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Digitonin-Cholesterol Complex Formation: Effects of Varying the Length of the Side-chain

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A method was designed to study complex formation between saponin and cholesterol in aqueous ethanol, and was applied, with some modifications, to examine the ability of cholesterol analogues bearing various lengths of side-chain to form complexes (digitonides) with digitonin. The results indicated that all the analogues were capable of forming digitonides regardless of the length of the side-chain, but the effectiveness of digitonide formation increased as the length of the side-chain increased. A plausible structural model is proposed for the digitonide in order to interpret these results.

Keywords—digitonin; saponin; digitonide; molecular complex; cholesterol; cholesterol analogue

It is well known that the majority of saponins interact with sterols to form tightly bound non-covalent complexes of great insolubility in aqueous ethanol.¹⁾ The interaction with sterols may be involved in the physiological action of saponins.²⁾ Hemolysis might be explained by the interaction of saponins with cholesterol in the erythrocyte membranes causing a permeability change in the cell membranes and allowing the leakage of cellular contents. Toxicity to fish and antifungal activity of saponins may also arise from such effects on membrane permeability. However, some experimental results which argued against this hypothesis were reported.³⁾

Previously we reported that digitonin,^{4,5)} a strongly lytic saponin, actually formed a rigid, immobilized complex with cholesterol in model membranes, and the occurrence of such a complex, which is similar to "digitonide" in aqueous ethanol, strongly perturbed the lipid bilayers; this finding suggested that the complex formation might be a factor in hemolysis induced by digitonin.⁶⁾ However, the mechanism of the digitonin-cholesterol interaction and the nature of the resulting complex (digitonide) are not yet clearly understood.

We report here a method to determine complex formation between digitonin and chole-

$n=0$ C22
 $n=1$ C23
 $n=2$ C24
 $\vdots$
 $n=7$ C29
 $n=8$ C30
 $n=10$ C32
 $n=12$ C34

digitonin R=Xyl-Glc-Gal-
Glc-Gal

desgluco-
digitonin R=Xyl-Glc-Gal-
Gal

glucosyl-
galactosyl-
digitogenin R=Glc-Gal-

Chart

terol (including defined stoichiometry) and the application of the method for examining the effects of the side-chain of sterol molecules on the interaction with digitonin. The results are interpreted in terms of a plausible structural model of digitonide.

Results and Discussion

Characterization of Digitonin and Digitonide

It is well known that commercial "digitonin" is a complex mixture of structurally related saponins.⁴⁾ In the present study, digitonin and desglucodigitonin were separated by using DCC^{7,8)} from commercial "digitonin." Although digitonin and desglucodigitonin obtained here appeared to be homogeneous by DCC and HPTLC, ¹³C-NMR spectra indicated that the samples contained small amounts of isomers at C-22 and C-25 (less than 10%).⁹⁾ We did not attempt to separate the epimers and used the samples for the following experiments.

In order to exclude the possibility that the precipitates formed on addition of digitonin to cholesterol solution resulted from the cocrystallization of the species, or less likely, from simple alteration of the solubility, we compared the powder diffraction patterns of the precipitates with those of digitonin and cholesterol. The powder pattern of the precipitates is not simply an addition of the patterns of digitonin and cholesterol but is completely different. The results clearly indicate that the complex has a unique crystal structure different from those of digitonin and cholesterol. This suggestion is in good agreement with the fact that the melting point of the complex is relatively sharp (214–218°C) and is not lower than that of cholesterol (150°C), whereas digitonin alone and on mixed fusion with cholesterol gave melting points of 265–270°C and 146°C, respectively.

Digitonide formation has been studied mostly by visual determination of the precipitates.¹⁰⁾ We designed a method to ensure complex formation in which uncomplexed radioactive cholesterol in the supernatant was determined after the removal of the insoluble complex by centrifugation.

Fig. 1. Plots of the Amounts of Cholesterol in the Supernatant vs. Those of added Glycosides

The amounts of glycosides are expressed in molar ratios with respect to cholesterol. ○—○: digitonin, ●—●: desglucodigitonin, △—△: glucosylgalactosyldigitogenin.

