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THE ENTRAPMENT OF A HUMAN INSULIN-DEAE DEXTRAN COMPLEX IN DIFFERENT COMPOUND LIPOSOMES

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ABSTRACT

Human insulin and a human insulin-DEAE (diethylaminoethyl) dextran complex entrapped in liposomes of various lipid compositions were studied. DMPC (dimyristoyl phosphatidylcholine) and E 200 H (Epikuron 200 H = hydrogenated soy lecithin) were the two phospholipids used and the liposome systems were phospholipid only, phospholipid/cholesterol compound in the molar ratios of 9:1, 8:2 and 7:3 as well as a positively charged liposome system of phospholipid/cholesterol/stearylamine compound in the molar ratio of 7:2:1. Liposomes were prepared by a chloroform film method and passed through a high pressure homogenizer. The percentage of entrapment of insulin in liposomes were determined by ultracentrifugation. Insulin was assayed by a modified Lowry

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method with protein precipitation. Positively charged liposomes gave the highest percentage of entrapment of approximately 76 and 58% with the uncomplexed human insulin system, and approximately 24 and 28% with the complexed human insulin system of the charged DMPC and E 200 H phospholipid liposomes respectively. In all cases, except the positively charged liposome system, the percentage of entrapment of the human insulin-DEAE dextran complex into liposomes was higher about 2-3% in a DMPC liposome system and about 2-8% in a E 200 H liposome system more than that of the uncomplexed insulin. Not only the increase of cholesterol contents in liposomes increased the percentage of entrapment of insulin, but also the homogenization of liposomes appears to increase the percentage of entrapment as well.

The permeability kinetics of human insulin and human insulin-DEAE dextran complex from positively charged liposomes of both DMPC and E 200 H phospholipid was studied at 37° C in 0.067 M phosphate buffer, pH 7.4. All liposome systems appear to show a biphasic first order release kinetics of human insulin. The complexed human insulin system gave a release half life of about 2 times longer than the uncomplexed system in the first phase of DMPC liposomes and in the second phase of E 200 H liposomes. In the first phase of E 200 H liposomes, the complexed system showed a release half life of approximately 3 times longer than the uncomplexed insulin liposome system. However, in the second phase of DMPC liposomes, the release half life of the complexed and uncomplexed human insulin showed no significant differences. The human insulin-DEAE dextran complex liposomes showed about 14% and 7% human insulin bound to the liposome surface while the uncomplexed human insulin showed only 22% and 19% in DMPC and E 200 H liposome systems respectively.

INTRODUCTION

Many attempts have been made in developing orally applicable insulin. The large body of literature concerned has been on the

entrapment of plain insulin in liposomes (1-10). In some cases, liposomes have been showed to protect insulin from degradation in the gastrointestinal tract (10-12). However, the ability of liposomal encapsulation for insulin to facilitate oral absorption seems to be questioned (1, 4, 6, 8 ,9, 13). The main problem seems to be the instability of the liposomes to retain the entrapped hormone in the gastrointestinal system (13). This appears to be caused by the rapid leakage of insulin from the liposomes (1). The complexation of human insulin to DEAE (diethylaminoethyl) dextran has been previously studied in our laboratory. DEAE dextran polymers have also been complexed with methotrexate and this complex can be effectively encapsulated in liposomes (14, 15). The entrapment of DEAE dextran-human insulin complex in liposomes instead of human insulin alone may be a better system in solving the leakage of the entrapped human insulin, and the low efficiency of the hormone in liposomes. The large molecular size of the dextran-insulin complex may effectively preclude the diffusion of insulin from liposomes. It has been reasoned that appropriate changes in the compositions of insulin containing liposomes could render liposomes more resistant to phospholipase and detergent attack in the gut thus allowing a more efficient transport of liposomal insulin into the peripheral (16). Moreover, it has been shown that a considerably reduce of blood glucose level in diabetic rats and in some extent in normal rats can be obtained by using certain semi-synthetic phospholipids, which at the temperature of the body are more resistant to pancreatic phospholipases or to detergents, to prepare liposomes (17,18).

