Interaction between Cholesterol and Calcium Ions in Solution

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Dialysis rates of cholesterol, calcium chloride dihydrate and of their mixture in 90% aqueous dioxane through Visking cellulose membrane were characterized by half-escape times ($t_{1/2}$) of 2.2, 1.0 and 10.5 hours, respectively. Slow dialysis rate observed with the mixture was due to complex formation between four molecules of cholesterol and two molecules of calcium chloride dihydrate, molecular weight 1800 to 2000. The association constant for this complex in 90% aqueous dioxane was estimated to be 3.9×10^{14}. Rates of dialysis obtained with a natural protein membrane were in the reverse order to those obtained with cellulose membrane. Half-escape times for cholesterol, calcium chloride dihydrate and for their mixture were 0.6, 6.7 and 1.4 hours, respectively. Determinations of milliosmolality of the three solutions by freezing point depression indicated that in the mixture there were fewer osmotically active particles than in their separate solutions of the same molarity, also suggesting formation of the complex which was detected by dialysis experiments.

Introduction

Cholesterol and Ca^{2+} ions are known to form under certain conditions a solid molecular complex consisting of two molecules of cholesterol per one Ca^{2+} ion [1]. The complex has distinct physico-chemical properties and is stable in non-polar solvents. In hydroxylic solvents it dissociates slowly into components. Cholesterol appears to be coordinated to Ca^{2+} *via* its hydroxyl group because no complex is formed with acetylated cholesterol or with cholestenone. Furthermore, the hydroxyl group has to be in β configuration since no complex is formed with epicholesterol in which it is in α configuration.

The biological significance of cholesterol-Ca^{2+} ion interaction is presently unknown, but it might be of importance in the development of arteriosclerosis [2]. It is generally believed that low-density lipoproteins of plasma can penetrate endothelial layer of artery and deposit cholesterol and its esters within the intima and media of the arterial wall. The arterial intima also contains numerous elastin fibers and other macromolecules which are able to accumulate Ca^{2+} ions. The elastin fibers are composed of α-elastin, a protein whose amino acid composition is quite unusual. About 33% are glycine residues, 60% are hydrophobic amino acid residues and the

remaining 7% are other amino acids. The high content of hydrophobic amino acids and of glycine is thought to be responsible for hydrophobic stabilization of protein subunits in elastin fibers and probably for the accumulation of cholesterol. It is possible that a high concentration of cholesterol and of Ca^{2+} ions in a relatively hydrophobic region of the arterial intima can lead to a type of cholesterol-Ca^{2+} ion interaction described in this paper.

The purpose of the present investigation was to determine whether cholesterol and Ca^{2+} ions are able to form complexes in solution. As a solvent we chose 1,4-dioxane-water mixture (9:1, by volume) whose composition is close to that of the dioxane-water azeotrope which consists of 81.6% of dioxane and 18.4% water (b. p. 87.8 °C). We reasoned that in a 9:1 dioxane-water mixture the concentration of water is sufficiently low to permit complex formation between cholesterol and Ca^{2+}. We have observed complex formation in this solvent and studied its properties by quantitative dialysis technique and by osmometry.

Materials and Methods

The following chemicals were used: cholesterol (mol. wt. 387) from Aldrich Chemical Company, Inc.; calcium chloride dihydrate (mol. wt. 147) from Fisher Scientific Company; 1,4-dioxane (mol. wt. 88.1, b. p. 101 °C, m. p. 11 °C) from Fisher Scientific Company, glass-distilled water.

One set of dialysis experiments was performed with 18/32 inch Visking cellulose dialysis tubing using Craig's analytical dialysis cell [3]. Samples (0.5 ml) of 0.01 M $CaCl_2 \cdot 2 H_2O$ in 9:1 dioxane-water were dialyzed against 5 ml of the same solvent. Samples (0.5 ml) of 0.026 M cholesterol in 9:1 dioxane-water (1% solution) were dialyzed against 5 ml of the same solvent. Samples (0.5 ml) of 0.01 M $CaCl_2 \cdot 2 H_2O$ — 0.0026 M cholesterol in 9:1 dioxane-water were dialyzed against 5 ml of 0.0026 M cholesterol in the same solvent. Outside solvent was changed at 0.5, 1, 2 and 4 hours from the start of the dialysis experiment. Cholesterol content of dialysates and of retentates was determined by the method of Zlatkis *et al.* [4]. The Ca^{2+} content was determined by the method of atomic absorption spectrometry using Perkin-Elmer Atomic Absorption Spectrometer Model 303. The emission line of Ca^{2+} at 2112 Å was used. The dialysis results were expressed as a semilogarithmic plot of % material remaining inside as a function of dialysis time — a procedure originally worked out by Craig *et al.* [5]. In another set of dialysis experiments a natural protein membrane (Naturalamb prophylactic membrane made from defatted lamb intestine, Young Drug Products Corporation) was used. The dialysis assembly consisted of a glass plunger which loosely fitted into the membrane leaving a space for the solutions (1 ml) to be dialyzed and an outside glass tube containing 15 ml of solvent against which dialysis was performed. The composition of dialyzing solutions, times at which outside solvents were changed and analysis for cholesterol and Ca^{2+} were the same as described for cellulose membrane experiments.