Fig. 1 shows the amount of uncomplexed cholesterol in the supernatant (molar ratio with respect to glycoside) as a function of added glycoside. Addition of digitonin and desglucodigitonin up to equimolar ratio reduced the amount of cholesterol in the supernatant, and no significant amount of cholesterol was observed in the supernatant above equimolar ratio. These results imply the formation of an insoluble complex consisting of cholesterol and digitonin or desglucodigitonin. In contrast to this observation, on addition of glucosylgalactosyldigitogenin, no insoluble complex appeared. Since there is a distinct break at the point of 1.0 in the curve and the slope of the curve, before the break, is 1.0, it is apparent that an equimolar complex is actually formed from digitonin and desglucodigitonin and is extremely insoluble.

The formation of an equimolar complex was suggested by Windaus¹¹⁾ based on the elemental analysis of digitonide, and some other methods have been designed to study complex formation.¹⁰⁾ The technique described here appears to have advantages over those described

previously, in that it has comparable sensitivity and reliability for determining the ability of saponins to form complexes and can also determine their stoichiometry.

Effects of Varying the Length of Side-chain

Structural characteristics required for sterols to form digitonides have been extensively studied.¹⁰⁾ The results suggest that the steroid ring system with a 3β -hydroxy group is essential for the formation of such a complex, but the hydrocarbon side-chain at C-17 is much less critical. We examined the effects of various lengths of side-chain on the ability to form digitonides. Cholesterol analogues bearing side-chains of various lengths and lacking the terminal methyl branch were synthesized; their structures are given in the chart. The cholesterol analogues are referred to by the total number of carbon atoms in them as proposed by Suckling and Boyd.¹²⁾ In order to examine whether cholesterol analogues with a shortened or lengthened side-chain have the ability to form digitonide, experiments similar to those in Fig. 1 were carried out in which ^3H -labeled C22 and C32 were allowed to interact with digitonin. The formation of digitonides with equimolar stoichiometry was clearly demonstrated, and the solubility of C22-digitonide was found to be slightly higher than those of C32- and cholesterol-digitonides, suggesting a relatively weak interaction between C22 and digitonin.

It would be interesting to know how long a sterol side-chain is required for maximum interaction with digitonin. The comparative abilities of various analogues to form digitonide with respect to cholesterol were examined by means of competition experiments in which digitonides were prepared in the simultaneous presence of equimolar amounts of digitonin and labeled cholesterol in addition to various amounts of each of the unlabeled analogues. After removal of insoluble material by centrifugation, the amount of cholesterol in the supernatant was determined. Fig. 2 shows the results obtained for cholesterol (control), Δ^4 -cholesten-3-one (control) and the analogues with shorter side-chain lengths than that of cholesterol. On increasing the molar ratios of analogues to cholesterol, all the analogues tested here caused an

Fig. 2. Competitive Experiments between Labeled Cholesterol and Unlabeled Analogues with Reduced Side-chains for the Formation of Digitonide

The mixture consisted of equimolar amounts of cholesterol and digitonin in addition to various amounts of analogues. The amounts of sterols are expressed in molar ratios with respect to digitonin. $\circ-\circ$: cholesterol, $\bullet-\bullet$: Δ^4 -cholesten-3-one. Data points for other sterols are omitted for simplicity.

Fig. 3. Competitive Experiments between Labeled Cholesterol and Unlabeled Analogues with Lengthened Side-chains for the Formation of Digitonide

The mixture consisted of equimolar amounts of cholesterol and digitonin in addition to various amounts of analogues. The amounts of sterols are expressed in molar ratios with respect to digitonin. $\circ-\circ$: cholesterol. Data points for other sterols are omitted for simplicity.