In order to solve the problem of instability of insulin liposomes, modifications may be done on the nature or compositions of lipids to form liposomes or the hormone itself. DMPC (dimyristoyl phosphatidylcholine) and E 200 H (Epikuron 200 H, a hydrogenated soy lecithin) are two phospholipids used in the present study. E 200 H has been characterized by TLC, GC and DSC in our laboratory. It contains 2% of lysolecithin and fatty acids of about 75% stearic acid and 25% palmitic acid. E200H has the transition temperature of approximately 52° C. It is not too expensive and very stable to

oxidation. By the addition of suitable amount of a substance, e.g. cholesterol, the transition enthalpy of E 200 H can be decreased and a more stable liposome can be obtained. DMPC which has a transition temperature of about 20° C is more expensive than E 200 H. Its transition temperature is also rather low. In this study, effects of different lipid compositions on human insulin and the human insulin-DEAE dextran complex entrapment in liposomes have been performed.

MATERIALS

Human insulin and standard human insulin (Hoechst AG, D-6230 Frankfurt/ Main), DEAE-dextran (average M.W. 500,000, Pharmacia GmbH, D-7800 Freiburg), Dimyristoyl phosphatidylcholine (DMPC, Lipoid KG, D-6700 Ludwigshafen 24), Epikuron 200 H (E 200 H, hydrogenated soy lecithin, Lucas Meyer GmbH & Co., D-2000 Hamburg), cholesterol (Croda GmbH, 4054 Nettetal 2-Herrenpfad) and stearylamine (Sigma Chemical Company, St. Louis, Mo 63178, U.S.A.) were used as received. A protein assay kit was purchased from Sigma Diagnostics, P.O.Box 14508, St. Louis, Mo 63178, U.S.A. All other chemicals were of reagent grade and used as obtained.

METHODS

Preparation of Human Insulin Complex

Ten milliliters of human insulin (2mg/ml)-DEAE dextran (0.05 g/ml) complex solution were prepared in 0.067 M phosphate buffer at pH 7.4 and incubated in a controlled temperature water bath at 37° C for one hour.

Preparation of Liposomes

Liposomes were prepared in the first step by the chloroform film method (14, 15, 19, 20). DMPC and E200H were two phospholipids

used in liposome preparations. Liposome systems studied were phospholipid only, phospholipid/cholesterol in the molar ratios of 9:1, 8:2 and 7:3, and phospholipid/cholesterol/stearylamine in a molar ratio of 7:2:1. A total phospholipid mixture weight of 50 mg was dissolved in about 5 ml of chloroform in a flask. Rotary evaporation of the chloroform at 37° C on a Büchi Rotavapor left a thin film on the wall of the flask. An amount of 7.5 ml of human insulin or human insulin-DEAE dextran complex solution was added to the film which was collapsed into multilamellar liposomes by vortexing for 15 minutes at 37° C (DMPC liposome systems) or 53° C (E 200 H liposome systems) with the aid of 0.5 mm glass beads. The multilamellar liposome dispersion was finally passed through a Gaulin MICRON LAB 40 high pressure homogenizer (Hochdruck Homogenisator, Type MICRON LAB 40, APV Gaulin GmbH, D-2400 Lübeck, W. Germany) at 80 MPa for 3 cycles in order to obtain a more uniform unilamellar liposome dispersion. The dispersion was further incubated at 37° C (DMPC liposome systems) or 53° C (E 200 H liposome systems) for 15 minutes.

Entrapment Determination of Human insulin in Liposomes

The resulting liposomes were separated from the unentrapped human insulin and DEAE dextran by centrifugation at 50,000 g, 4° C for 10 minutes on a centrifuge (RC-5 Superspeed Refrigerated Centrifuge, Du Pont de Nemours GmbH, Dieselstrasse 18, D-6350 Nauheim 1). In order to ensure complete removal of the unentrapped human insulin and DEAE dextran, the residues or pellets were twice washed with 7.4 pH phosphate buffer and recentrifuged. Human insulin was assayed by a modified Lowry method with protein precipitation (21, 22) using a protein assay kit and a standard curve constructing from standard human insulin. The amount of human insulin in the pellets and in the supernatant liquid were used to determine the percentage of human insulin entrapped in the liposomes. Only the freshly prepared samples were used in this study and at least three different sample preparations were run for each liposome system.

