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THERMODYNAMIC CHARACTERISTICS OF A HUMAN INSULIN-DEAE-DEXTRAN COMPLEX ENTRAPPED IN LIPOSOMES

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ABSTRACT

Binding parameters were measured for a study of in vitro complexation interaction of human insulin with diethylaminoethyl (DEAE) dextran polymer by a diaultrafiltration (rapid dialysis). The complex was found to have appearent affinity constants of 22.9, 3.2 and 1.5 M^{-1} at 25° C, 37° C and 45° C respectively in 0.067 M phosphate buffer at pH 7.4. The complexation reaction was found to be exothermic with an enthalpy change (ΔH) of 26 kcal/mole. Differential scanning calorimetry (DSC) was used to obtain the

temperatures and enthalpies for the insulin denaturation process in the presence of the DEAE-dextran polymer and liposomal membranes. The human insulin-DEAE-dextran complex appears to have a higher denaturation temperature with a higher denaturation enthalpy than the free human insulin in 0.067 M phosphate buffer at pH 7.4 solution. The uncomplexed human insulin entrapped in dimyristoyl phosphatidylcholine (DMPC) liposomes and neutral liposomes (DMPC/cholesterol = 1:1) did not differ significantly in temperature and enthalpy of denaturation from the unentrapped protein. Conversely, the complexed insulin entrapped in liposomes indicated an unchanged denaturation temperature but a lower denaturation enthalpy compared to the complexed human insulin not entrapped. The complexed human insulin entrapped in positively charged liposomes prepared from DMPC/cholesterol/stearylamine in the molar ratio of 7:2:1 showed a slight change in denaturation temperatures and lower denaturation enthalpies than the complexed protein not entrapped whereas the uncomplexed human insulin gave three peaks of different denaturation temperatures and enthalpies.

INTRODUCTION

Recently, many types of insulin have been developed, for example, insulin from a variety of animals, insulin conjugated with a polymer (1), and a human insulin which can be obtained either from enzymatic modification of insulin from pork pancreas or from biotechnology using recombinant DNA. Although human insulin has the advantage over other insulins in being less immunogenic so that lower incidences of resistance can be developed (2-10), the only effective route of administration as with other insulins is by injection. Injection is known to be an inconvenient and painful route especially for the diabetic child who requires a daily injection.

Several modifications of human insulin have been performed in order to obtain a long acting or an oral delivery system. However, the work involving the structure of the hormone may affect its activity, since the insulin was chemically modified by being covalently bonded to many polymers (1, 11, 12). Liposome-entrapped insulin could be used to prolong the hypoglycemic action of insulin (13) and be administered orally (14-22). Also, the conjugation of insulin to the surface of the liposome allows for the potential use of insulin as a transporting molecule to deliver the liposome-entrapped drug to insulin receptor rich tissues (23-25). In our laboratory, human insulin has been complexed with DEAE-dextran polymer and entrapped in liposomes for the purpose of developing an oral insulin (26, 27). The proper conditions of the complexation are 37° C, in pH 7.4 of 0.067 M phosphate buffer, yielding the maximum fraction bound of 0.02 (26). The highest percentage of entrapment of the complexed and the uncomplexed hormone was demonstrated in positively charged liposome systems (27). Since the complexation is obtained simply by mixing the reacting solutions and the complex is formed mainly by electrostatic interactions, the activity of the human insulin is not affected by the modification.

For oral administration, the complexation of human insulin to DEAE-dextran or the entrapment of the complex in liposomes may present a more stable system than the uncomplexed and the unentrapped human insulin. It is thus essential to study the interactions of human insulin with DEAE-dextran polymer as well as with the liposomal membranes. In the present study, thermodynamic characteristics of the interactions are studied by a diaultrafiltration and a thermal analysis method. Methods of dialysis and ultrafiltration have been sucessfully used to study the binding of small molecules to macromolecules (28-30).