increase of the amount of cholesterol in the supernatant, whereas no significant effect was observed for Δ^4 -cholesten-3-one. The results indicate that these analogues are able to compete efficiently with cholesterol for the formation of digitonide. Among the six analogues studied so far, C27 is the most effective, though it is less effective than cholesterol itself, while C22, which has the shortest length of side-chain, is the least effective. Moreover, a marked difference was seen between the curves of analogues with longer and shorter side-chains. While the amount of the labeled cholesterol increased in a linear fashion with increasing contents of longer side-chain analogues, the curves of shorter side-chain analogues are essentially convex; in particular, the plots of C22 and C23 are roughly linear up to the analogue/cholesterol ratio of 0.6, and maintain an almost constant value of about 0.25 above that ratio. Since the mixture contains equimolar amounts of cholesterol and digitonin, the amount of free cholesterol in the supernatant is equal to the amount of analogue incorporated into digitonide. The results shown in Fig. 2 suggest that C22 and C23 were only incorporated into digitonide up to a molar composition of 20 mol% sterol, and the ultimate composition of the digitonide formed under these conditions was 0.1 C22 or C23, 0.4 cholesterol and 0.5 digitonin. This composition was further examined by means of the following experiments, where ^3H -labeled C22 and ^{14}C -cholesterol were utilized simultaneously in order to determine the amounts of both sterols in the supernatant. The results demonstrated that at least 0.15 equivalent mole of C22 was incorporated into insoluble digitonide and a considerable amount of cholesterol (0.24) was detected in the supernatant. The discrepancy of these values is due to the incomplete precipitation of C22-digitonide; as described earlier, the solubility of C22-digitonide is higher than that of cholesterol-digitonide.

It is apparent from Fig. 2 that the effectiveness of digitonide formation parallels the length of the side-chain; the effectiveness decreased as the length of the side-chain was reduced. A parallel relationship between the effectiveness and the length of the side-chain was also observed for the interaction between desglucodigitonin and these analogues (data not shown). The results obtained for desglucodigitonin also indicated that C27 was more effectively incorporated into desglucodigitonin-complex than cholesterol.

The results obtained here encouraged us to examine analogues with lengthened side-chains. The competitive experiments described above were performed in 63% aqueous ethanol solution in order to minimize the solubility of the digitonides formed. Due to the low solubility of the analogues bearing longer side-chains, similar precipitation experiments were carried out in 83% aqueous ethanol in which the solubility of the analogues was sufficiently high. C27, C28 and C29 showed approximately the same effectiveness as cholesterol, while analogues with longer side-chains were less effective (Fig. 3). It is interesting that the curves of longer side-chain analogues resemble those observed for shorter side-chain analogues in that they display breaks and plateau values. Furthermore, the plateau values were found to be dependent on the length of side-chain. Additional experiments were performed in an effort to elucidate the molar composition of digitonide containing cholesterol and C32 using a method similar to that described for C22. It was found that the addition of C32 produced no significant alteration of the amount of C32 or cholesterol in the supernatant at high C32/cholesterol ratios, indicating that, probably due to the simultaneous presence of cholesterol, added C32 solely formed precipitates without competing (data not shown). Further experiments were carried out in aqueous ethanol containing a small amount of water to ensure complete solubilization of free sterols. The results clearly demonstrated that C32 was more effectively incorporated into digitonide than cholesterol. This observation is consistent with that presented in Fig. 3, where much steeper slopes were seen for C32 (and also C34) than cholesterol at lower analogue/cholesterol ratios. Taken in conjunction with the results obtained for shorter analogues (Fig. 2), it is concluded that effectiveness of digitonide formation increases as the length of side-chain is increased.

As described earlier, it is likely that the formation of the rigid, immobilized digitonin-

cholesterol complex in the membranes is related to the hemolysis induced by digitonin.⁶⁾ The interaction between digitonin and analogues in model membranes has been examined by measuring the permeability properties of liposomes constituted from analogues bearing shorter side-chains (C22—C27).¹³⁾ The order of the sensitivities of analogues towards digitonin in liposomes was essentially parallel to that obtained in the present examination, as far as the shorter side-chain analogues are concerned. However, the sensitivities of longer analogues towards digitonin in liposomes decreased as the length of the side-chain was increased (C28—C34).¹⁴⁾ The inconsistency between the effectiveness of digitonide formation in aqueous ethanol and the sensitivity towards digitonin in liposomes might reflect a difference in the mode of the action of digitonin or in the stability of digitonides formed in model membranes from those in aqueous ethanol.