The Permeability Kinetics Study of the Release of Human Insulin from the Liposome Systems

The positively charged liposomes with lipid compositions of phospholipid/cholesterol/stearylamine in a molar ratio of 7:2:1 were separated from the unentrapped human insulin and DEAE dextran by ultracentrifugation. The pellets were washed twice, centrifuged at 50,000 g, 4° C for 10 minutes and resuspended in pH 7.4 of 0.067 M phosphate buffer. The dispersion was incubated in a shaking water bath at 37 ° C. Two samples of 0.2 ml were taken at each time interval from 0 to 25 hours. For each sample, the human insulin remaining in the liposomes was immediately separated from the free released human insulin in the system by ultracentrifugation. Human insulin in the supernatant liquid and pellets were determined by the modified Lowry method with protein precipitation. The percentage of human insulin remaining in the liposomes at various sampling time intervals was calculated.

RESULTS AND DISCUSSION

The Lowry procedure with protein precipitation has been used for quantitation of human insulin in this study. The recovery of human insulin was found to be more than 96%. The hormone concentration was determined from a calibration curve of standard human insulin.

Entrapment Determination of Human Insulin in Liposomes

The average percentage of entrapment of human insulin (both uncomplexed and complexed systems) in DMPC and E 200 H liposome systems before and after homogenization were shown in Table 1. In all cases, the standard deviation of less than 9% and 7% were obtained in DMPC and E 200 H liposome systems respectively. The percentage of entrapment of human insulin appears to increase with the increased amount of cholesterol. For an uncomplexed and

TABLE 1

Comparison of Mean Percent and Standard Deviation of Entrapment of Human Insulin and Human Insulin-DEAE Dextran Complex in DMPC and E 200 H Liposome Systems of 50 mg Total Lipids

Lipid	Before Homogenization		After Homogenization	
Composition	UNCPX	CPX	UNCPX	CPX
DMPC/Ch/SA				
1:0:0	2.56± 0.20	4.32± 0.24	3.79± 0.21	7.11± 0.43
9:1:0	2.50± 0.34	5.59± 0.39	4.02± 0.23	5.71± 0.50
8:2:0	5.45± 0.45	7.00± 0.16	6.85± 0.34	8.63± 0.40
7:3:0	6.89± 0.50	9.37± 0.65	7.39± 0.52	9.39± 0.62
7:2:1	80.21± 5.78	24.13± 1.09	75.84± 2.43	23.86± 1.05
E 200 H/Ch/SA				
1:0:0	5.52± 0.15	9.58± 0.53	7.10± 0.25	9.68± 0.64
9:1:0	5.04± 0.16	8.78± 0.43	6.96± 0.19	9.18± 0.51
8:2:0	5.26± 0.22	9.67± 0.48	8.10± 0.26	9.90± 0.63
7:3:0	7.14± 0.32	15.12± 0.65	9.68± 0.50	15.60± 0.74
7:2:1	71.42± 4.21	30.05± 1.56	58.21± 2.62	27.91± 1.94

Note: DMPC = Dimyristoyl Phosphatidylcholine, Ch = Cholesterol, SA=Stearylamine, E 200 H = Epikuron 200 H, UNCPX = Uncomplexed Human Insulin Liposome Systems, CPX = Complexed Human Insulin-DEAE Dextran Liposome Systems. All lipid compositions are in molar ratios and the lipid composition in the molar ratio of 7:2:1 is a positively charged liposome system.

homogenized system, the entrapment were approximately 4% to 4% to 7% to 7.5% in DMPC liposome systems and were 7% to 7% to 8% to 10% in E 200 H liposome systems as the cholesterol contents increased stepwise from 0% to 10% to 20% to 30% in molar ratios respectively. Incorporation of cholesterol in liposomes may stabilize liposomes by causing denser phospholipid bilayers and thus reducing

the leakage of the entrapped hormone. Homogenization of liposomes indicated a increase of percentage of entrapment of human insulin in liposomes in some extent since a more uniform unilamellar liposome system was obtained after homogenization. Although a ratio of volume of entrapment to lipid amount in unilamellar liposomes is lesser than in multilamellar liposomes, steric hindrance effects of the hormone and the complex to be entrapped in liposomes may decrease and can be more effectively entrapped in unilamellar liposomes than in multilamellar liposomes. It has been shown in our laboratory that the average size of liposomes of approximately 0.030 μ in diameters by a negative staining electron microscopic method was obtained when homogenized for 3 cycles (23).