Lipid-protein interactions have been studied intensively over the past years using a variety of techniques (31). Spectroscopic techniques can give information on the dynamic and conformational

properties of these molecules (32, 33). Calorimetric measurements can be used to gain insight into their thermodynamic properties (34). Many calorimetric measurements have been made to determine the thermodynamic parameters for the denaturation process of protein such as the reduction of insulin (35). Thermal techniques, in particular, differential scanning calorimetry (DSC) have become standard for studying the thermally-induced transition of biological membranes and bilayers of model lipids from an ordered, crystalline-like state at low temperature (gel phase) to a more disordered liquid crystalline state at high temperature (34, 36). Biological membranes are composed of lipids, proteins and some polysaccharides. The DSC technique is useful for studying the interactions of the complexation of human insulin with DEAE-dextran and the entrapment of the complex in liposomes. The result of the study of thermodynamic characteristics of a human insulin-DEAE-dextran complex entrapped in liposomes can not only be applied to an evaluation of the complex and the complex in liposomes, but also to the understanding of the fate of this developed delivery system in in vivo experiments as well.

MATERIALS

Human insulin (Hoechst AG, D-6230 Frankfurt/M. 80), DEAE-dextran (M.W. 500,000, Pharmacia GmbH, D-7800 Freiburg), Dimyristoyl phosphatidylcholine, DMPC (Lipoid KG, D-6700 Ludwigshafen 24), cholesterol (Croda GmbH, 4054 Nettetal 2-Herrenpfad) and stearylamine (Sigma Chemical Company, St. Louis, Mo 63178, USA) were used. All other chemicals were of reagent grade and used as obtained.

METHOD

Diaultrafiltration (Ultradialysis, Rapid Dialysis) Study

Figure 1 is a schematic diagram of the rapid dialysis apparatus. It provides for the introduction of 1 to 50 ml of the experimental solution into an Amicon ultrafiltration cell which is then forced to filter through an ultrafiltration membrane (Diaflo^{R}, molecular weight cut off 30,000, Amicon GmbH, D-5810 Witten) under pressure controlled by a N_2 gas tank. At constant pressure, the volume of the sample solution in the cell is always constant and is supplied by a pressurized buffer reservior.

In the experiment, different human insulin-DEAE-dextran complex solution concentrations prepared at 37° C, 1 hour in 0.067 M phosphate buffer pH 7.4, were placed into the cell, which was well-stirred and thermostatted at 25° C, 37° C and 45° C by a controlled temperature bath at pressure of 1 bar. The ultrafiltrate samples at a flow rate of about 3 to 4 min/ml were collected at 10 to 15 min intervals by an automatic fraction collector (Foxy, Lincoln, Nebraska, USA). The fractions were collected until equilibrium was reached, i.e., there was no change in the concentration of human insulin in the fractions collected. Human insulin, which has the molecular weight of about 6,000, was forced through the membrane rapidly; the complex and the DEAE-dextran polymer, which has an average molecular weight of approximately 500,000, are retained in the cell. At equilibrium, the uncomplexed drug concentration (D_f) is equal on both sides of the membrane. Drug concentrations in the sample collected were determined spectrophotometrically at 276 nm. The concentration of unbound human insulin (D_f) was calculated from summation of amount human insulin present in the fractions collected. After dialysis, the concentration of human insulin-DEAE-dextran complex (DP) was calculated for four known different initial drug concentrations in the cell (D_c) using the relation :