The effects of the length of the sterol side-chain in the lipid-sterol interaction have been extensively examined by using a variety of techniques, *e.g.* EPR,¹²⁾ measurements of permeability properties,¹³⁾ and growth supplements for yeast,¹⁵⁾ in model and biological membranes. By all these criteria, the order of effectiveness is the same: cholesterol caused the greatest effect, the analogues with shorter or longer side-chains than cholesterol being less effective. The maximum interaction with phospholipid has been interpreted in terms of the maximum sterol-acyl chain contact resulting from the geometrical fit between phospholipid and cholesterol in the bilayers.

A Plausible Model of Digitonide

Although the correlation between digitonide formation and the structure of steroids is well documented,¹⁰⁾ relatively few studies have been directed towards understanding the molecular mechanism of the interaction of saponin with cholesterol. A hypothetical micellar type arrangement of saponin and cholesterol was proposed for the structure of saponin-cholesterol complex by Clauert *et al.*¹⁶⁾ and later also by Seeman¹⁷⁾ based on electron micrographic investigations. The principal feature of the molecular architecture of the model is a circular arrangement of saponin molecules with the hydrophilic sugar regions facing towards the center. Cholesterol molecules are localized on the outside of the circular structure and are in association with the hydrophobic aglycone portion of the saponin in a 1:1 molar ratio. In this model the interaction can be ascribed to the attractive forces between hydrophobic aglycone and cholesterol. If such a force played an important role in the interaction, it would be predicted that the analogues would compete with cholesterol in a manner directly proportional to the increase in molar ratios of analogues. Our finding obtained for analogues with shorter side-chain (Fig. 2) was not compatible with this model. However, the possibility that digitonin interacts with cholesterol by a different mechanism from those of other saponins has to be taken into account.

On the basis of the available data, a more plausible explanation for the interaction is that digitonide belongs to a class of clathrate compounds in which digitonin plays the role of host and sterols are guests. Digitonin molecules are arranged in such a manner as to form a hydrophobic cavity or channel, capable of enclosing molecules of the size of cholesterol in equimolar ratio. Based on this hypothetical model, one would consider that the structural stability of the digitonide is, at least partially, dependent on the steric packing of guest molecules, since the shape of the cavity or channel, in which the guest molecules are retained, is determined by the architecture provided by the arrangement of digitonin molecules. Evidence in favor of this hypothesis has been presented by Windaus *et al.*¹⁸⁾ They reported that the formation of insoluble digitonide was not restricted to sterols, but many non-steroidal compounds, *e.g.* phenol and terpene alcohols, were able to form *loosely* bound complexes.

According to the model presented here, sterols with a rigid tetracyclic nucleus but lacking a flexible side-chain would exhibit relatively weak interaction with digitonin, since the failure of precise geometrical fit would lead to a looser packing in the cavity or channel. In support

of this, analogues bearing a shorter side-chain were shown to be inferior to longer side-chain analogues in terms of effectiveness of digitonide formation. Moreover, the degree of complex formation increased as the length of side-chain was increased. The order probably reflects differences in the degree of steric packing of these guest molecules arising from the sterol-sterol interaction. Longer side-chain might favor the geometrical fit, since these analogues have the freedom to alter their shapes and sizes by extending, bending or folding the long flexible alkyl chains. The most striking result presented in this publication is the appearance of an upper limit of competition observed for the analogues with shorter side-chain (Fig. 2). Such an unusual feature might be attributed to the formation of "mixed digitonides" in which cholesterol and each shorter analogues may pack together in the cavity or channel in such a way as to optimize the sterol-sterol interaction. The observed ultimate sterol composition in the "mixed digitonides" may reflect the achievement of the maximum geometrical fit which could be provided by mixing of cholesterol and respective analogues.

While we are unable to give a detailed molecular explanation for these phenomena, all the available experimental results can be interpreted by assuming that digitonide possesses a cavity or a channel which can be filled by molecules of various shapes.

Experimental

Isolation of Digitonin and Desglucodigitonin—In a typical experiment, commercial "digitonin" (300 mg), purchased from Merck and Co. Inc., (West Germany), was separated by a hand-built DCC apparatus¹⁹⁾ in an ascending mode using the solvent system of CHCl_3 -MeOH- H_2O (volume ratio; 50: 60: 40). The fractions containing digitonin were combined, concentrated and then again subjected to DCC for further purification using the solvent system of CHCl_3 -MeOH- H_2O -*n*-PrOH (volume ratio; 45: 60: 40: 5). Crystallization of the product from aqueous ethanol gave colorless needles, mp 265–270°C. Yield, 170 mg. Desglucodigitonin was separated using the same methods but could not be obtained in crystalline form. Yield, 50 mg.