In all cases, except the positively charged liposome system, E 200 H liposome systems seem to give a higher percentage of entrapment of insulin than DMPC liposome systems. E 200 H may form a more stable liposome than the DMPC does. E 200 H has a transition temperature of 32° C higher than that of DMPC.

The percentage of entrapment of human insulin in the complexed liposome systems was about 2-3% in DMPC liposome systems and about 2-8% in E 200 H liposome systems higher than in the uncomplexed liposomes. Complexation of human insulin to DEAE dextran appears to facilitate the entrapment of insulin in liposomes. The large molecular size of the complex may effectively preclude the diffusion of insulin from liposomes.

Positively charged liposome systems indicated an increase of entrapment of human insulin by incorporation of stearylamine. This agreed with previous works (3-4). This system demonstrated the highest percentage of entrapment of approximately 80 and 72% in the uncomplexed system and approximately 24 and 30% in the complexed system of DMPC and E 200 H liposomes respectively. At pH 7.4, human insulin is expected to have negative charges which can bind to positive charges of stearylamine incorporated in liposomes thereby enhancing the entrapment. However, complexation of human insulin with DEAE dextran and/or homogenization of

liposomes does not appear to increase the percentage of entrapment of insulin in these liposomes. This may be due to the possible adsorption of human insulin on the surface of positively charged liposome systems especially in the uncomplexed systems. Homogenization may reduce the adsorption of human insulin on liposome surfaces since unilamellar liposomes may have a higher steric hindrance effect on surface adsorption of human insulin.

The Permeability Kinetics Study of the Release of Human Insulin from the Liposome Systems

It has been suggested a two-compartment first order kinetic model can be used to describe the diffusion of a drug from a multilamellar liposome system where there is fast diffusion from the outer layer and slower diffusion from the inner layers (24). This model can also be applied to a small or unilamellar liposome system where the surface adsorbed drug and the drug entrapped in the liposomes represent the two compartments (13). There is evidence that adsorption of the materials on liposome surface may occur (10, 25, 26).

The kinetics of human insulin release from human insulin and human insulin-DEAE dextran complex positively charged liposomes of DMPC and E 200 H systems were shown in Figure 1 and 2. All cases appear to have a biphasic first order kinetic release of human insulin from the liposomes. The first rapid release is probably due mainly to the desorption of the adsorbed human insulin on the surface of the liposome and, to a lesser extent, to the diffusion of the entrapped human insulin. It lasted until 2 hours. The second slower release is assumed to be solely from the diffusion of the entrapped hormone through the lipid bilayers. The half lives of human insulin released from both human insulin and human insulin-DEAE dextran complex liposomes were demonstrated in Table 2. The half life for the human insulin-DEAE dextran liposome system was approximately 2 times longer than that of the uncomplexed liposome system in the first phase of DMPC liposomes and in the second phase of E 200 H liposomes. In the first phase of E 200 H liposome