$$(DP) = (D_c) - 2(D_f)$$

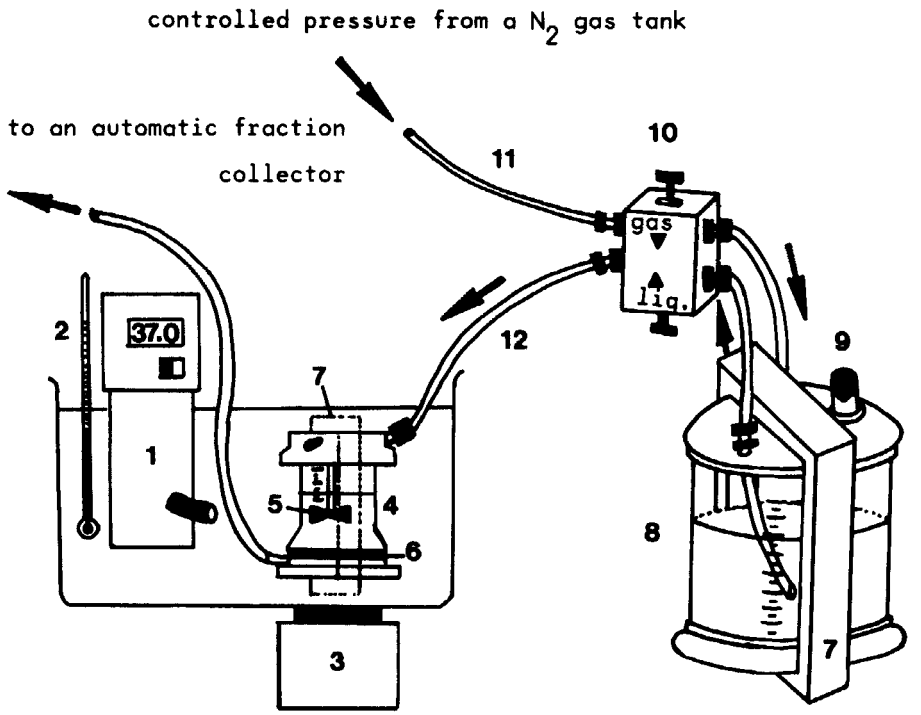

FIGURE 1

Schematic Representation of the Amicon Rapid Dialysis Apparatus. 1. Heater 2. Thermometer 3. Magnetic Stirrer 4. Amicon Ultrafiltration Cell 5. Stirrer 6. Ultrafiltration Membrane (Diaflo^R) 7. Strap Assembly 8. Pressurized Buffer Reservoir 9. Pressure Relief Valve Assembly 10. Selector 11. Gas Inlet 12. Liquid Outlet.

The data were then analyzed according to the method of Klotz (37) which uses a plot of the equation :

$$1/r = (1/nK)(1/D_f) + 1/n$$

K, the apparent affinity constant, for the drug-dextran polymer complex and n, the number of drug binding sites (in moles of drug) per reference "unit" of polymer, were determined; r is the number of moles of the complexed human insulin per "reference unit" of dextran. DEAE-dextran has 3 glucose units with 1 affiliated diethylaminoethyl group with a unit weight of 619.5. The method

assumes a single class of independent binding sites of insulin on the DEAE-dextran molecules. From a van't Hoff plot of log K versus 1/T, ΔH (the enthalpy change for the interaction) was obtained from the slope: $\Delta H = -\text{slope}(2.303)R$, where R is a gas constant = 1.987 cal/mole degree. ΔG and ΔS were computed from the relationships : $\Delta G = -RT(\ln K)$ and $\Delta S = (\Delta H - \Delta G)/T$, respectively.

Differential Scanning Calorimetry (DSC) Study

The temperatures (T_m's) and enthalpies (ΔH_{cal}'s) of denaturation were determined using a differential scanning calorimeter (DASM-4M, Mashpriborintorg, Moscow, USSR) at heating rates of 1° K/min. Some samples were also separately run by a DASM-1M differential scanning calorimeter (Mashpriborintorg, Moscow, USSR) at heating rate of 0.75° C/min. $\Delta H_{v.H.}$ (the van' t Hoff transition enthalpy) is estimated directly from the half height width ($\Delta T_{1/2}$) of the calorimetric curve by using the equation (38) :

$$\Delta H_{v.H.}\ (\text{cal/mol}) \simeq (6.9\ T_m^2)/\Delta T_{1/2}$$

The cooperative unit size (c.u.) is estimated from the ratio $\Delta H_{v.H.}/\Delta H_{cal}$; the value falls within 10% of the real value.

RESULTS AND DISCUSSION

Analysis of Diaultrafiltration Study

The observed n and K values at different temperatures were obtained from the Klotz plot of 1/r versus $1/D_f$ (Table 1).