Preparation of Glucosylgalactosyldigitogenin—A solution of digitonin (1 g) in 50% aqueous ethanol (50 ml) containing 5% sulfuric acid was heated at 60°C for 24 h. After neutralization of the solution with NaHCO_3 solution, ethanol was evaporated off and the mixture was diluted with water and extracted with *n*-butanol. The *n*-butanol layer was washed with water and concentrated *in vacuo* to give a residue which was separated by DCC using the solvent system of CHCl_3 -MeOH- H_2O (50: 60: 40) in the ascending mode. Evaporation of the solvent gave 180 mg of the desired prosapogenin as an amorphous powder. The structures of these saponins and prosapogenin were confirmed by chemical analysis of their acid hydrolysates and also by determination of the ^{13}C -NMR spectra. A detailed account of the ^{13}C -NMR spectra of these glycosides will be published elsewhere.

Synthesis of Cholesterol Analogues—Cholesterol analogues were synthesized starting from 23,24-bis-norcholenic acid (Steraroids Inc., U.S.A.) by the reported methods with slight modifications.²⁰⁾ They were characterized by IR, ^1H - and ^{13}C -NMR and mass spectroscopy, as well as elemental analysis.

^3H -Labeled Analogues— ^3H -Labeled C22 was prepared from 3 β -*O*-tetrahydropyranyl-22-tosyloxy-bisnorchol-5-ene²⁰⁾ by reduction with LiAl^3H_4 (Radiochemical Centre), followed by acid hydrolysis, and the product was purified by column chromatography and recrystallization from aqueous ethanol. ^3H -Labeled-C32 was prepared from the corresponding ketone, which was synthesized basically according to Fieser,²¹⁾ by reduction with LiAl^3H_4 in ether, and the product was purified by column chromatography followed by recrystallization from aqueous ethanol.

X-Ray Powder Diffraction—Taken on a Rigakudenki D-3F diffractometer. Target, Cu; filter, Ni; voltage, 30 kV; current, 10 mA; detector, G, M. counter; count range, 2000 cps; time constant, 0.5 s; scanning speed, 4°/min; chart speed, 40 mm/min.

Cholesterol, $2\theta_{\text{CuK}\alpha}$ [degree (d : Å)]: 5.0 (17.77), 10.2 (8.69), 12.4 (7.16), 12.3 (6.34), 14.6 (5.86), 16.4 (5.39), 17.4 (5.10), 18.1 (4.89), 18.6 (4.76), 20.4 (4.35), 21.7 (4.10), 22.6 (3.84), 26.6 (3.61), 25.9 (3.44), 27.8 (3.20).

Digitonin, $2\theta_{\text{CuK}\alpha}$ [degree (d : Å)]: 6.0 (14.76), 8.0 (11.02), 9.3 (9.48), 10.8 (8.16), 11.7 (7.54), 12.4 (7.13), 13.1 (6.75), 14.3 (6.17), 15.5 (5.71), 17.2 (5.17), 17.8 (4.99), 18.3 (4.84), 19.0 (4.66), 20.1 (4.41), 21.0 (4.23), 23.0 (3.87), 24.6 (3.62), 25.0 (3.55), 25.8 (3.46), 26.6 (3.35), 23.8 (3.10), 29.5 (3.02).

Cholesterol-digitonide, $2\theta_{\text{CuK}\alpha}$ [degree (d : Å)]: 6.0 (14.82), 7.5 (11.83), 8.9 (9.92), 11.3 (7.86), 16.2 (7.24), 14.0 (6.32), 15.5 (5.71), 16.1 (5.19), 17.9 (4.97), 18.2 (4.88), 20.1 (4.41), 20.6 (4.30), 21.0 (4.23), 22.7 (3.92), 24.2 (3.67), 26.1 (3.41), 28.6 (3.12).

