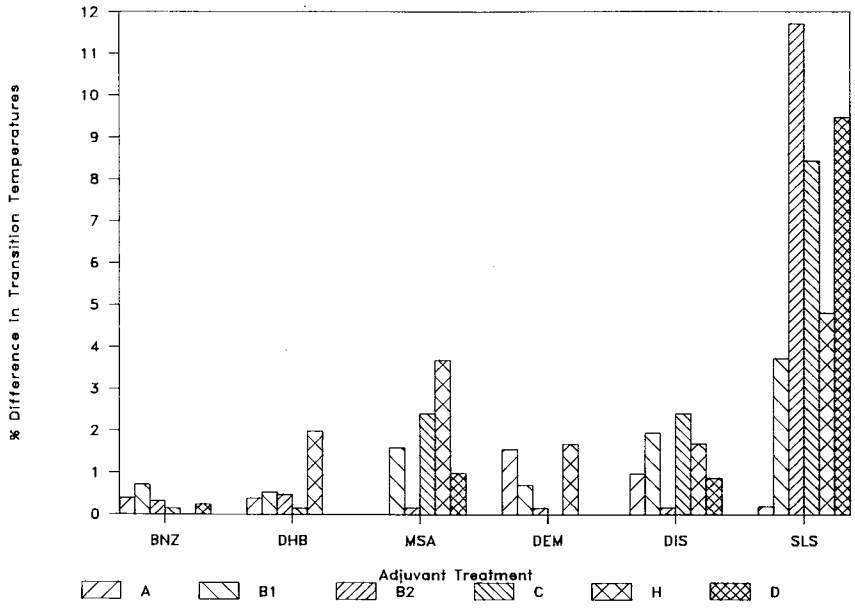

Fig. 4. Transition temperature reversibility. Percentage difference in the transition temperatures between control and washed RBC membranes which were previously treated with adjuvants at the highest concentration used in this study. The letters correspond to the transitions, and the abbreviations represent the adjuvants as follows: BNZ, sodium benzoate; DHB, sodium 3,5-dihydroxybenzoate; MSA, sodium 5-methoxysalicylate; DEM, diethyl maleate; DIS, sodium 3,5-diiodosalicylate; and SLS, sodium lauryl sulfate.

Table I. Concentration of Adjuvant Required to Shift the Transition Temperature 1°C[a]

Adjuvant[b]	Transition A	B_1	B_2	C
BNZ	96.70	37.72	41.31	98.50
	−0.97[c]	−0.84	−0.99	−0.38
DHB	54.45	224.21	191.30	31.79
	−0.98	−0.89	−0.97	−0.34
MSA	21.24	11.00	18.47	39.06
	−0.99	−0.99	−0.97	0.34
DEM	11.31	9.55	4.36	4.77
	−0.98	−0.94	−0.93	−1.00
DIS	0.46	0.33	0.29	0.33
	−0.99	−0.89	−0.99	−0.87
SLS	0.81	0.14	0.13	0.05
	−0.97	−0.94	−0.95	−0.98

[a] Units of adjuvant concentration are m*M*. The concentrations were obtained from the linear relationship between transition temperature and adjuvant concentration. The transition temperatures were adjusted for osmotic differences.

[b] BNZ, Na benzoate; DHB, Na 3,5-dihydroxybenzoate; MSA, Na 5-methoxysalicylate; DEM, diethyl maleate; DIS, Na 3,5-diiodosalicylate; SLS, Na lauryl sulfate.

[c] The correlation of linearity, *r*, was also obtained from the transition temperature vs concentration relationship.

correlation with the adjuvant GI absorption promotion potency (3,5). This transition is associated with the partial unfolding of spectrin, the major protein of the RBC cytoskeleton (12,17). In the RBC cytoskeleton, spectrin binds with both actin and ankyrin (18). Actin is also bound to protein band 4.1, and the spectrin–actin–band 4.1 complex results in the cytoskeleton meshwork of the RBC (18). Spectrin-bound ankyrin (bands 2.1, 2.2, 2.3) also binds to the integral membrane protein band 3 to form a bridge between the spectrin–actin–band 4.1 cytoskeletal complex and the membrane lipid bilayer (18).

A homologue to RBC spectrin is found in intestinal epithelial cells (21). This protein holds actin filaments in place to give the microvilli of the intestine their structure (18). Since intestinal epithelial cells contain a spectrin-like protein (18–20), it is tempting to speculate that adjuvant might physically interact with this protein like they do with RBC spectrin. An experiment which could investigate this possiblity further would involve isolating both the RBC spectrin and the intestinal homologue of spectrin and observing their respective protein–adjuvant interactions calorimetrically.

Adjuvant Effects on the C Transition. In contrast to the A transition, the shifts in the C transition relative to control did not correlate as well with adjuvant concentrations. This lack of correlation was unexpected since the C transition represents the membrane-spanning portion of band 3 protein and has some lipid involvement (11,12). The protein is also responsible for anion transport in the RBC membrane (21,22). On the other hand, C transition downward shifts did correlate with the more potent adjuvants, DEM, DIS, and SLS. These adjuvants are much more lipophilic than BNZ, DHB, and MSA, the other three adjuvants studied. Since the C transition involves protein–lipid interactions (12,23,24), alterations in membrane fluidity might play a role in adjuvant activity. Potent adjuvants may decrease lipid structure in biological membranes and thus promote permeability changes.