At pH 7.4, human insulin has a net charge number of -2 and a pKa of about 5.2. The DEAE-dextran used in this study has 3 glucose units and 1 positively charged diethylaminoethyl group per 1 reference unit. At physiological pH, one might expect that the

TABLE 1

Data for the Klotz Plot at Different temperatures

Temperature (° C)	1/r ref.unit/moles	$1/(D_f)$, X 10^3 M^{-1}
25	265.96	21.35
	486.97	52.96
	336.27	25.44
	412.08	38.18
37	331.50	9.26
	341.72	12.86
	480.05	18.42
	587.96	19.42
45	381.92	9.39
	419.72	7.44
	441.99	10.33
	468.57	14.95

TABLE 2

Binding Parameters of the Complexation of Human Insulin to DEAE-dextran from a Diaultrafiltration Study

Temperature (° C)	Numbers of Binding Sites (n, X 10^{-5} moles insulin/ref.unit)	Apparent Affinity Constant K (M^{-1})
25	668	22.9
37	1286	3.2
45	2019	1.5

primary interaction between human insulin and the polymer would be electrostatic. The predicted stoichiometry for a simple charge-charge interaction would be 2 reference units for each human insulin molecule. Thus, n, which has been defined as the number of independent drug binding sites of the same class per reference unit of DEAE-dextran polymer, should be equal to 0.5 for such an interaction. However, for each system, the observed n values obtained were much less than 0.5 (Table 2). This finding agrees with those of our previous experiments indicating that the fraction of insulin bound as a complex is very low (26). Steric hindrance, induced by the quaternary and tertiary structures of human insulin and the dextran molecules, appears to significantly affect binding by precluding the potential charge-charge interaction. Moreover, X-ray crystallographic analysis has revealed the three-dimensional structure of insulin at a resolution of 1.9 Å. Insulin has a compact three-dimensional structure, with only the amino acids and the carboxyl termini of the B-chain extending beyond the rest of the protein. The A-chain is nested between the extended arms of the B-chain. A non-polar core is formed by burried aliphatic side chains from both chains (39). Thus, only the negative carboxyl group of the B-chain can interact with the polymer.

From Table 2, it can be seen that as the temperature was increased from 25° C to 45° C, n increased from 668 X 10^{-5} to 2019 X 10^{-5} whereas the apparent affinity constants decreased from 22.9 to 1.5. Apparently, more molecules become accessible as the temperature increases. The van' t Hoff plot of log K versus 1/T was constructed as shown in figure 2. The Gibb's free energy (ΔG) and the entropy change (ΔS) were calculated (Table 3). The apparent affinity constants decreased with increasing temperature, demonstrating that the binding process was exothermic. The entropy change on binding was negative due to the increased ordering of the system induced by the complexation process.