Determination of the Stoichiometry of the Complex between Cholesterol and Digitonin, Desglucodigitonin

and Glucosylgalactosyldigitogenin (i)—To 1.0 ml of ^{14}C -cholesterol (Radiochemical Centre) solution in ethanol ($2.39\ \mu\text{mol/ml}$; $1.62 \times 10^5\ \text{dpm/ml}$) was added 2.0 ml of a solution of glycoside in 50% aqueous ethanol (0—2.5 equivalent mole of glycoside with respect to cholesterol). The mixture was allowed to stand at room temperature for 2 h, and centrifuged at 3000 rpm for 20 min. Then 100 μl of the supernatant was removed for radioactivity measurements. The amount of cholesterol which was not incorporated into the insoluble complex was calculated from the radioactivity in the supernatant. The results are plotted in Fig. 1.

Digitonide Formation with C22 (ii)—Various amounts of ^3H -C22 solution in ethanol (1.0 ml) ($4.61 \times 10^5\ \text{dpm}/\mu\text{mol}$); 0—1.6 molar ratio with respect to digitonin) were added to 1.0 ml of digitonin solution ($1.29\ \mu\text{mol/ml}$) in 50% aqueous ethanol. Samples were allowed to stand at room temperature for 2 h. After centrifugation (3000 rpm, 20 min), 100 μl of the supernatant was removed for radioactivity measurements. The amount of C22 remaining in the supernatant was calculated from the radioactivity. The amounts of C22 in the supernatant (the amount of added C22) expressed in molar ratios with respect to digitonin were as follows: 0.03 (0.24), 0.03 (0.48), 0.07 (0.74), 0.14 (0.98), 0.27 (1.22), 0.52 (1.47).

Digitonide Formation with C32 (iii)—Procedure (ii) was used with minimum modifications. Various amounts of ^3H -C32 in ethanol were added to digitonin solution in ethanol and then the mixture was diluted by adding a small amount of water to give a final ethanol/water ratio of 20. The amounts of C32 in the supernatant (the amount of added C32) expressed in molar ratios with respect to digitonin were as follows: 0.02 (0.31), 0.04 (0.47), 0.05 (0.62), 0.07 (0.77), 0.09 (0.93), 0.25 (1.09), 0.38 (1.25), 0.56 (1.41).

Competitive Digitonide Formation Experiments with Cholesterol Analogues bearing Shorter Side-chains (iv)—A solution containing 0—1.1 equivalent mol of analogue with respect to cholesterol in ethanol was mixed with ^{14}C -cholesterol solution in ethanol and the mixture was added to digitonin solution (equimolar with respect to cholesterol) in aqueous ethanol (50%). The final ethanol/water ratio in the medium was adjusted to 2.0 by adding water. The amount of cholesterol in the supernatant was determined, after centrifugation, by measuring the radioactivity in the supernatant. The results are shown in Fig. 2.

^3H -Labeled C22 was also utilized and the amounts of C22 and cholesterol in the supernatant were calculated based on the radioactivity of these sterols. The amount of C22 and cholesterol in the supernatant (added C22) were as follows: 0, 0.03 (0); 0.05, 0.07 (0.14); 0.16, 0.11 (0.03); 0.29, 0.16 (0.44); 0.46, 0.18 (0.59); 0.61, 0.22 (0.74); 0.73, 0.24 (0.88); 0.88, 0.24 (1.03); 1.08, 0.24 (1.17).

Competitive Experiments with Longer Side-chain Analogues (v)—These experiments were carried out by procedure (iv) except that ethanolic solution of digitonin was used and the final ethanol/water ratio in the medium was adjusted to 5.0 by adding water before centrifugation. The results are shown in Fig. 3. ^3H -Labeled C32 was used and in this particular experiment the final ethanol/water ratio was adjusted to 12.5. The amounts of C32 and cholesterol in the supernatant (added C32) were as follows: 0, 0.15 (0); 0.02, 0.23 (0.09); 0.07, 0.28 (0.18); 0.09, 0.37 (0.28); 0.11, 0.41 (0.37); 0.14, 0.44 (0.46); 0.15, 0.49 (0.55); 0.16, 0.47 (0.64); 0.17, 0.49 (0.74); 0.16, 0.52 (0.83).

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