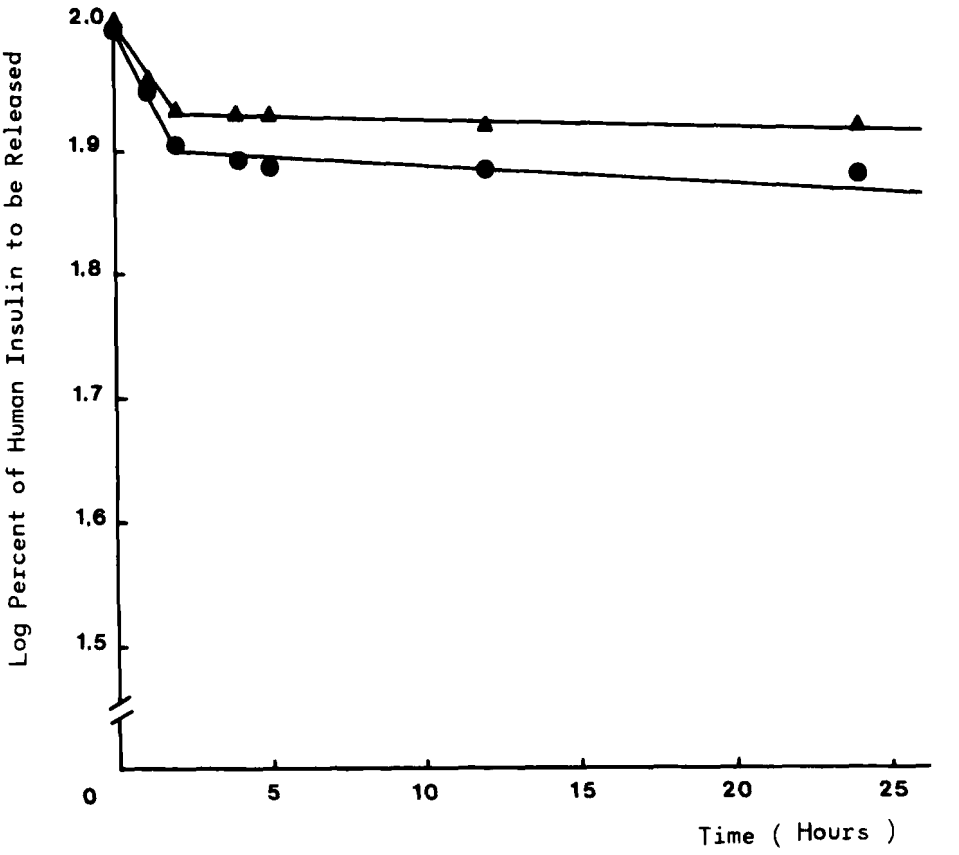

FIGURE 1

The Characteristic Release of Human Insulin from Positively Charged DMPC Liposome Systems in 0.067 M Phosphate Buffer, pH 7.4 at 37° C. ● and ▲ Represent the Human Insulin and the Human Insulin-DEAE Dextran Complex Liposome Systems Respectively.

systems, the complexed insulin gave an approximate release half life of 3 times longer than the uncomplexed system. However, about the same half life was obtained both in the uncomplexed and complexed systems in the second phase of DMPC liposome systems.

As mentioned earlier, there seems to occur adsorption of human insulin on the surface of positively charged liposome systems, and