Analysis of the DSC Study

Table 4 gives the results of the DSC study of human insulin

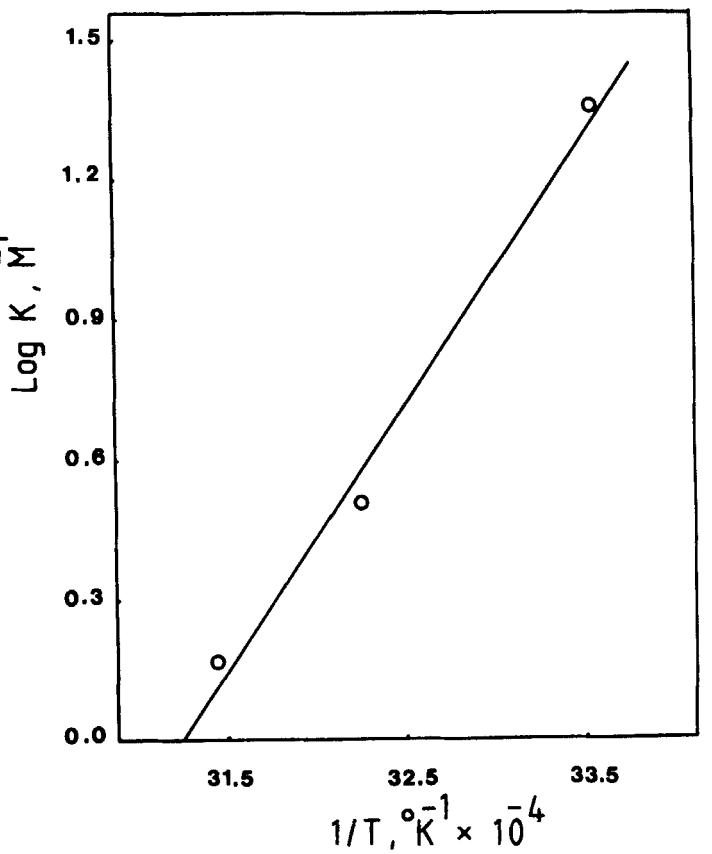

FIGURE 2

The van't Hoff Plot of Log K versus 1/T for Human Insulin-DEAE-Dextran Complex in 0.067 M Phosphate Buffer at pH 7.4.

TABLE 3

Thermodynamic Parameters of Interaction of Human Insulin with DEAE-dextran from a Diaultrafiltration Study

Temperature (° C)	ΔH kcal/mole	ΔG kcal/mole	ΔS cal/mole ° K
25	-26.27	-1.85	-81.95
37	-26.27	-0.72	-82.44
45	-26.27	-0.25	-81.82

in different experimental systems. The human insulin-DEAE-dextran complex solution was prepared by simple mixing at 37° C for 1 hour (26). Liposome samples were prepared by a chloroform film method in which the lipid mixture was dissolved in chloroform. A thin film was obtained after drying in vacuum and a drug or drug-complex solution was added to produce a liposome preparation (27). The human insulin concentrations were determined by a Lowry method with protein precipitation. Protein recovery in the presence of lipids and dextran polymers was about 90% to 95%.

The denature temperature (T_m) of human insulin in 0.067 M phosphate buffer at pH 7.4 (Sample No. 1) as determined by a DASM-1M calorimeter, showed a slightly higher value than that determined by the DASM-4M calorimeter which is a newer version of the DASM-1M. In contrast, the denature enthalpy (ΔH_{cal}) and the cooperative unit size (c.u.) seemed to stay almost invariant. In the case of true first-order transition, the c.u. value approaches infinity while this value is unity when there is a completely noncooperative equilibrium (34). Since human insulin showed the value of $\Delta H_{v.H.}$ which is about 2 (i.e. a c.u. value) more than the ΔH_{cal}, there may have some degree of cooperativity between the human insulin molecules.

When human insulin was complexed with DEAE-dextran polymer (samples No. 2 and 3), both T_m and ΔH_{cal} were higher than the uncomplexed human insulin, possibly indicating that a more thermally stable complex was obtained. Urea, which is known to have a role in unfolding protein chains, appears to have no effects on the thermodynamic parameters of the complexed protein at 0.0042 M concentration (sample No. 2). The c.u. of the complex was also significantly decreased indicating a more non-cooperative equilibrium.

Liposomes prepared from DMPC only (sample No. 4) gave a T_m of about 24.0° C, which is the same main transition temperature of the phospholipid previously reported (34). In neutral blank liposomes (sample No. 7) which has 50 mol% of the cholesterol