Milliosmolality (milliosmoles per kg of solution) of various solutions of cholesterol, calcium chloride dihydrate and cholesterol plus calcium chloride dihydrate mixtures in 9:1 dioxane-water was measured with Fiske Osmatic-Automatic Osmometer Model 130 using commercial glass caps containing 0.2 — 0.4 ml of a solution to be measured. This apparatus utilizes the principle of freezing point depression to determine osmolality in the range 0 — 2000 milliosmoles/kg. The precision of the measurement is ± 3 mOs/kg. The following solutions were prepared for osmolality measurements: cholesterol in 9:1 dioxane-water (0.0017 M, 0.0029 M, 0.0038 M, 0.0044 M, 0.005 M); calcium chloride dihydrate in 9:1 dioxane-water (same molarities as for cholesterol); cholesterol plus calcium chloride dihydrate mixture in 9:1 dioxane-water (0.01 M cholesterol — 0 M Ca^{2+}, 0.0083 M cholesterol — 0.0017 M Ca^{2+}, 0.0071 M cholesterol — 0.0029 M Ca^{2+}, 0.0063 M cholesterol — 0.0038 M Ca^{2+}, 0.0056 M cholesterol — 0.0044 M Ca^{2+}, 0.005 M cholesterol — 0.005 M Ca^{2+}, corresponding to cholesterol/Ca^{2+} ratios of ∞, 4.9, 2.4, 1.7, 1.3 and 1.0).

Results and Discussion

The dialysis results obtained with Visking cellulose tubing are shown in Fig. 1 and in Table I. Calcium chloride dialyzed through the membrane with the shortest half-escape time ($t_{1/2}$, time necessary for 50% of material to escape through the
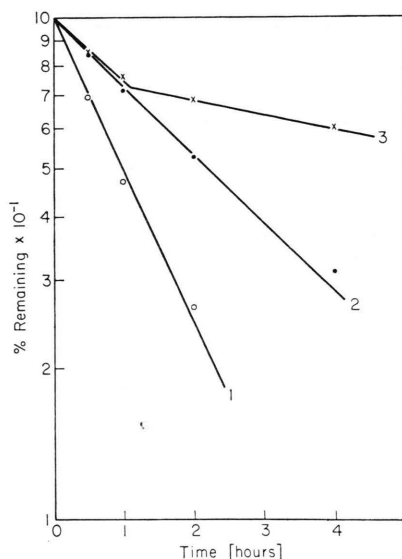

Fig. 1. Dialysis curves obtained at 25 °C with 18/32-inch Visking cellulose membrane using Craig's analytical dialysis cell [3]. Curve 1, 0.01 M calcium chloride dihydrate solution; curve 2, 0.026 M cholesterol solution; curve 3, 0.01 M calcium chloride dihydrate — 0.0026 M cholesterol solution. Solutes were dissolved in 90% aqueous dioxane and dialyzed against same solvent except for the mixture of $CaCl_2 \cdot 2 H_2O$ and cholesterol which was dialyzed against 0.0026 M cholesterol in 90% aqueous dioxane. Inside volume was 0.5 ml and the outside volume 5 ml.

membrane into the outside solvent) equal to 1 hour (curve 1). Half-escape time for cholesterol was 2.2 hours (curve 2). Dialysis of these substances obeyed a first order process characterized by straight lines on a semilogarithmic plot and $t_{1/2}$ values independent of the initial concentration of material to be dialyzed. A solution containing a mixture of 0.01 M $CaCl_2$ — 0.0026 M cholesterol showed quite a different behavior (curve 3). The dialysis curve had a sharp break indicating two first order dialysis processes [5]. The faster process was similar to that of cholesterol dialysis, and the slower one was characterized by a $t_{1/2}$ value of 10.5 hours cor-