Reversibility Experiments. One of the potential advantages of studying adjuvant–RBC membrane interactons with DSC is the capability of monitoring "reversible" interactions. Reversibility was defined as a return of adjuvant-induced transition temperature shifts to the original temperature after adjuvant treatment and subsequent washings with adjuvant-free buffer. All of the adjuvants at the concentrations tested, with one exception, were reversible (Fig. 4). Furthermore, the scans of the reversibility experiments were virtually superimposable with those of the control.

The adjuvant which caused irreversible changes at all concentrations was SLS. This was expected because of the membrane solubilizing properties of SLS. At sufficiently high concentrations, surfactants are capable of disturbing the membrane structure by dissolving the integral protein components of the membrane and increasing the fluidity of the membrane lipid bilayer (25). In addition, GI studies showed that SLS produced irreversible damage to the GI membrane and a continued absorption enhancing effect after the adjuvant was removed (7). In summary, we believe that DSC may provide a rapid method for monitoring the ability of different adjuvants to interact with membranes and for assessing the reversibility or irreversibility of this interaction. The method may have only limited utility, however, in searching for new absorption promoters or for extracting new information regarding their mechanisms of action.

ACKNOWLEDGMENT

The work reported here is based wholly upon research performed in partial fulfillment of the requirements for the degree of doctor of philosophy in pharmaceutics at the Philadelphia College of Pharmacy and Science.

REFERENCES

1. T. Nishihata, J. H. Rytting, A. Kamada, and T. Higuchi. *Diabetes* 30:1065–1067 (1981).
2. S. Yoshioka, L. Caldwell, and T. Higuchi. *J. Pharm. Sci.* 71:593–594 (1982).
3. T. Hishihata, J. H. Rytting, and T. Higuchi. *J. Pharm. Sci.* 71:865–868 (1982).
4. T. Nishihata, C. Lee, B. T. Nghiem, and T. Higuchi. *J. Pharm. Sci.* 73:1523–1525 (1984).
5. T. Nishihata, H. Takahata, and A. Kamada. *Pharm. Res.* 2:307–309 (1985).
6. H. Kajii, T. Horie, M. Hayashi, and S. Awazu. *J. Pharm. Sci.* 75:475–478 (1986).
7. T. Nishihata, J. H. Rytting, and T. Higuchi. *J. Pharm. Sci.* 70:71–75 (1981).
8. J. T. Dodge, C. Mitchell, and D. J. Hanahan. *Arch. Biochem. Biophys.* 100:119–130 (1963).
9. S. R. Davio and P. S. Low. *Biochemistry.* 21:3585–3593 (1982).
10. J. Holinej. Ph.D. thesis, Philadelphia College of Pharmacy and Science, Philadelphia, 1988.
11. J. F. Brandts, R. D. Taverna, E. Sadasivan, and K. A. Lysko. *Biochim. Biophys. Acta.* 512:566–578 (1978).
12. J. F. Brandts, L. Erickson, K. A. Lysko, T. Schwartz, and R. D. Taverna. *Biochemistry* 16:3450–3454 (1977).
13. E. Freire and R. L. Biltonen. *Biopolymers* 17:463–479 (1978).
14. E. Freire and R. L. Biltonen. *Biopolymers* 17:497–510 (1978).
15. S. N. Orlov, V. L. Shnyrov, G. G. Zhadan, I. S. Litvinov, P. V. Gulak, N. I. Poludin, and Y. V. Postnov. *Bull. Exp. Biol. Med.* 96:55–56 (1983).

16. M. Minetti, M. Ceccarini, A. M. Di Stasi, and V. T. Marchesi. *J. Cell. Biochem.* **30**:361–370 (1986).
17. J. Palek. *Semin. Hematol.* **20**:139–242 (1983).
18. A. B. Fulton. *The Cytoskeleton: Cellular Architecture and Choreography,* Chapman and Hall, New York, 1984, pp. 24–26, 40–41.
19. V. Bennett. *Annu. Rev. Biochem.* **54**:273–304 (1985).
20. J. R. Glenney and P. Glenney. *Eur. J. Biochem.* **144**:529–539 (1984).
21. J. W. Snow, J. F. Brandts, and P. S. Low. *Biochim. Biophys. Acta* **512**:579–591 (1978).
22. J. W. Snow, J. Vincentelli, and J. F. Brandts. *Biochim. Biophys. Acta.* **642**:418–428 (1981).
23. K. S. Krishnan and J. F. Brandts. *Mol. Pharmacol.* **16**:181–188 (1979).
24. S. R. Davio and P. S. Low. *Arch. Biochem. Biophys.* **218**:421–428 (1982).
25. T. Kondo. *Adv. Colloid Interface Sci.* **6**:139–172 (1976).