TABLE 4

Calorimetric Data from Insulin in Different Experimental Systems

Sample No.	Sample Descriptions	Insulin Conc. (mg/ml)	T_m (° C)	ΔH_{cal} kcal/mol
1	Human Insulin in Phosphate	0.963, 2.11*	63.1	16.9
	Buffer at pH 7.4	1.00*, 1.310*	64.0	15.8
			68.4[a]	16.3[a]
			68.5[a]	16.4[a]
			68.2[a]	17.7[a]
2	Human Insulin-DEAE-Dextran	0.863	70.1[b]	25.7[b]
	Complex with 0.0042 M Urea		70.0[b]	25.9[b]
3	Human Insulin-DEAE-Dextran	1.00, 1.211*	68.0[b]	27.2[b]
	Complex without Urea		71.5[a b]	26.4[a b]
4	DMPC Liposomes	NA	24.0[c]	NA
			24.1[c]	NA
5	Human Insulin/DMPC	1.767	67.2	17.3
	Liposomes		64.0[d]	4.7[d]
6	Human Insulin-DEAE-Dextran	1.853	71.7	12.4
	Complex/DMPC Liposomes		73.6[d]	6.2[d]
			71.8	12.6
7	Neutral Liposomes	NA	NO[c]	NA
8	Human Insulin/Neutral	1.925	68.7	15.9
	Liposomes		63.4[d]	8.9[d]
9	Complexed Human Insulin/	1.950	72.3	11.4
	Neutral Liposomes		72.6[d]	9.0[d]
10	Positively Charged	NA	39.1[c]	NA
	Liposomes			
11	Human Insulin/Positively	0.832*	71.8[a e]	5.1[a e]
	Charged Liposomes		76.2[a f]	-1.3[a f]
			86.1[a g]	10.7[a g]
12	Complexed Human Insulin/	1.900, 0.980*	74.1	18.0
	Positively Charged Liposomes		75.5[d]	8.2[d]
			73.5[a]	16.8[a]

added, the T_m had diappeared. When cholesterol was added to a concentration of 20 mol% in the presence of stearylamine (sample No.10), the T_m of the liposomes was increased to 39° C. Since DMPC has a low transition temperature of about 24° C, addition of cholesterol with the rigid steroid ring system leads to more rigid lipid bilayers. The increase in T_m of DMPC caused by cholesterol has been previously demonstrated (40).

Human insulin entrapped in DMPC and neutral DMPC liposomes (samples No. 5 and 8) showed no difference in T_m, ΔH_{cal} or c.u. compared to the free hormone. This result indicates that there are no specific interactions between human insulin and the liposomal membranes. Nevertheless, when the complexed human insulin was entrapped in the liposome systems (samples No. 6 and 9), although the T_m's were not changed from the complexed hormone not entrapped, the ΔH_{cal} values were about 2 times less than those of the complexed insulin not entrapped. As demonstrated earlier, the

Note: DMPC = Dimyristoyl Phosphatidylcholine. DMPC liposome is composed of DMPC only. Neutral liposome is composed of DMPC/cholesterol in the molar ratio of 1:1. Positively charged liposome is composed of DMPC/ cholesterol / stearylamine in the molar ratio of 7 : 2 : 1. Samples No. 2, 3, 6, 9 and 12 have 25 mg/ml DEAE-dextran and samples No. 4 to 12 have total phospholipid of 5 mg/ml. * are samples which were run with a DASM-1M calorimeter.

a = measured by a DASM-1M calorimeter

b = reference is a 25 mg/ml DEAE-dextran solution

c = T_m of lipid in blank liposomes

d = the second measurement of the above same sample without renewed fillment

e = the first peak of the same sample

f = the second peak of the above same sample

g = the third peak of the above same sample

binding process of human insulin to DEAE-dextran was an exothermic reaction with ΔH of 26 kcal/mole. In the presence of liposomes, ΔH from the binding process may be retained in the system, thereby probably reducing the heat for denaturation of the protein. The decreased ΔH_{cal} values may be also caused by an increased ordering of the complexed protein when it is entrapped in liposomes.

The second measurement without renewed fillment of the same samples (samples with superscripted d) showed that less than 100% of the protein was denatured during the first heating cycle. Less protein was irreversibly denatured when insulin was complexed with the DEAE-dextran polymer, since ΔH_{cal} decreased less at the second heating cycle for the complexed insulin than did the free insulin entrapped in liposomes. Thus, complexation of human insulin to DEAE-dextran polymer may protect the protein against thermal denaturation.

For human insulin and the complexed human insulin entrapped in positively charged liposomes (samples No. 11 and 12), the complexed protein gave the same T_m but a lower ΔH_{cal} than did the complexed insulin not entrapped. This difference may be attributable to the fact that it is only the denaturing of the fraction of the insulin complex which has interactions with the lipids and that the other fraction is denatured at other temperatures.