Table I. Dialysis of cholesterol, calcium chloride dihydrate and of their mixture in 90% aqueous 1,4-dioxane at 25 °C. Dialysis experiments were performed with 18/32-inch Visking cellulose membranes in a Craig's analytical dialysis cell [3] or with natural protein membranes prepared from defatted lamb intestine in a similar but larger cell. 0.026 M cholesterol solutions and 0.01 M calcium chloride dihydrate solutions in 90% aqueous dioxane were dialyzed against same solvent. 0.0026 M cholesterol-0.01 M calcium chloride dihydrate solutions in 90% aqueous dioxane were dialyzed against 0.0026 M cholesterol solution in 90% dioxane. Dialysates, obtained at 0.5, 1, 2 and 4 hours from the start of the dialysis experiment, were analyzed for cholesterol by the method of Zlatkis *et al.* [4] and for Ca^{2+} by atomic absorption spectrometry.

Sample	Half-escape time $(t_{1/2}$, [hours])		Molecular weight
	Cellulose m.	Protein m.	
Cholesterol	2.2	0.6	387
$CaCl_2 \cdot 2 H_2O$	1.0	6.7	147
Chol. plus $CaCl_2 \cdot 2 H_2O$	10.5	1.4	1800 — 2000 *

* Estimated from a plot of $t_{1/2}$ *versus* mol. wt.

responding to the escape of cholesterol — $CaCl_2$ complex through the membrane.

It has been shown previously by Craig and Pulley [6] that with a tight cellulose membrane there exists a nearly linear relationship between $t_{1/2}$ (in hours) and the molecular weight for an homologous series of cycloamyloses. Applying the same reasoning in our case, a molecular weight ranging between 1800 and 2000 was calculated for cholesterol — $CaCl_2$ complex. Such a complex could accommodate 4 molecules of cholesterol and two molecules of $CaCl_2 \cdot 2 H_2O$ giving a molecular weight of 1849 which is close to that calculated from the kinetics of dialysis. Moreover, this composition of the complex was in agreement with the analysis results of solid cholesterol — $CaCl_2$ complex for which it was established that there were two cholesterol molecules per one Ca^{2+} ion in the complex.

Dialysis curve of the type obtained for cholesterol and calcium chloride dihydrate mixture provides additional information [5]. On extrapolation to the ordinate of that portion of the curve which is identified with a slow dialyzing process one obtains the percentage of cholesterol-calcium chloride complex in the original mixture. Using this method of extrapolation to zero dialysis time it was found that approximately 78% of Ca^{2+} originally present in the mixture was complexed with cholesterol. From this information and from the knowledge of the initial concentrations of cholesterol and of calcium chloride

one can calculate an approximate association constant (K_a) for the process: 4 cholesterol + 2 $CaCl_2 \cdot 2 H_2O \rightleftharpoons$ (cholesterol)$_4$ — $(CaCl_2 \cdot 2 H_2O)_2$ complex. The value for K_a obtained in this way was 3.9×10^{14}. It may apply only to a 90% aqueous dioxane solution where the affinity of the components for one another is very high.
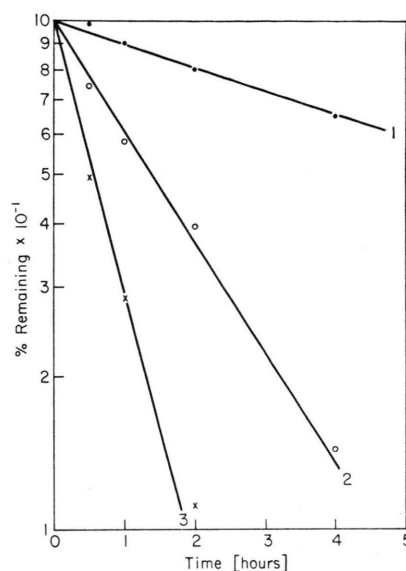

Fig. 2. Dialysis curves obtained at 25 °C with natural protein membrane (Naturalamb prophylactic membrane). Curve 1, 0.01 M calcium chloride dihydrate solution; curve 2, 0.01 M calcium chloride dihydrate — 0.0026 M cholesterol solution; curve 3, 0.026 M cholesterol solution. All solutes were dissolved in 90% aqueous dioxane and dialyzed against same solvent except for the mixture of $CaCl_2 \cdot 2 H_2O$ and cholesterol which was dialyzed against 0.0026 M cholesterol. Inside volume was 1 ml and the outside volume 15 ml.