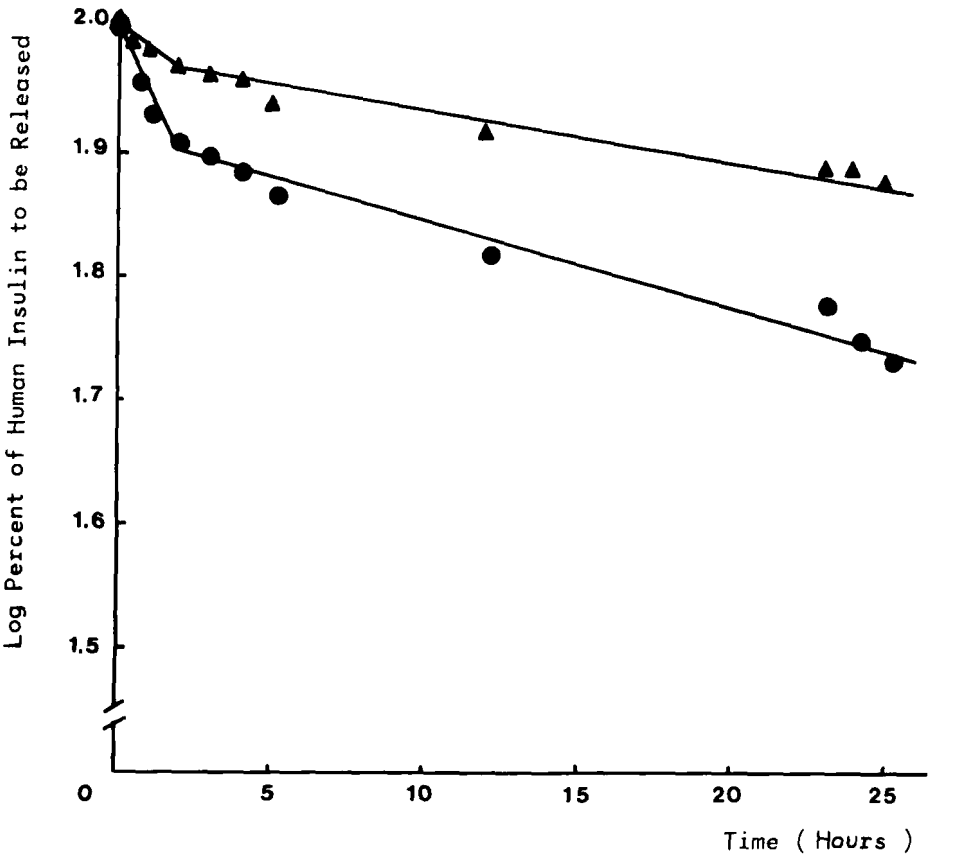

FIGURE 2

The Characteristic Release of Human Insulin from Positively Charged E200H Liposome Systems in 0.067 M Phosphate Buffer, pH 7.4 at 37° C. ● and ▲ Represent the Human Insulin and the Human Insulin-DEAE Dextran Complex Liposome Systems Respectively.

the release of human insulin from liposomes in the first phase is probably due mainly to a surface desorption process and, to a lesser extent, to a diffusion process. The second phase, however, is due primarily to diffusion of entrapped human insulin through the lipid membrane. The percentage of surface bound human insulin vs.

TABLE 2

Comparison of Half Lives for Human Insulin and Human Insulin-DEAE Dextran Complex Released from Positively Charged DMPC and E 200 H Liposome Systems

System	Half-Life (Hours)			
	Insulin Liposome		Complexed Insulin Liposome	
	1st Phase	2nd Phase	1st Phase	2nd Phase
DMPC	13.23	1062.38	22.65	1083.00
E 200 H	17.64	101.25	58.87	191.45

entrapped human insulin can be approximated quite easily with the amount of entrapped human insulin determined by extrapolation of the second phase curve to zero time and surface bound human insulin obtained by difference. The DMPC positively charged liposome system indicated a more surface adsorption of human insulin than the E 200 H liposome especially in the uncomplexed human insulin system as shown in Table 3. The human insulin-DEAE dextran liposomes showed about 14% and 7% human insulin bound to the liposome surface and about 22% and 19% on the uncomplexed human insulin of DMPC and E 200 H liposome systems respectively. In the complexed system, some negative charges of carboxyl groups in human insulin may be neutralized by the positively charged nitrogens of the DEAE dextran polymer thereby reducing the amount of available human insulin for surface adsorption.

TABLE 3

Comparison of the Mean Percent Adsorbed Human Insulin and Entrapped Human Insulin Remaining to be Released for Human Insulin and Human Insulin-DEAE Dextran Complex of Positively Charged DMPC and E 200 H Liposome Systems at Zero Time

System	Insulin Liposomes		Complexed Insulin Liposomes	
	Adsorbed	Entrapped	Adsorbed	Entrapped
DMPC	22.07	77.93	13.73	86.27
E 200 H	18.67	81.33	7.39	92.61

From an analysis of the above data, it appears that modifications done on the nature and compositions of lipid to form liposomes and the complexation of human insulin with DEAE dextran before entrapping in liposomes can have effects on the percentages of entrapment, and the preventation of the leakage of human insulin from liposomes. Although the presence of DEAE dextran does not increase the entrapment efficiency of human insulin in positively charged liposomes, it can significantly increase the half life of the delivery system. The study did not indicate any obvious advantages of positively charged DMPC liposomes over positively charged E 200 H liposomes in entrapping insulin-DEAE dextran complex. It might be better to use E 200 H instead of DMPC in the production of insulin liposomes in the future since E 200 H is not only less expensive than DMPC but it is also very stable to oxidation by hydrogenation of the unsaturated fatty acids. The high transition temperature of E 200 H makes it more physically stable.

Davenport and Schichiri have described that the size of liposomes should be small enough to come in sufficiency close contact with the intestinal mucosal surface to be adsorbed (27, 28). Liposomes prepared by high pressure homogenization method demonstrated in this study were very uniform in unilamellar of approximate average size of 0.030 μ in diameters by a negative staining electron microscopic method. Hence, human insulin or human insulin-DEAE dextran complex positively charged liposomes prepared from E 200 H phospholipid and by a high pressure homogenization method may be one of the promising delivery systems in the development for oral insulin. Nevertheless, a further in vivo study of these systems should be performed to clarify this comment.

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