For the uncomplexed human insulin entrapped in positively charged DMPC liposomes (sample No. 11), three peaks with different denaturation temperatures and enthalpies were observed in calorigrams (Figure 3). The first peak gave about the same T_m as the insulin not entrapped, whereas the second and the third peaks showed higher T_m values. Enthalpies of the first and third peak were endothermic, whereas the second peak was exothermic. The latter appears to be an exothermic reaction followed by an endothermic reaction. The denaturation may proceed as follows : 1) partial unfolding of insulin (first peak, endothermic), 2)

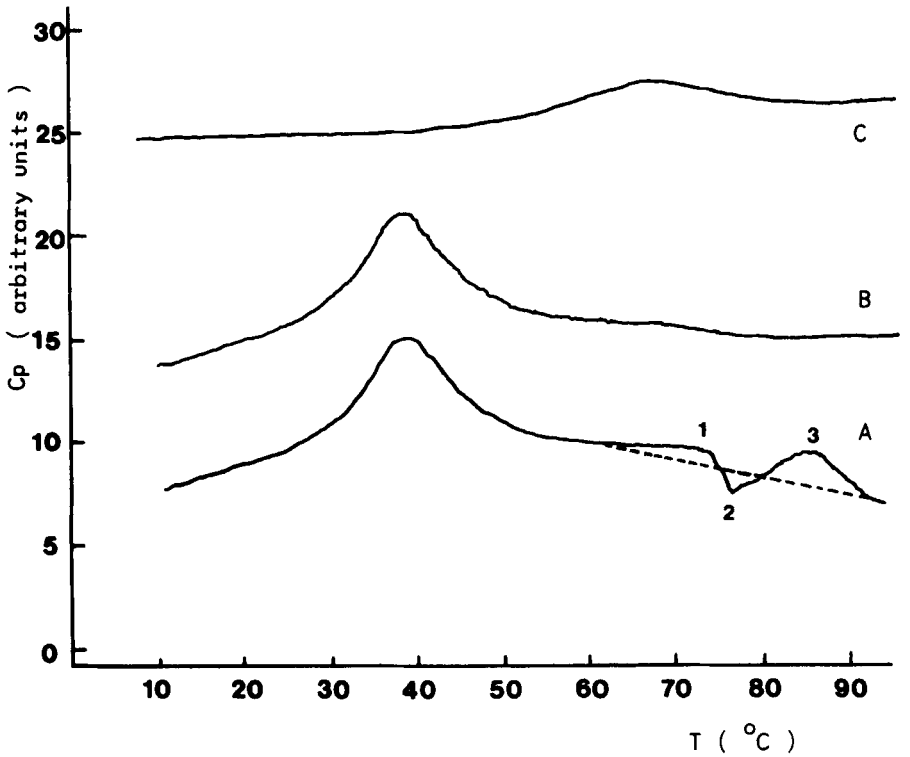

FIGURE 3

Differential Scanning Calorigrams by a DASM-1M Calorimeter of :

A : A Human Insulin (0.832 mg/ml) DMPC Positively Charged Liposome Dispersion (Sample No. 11).

B : A Positively Charged Blank Liposome Dispersion, DMPC/Cholesterol/Stearylamine in the Molar Ratio of 7:2:1 with Total Lipids of 2.5 mg/ml (Sample No. 10).

C : A Human Insulin (1.00 mg/ml) in 0.067 M Phosphate Buffer Solution (Sample No. 1).

electrostatic interaction of insulin with liposomal membranes (second peak, exothermic) and 3) complete denaturation (third peak, endothermic).

In conclusion, the results presented here suggest that the complexed human insulin entrapped in positively charged liposomes may be a better oral insulin system than free insulin entrapped in the same charged liposomes, not only has the highest percentage of the entrapment been previously demonstrated in this liposome system, but as we have shown here the complex is thermally more stable.

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