The results of dialysis experiments performed with natural protein membrane are shown in Fig. 2 and in Table I. The escape of solutes through this type of membrane is just in the opposite order to that obtained with cellulose membrane. Cholesterol alone dialyzed fastest with a $t_{1/2}$ value of 0.6 hours. A mixture of cholesterol and calcium chloride dihydrate, containing the complex, dialyzed with an intermediate value of $t_{1/2}$ (1.4 hours) and the $CaCl_2 \cdot 2 H_2O$ alone dialysed slowest with a $t_{1/2}$ value of 6.7 hours. It was observed that escape of $CaCl_2$ was helped by cholesterol through this type of membrane which was more hydrophobic than the cellulose membrane. In addition, the dialysis curve for the cholesterol — $CaCl_2 \cdot 2 H_2O$ complex was a straight line and did not show a break which was

observed with the cellulose membrane. This type of behavior was explained by Craig [5] to be due to poor membrane selectivity with respect to a "diffusional size" of a dialysing particle. Artificial mixtures of two components did show a break in the dialysis curve when tight and selective membrane was used but gave straight lines with less selective membranes.

The results of freezing-point depression measurements performed on 90% aqueous dioxane solutions of cholesterol, calcium chloride dihydrate and of their mixture were in agreement with the results obtained by quantitative dialysis. They are shown in Fig. 3. It can be seen that addition of $CaCl_2 \cdot 2 H_2O$
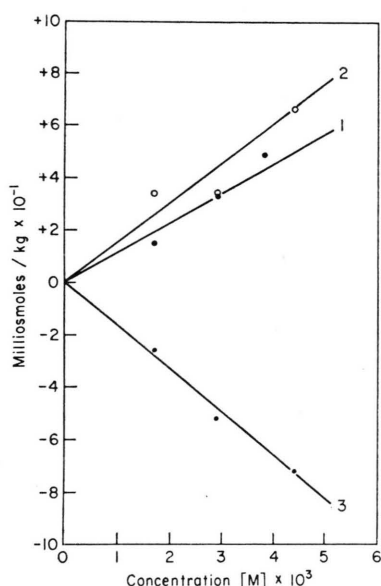

Fig. 3. Milliosmolality of 90% aqueous dioxane solutions containing increasing amounts of calcium chloride dihydrate (curve 1) and increasing amounts of cholesterol (curve 2). Curve 3 was obtained by starting first with a 0.01 M cholesterol solution in 90% aqueous dioxane and adding to it increasing quantities of 0.01 M calcium chloride dihydrate solution in the same solvent. Freezing point depressions were made on 0.2 to 0.4 ml solution samples with a Fiske Osmatic-Automating Osmometer, Model 130.

to 90% dioxane solution (curve 1) increased the milliosmolality of the resulting solution. The same was observed when cholesterol was added (curve 2). It is interesting, however, that $CaCl_2 \cdot 2 H_2O$ potentially containing five osmotically active particles when completely dissociated produced a smaller effect than did cholesterol at the same molar concentration. This might be due to binding of water molecules by Ca^{2+} ions thus lowering their osmotic activity. Curve 3 shows the effect of adding increasing quantities of $CaCl_2 \cdot 2 H_2O$ to the solution of cholesterol in 90% aqueous dioxane. It can be seen that $CaCl_2$ and cholesterol together lower the milliosmolality of the resulting solutions indicating that there are fewer osmotically active particles in the mixtures than in the separate solutions of the same molarity. This is consistent with the formation of cholesterol $- CaCl_2 \cdot 2 H_2O$ complex which was detected in dialysis experiments. As an example, one might consider the behavior of a solution containing 3 mM $CaCl_2 \cdot 2 H_2O$ (shown on abscissa) and 7 mM cholesterol (curve 3). The solution of this composition produced a decrease of 50 milliosmoles/ kg as compared with 10 mM cholesterol solution in the same solvent. On the other hand, if the components present in this solution were dissolved separately, their combined effect would have produced an increase of 143 milliosmoles/kg as compared to 90% aqueous dioxane. One can also see that the magnitude of these effects is much larger than that expected from a 10 mM solution (3 mM $CaCl_2 \cdot 2 H_2O$ + 7 mM cholesterol) suggestig that osmotic contribution of water, dissolved in dioxane may be different in the presence of Ca^{2+} ions and cholesterol.

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