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Interaction of Nitroglycerin With Human Blood Components

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Abstract □ Nitroglycerin is rapidly lost from solution when incubated with red blood cells or whole blood. The assumption that the loss is enzymatic in nature may not be true, since no major metabolite is detected during this incubation. Explanation on the basis of a chemical reaction is also difficult, since the products of the chemical hydrolysis of nitroglycerin are the same as the metabolic products. After an initial rapid loss, nitroglycerin disappearance at 37° follows an apparent first-order process in the concentration range of 10–480 ng/ml when incubated with washed red blood cells suspended in normal saline solution. The half-life for the reaction of the apparent first-order phase varies with the initial concentration and increases as the concentration increases (4 min at 10 ng/ml, 52 min at 480 ng/ml), suggesting a mixed kinetic mechanism. Metabolites of nitroglycerin (1,2- and 1,3-dinitroglycerin) react similarly to nitroglycerin in terms of an apparent initial, fast step, a secondary first-order dependence, and concentration-dependent rate effects; however, the rate of the reaction is much slower ($t_{1/2}$ = 33 min at 10 ng/ml) for the metabolite. These data suggest the possibility of a physical mechanism for the loss of nitroglycerin. Since the loss to red blood cells can be rapid, it seems that the mechanism should be delineated, and that the rate of disappearnce be considered in an analysis of the pharmacodynamics of the drug.

Keyphrases □ Kinetics—nitroglycerin loss to red blood cells □ Nitroglycerin—rate of loss to red blood cells □ 1,2- and 1,3-Dinitroglycerin—rate of loss to red blood cells □ Disposition—reactivity of nitroglycerin and its metabolites with blood

It has been reported that when nitroglycerin is incubated with whole blood (fresh or outdated), resuspended red blood cells, serum, or plasma, the drug is lost from solution by an apparent first-order process. A relatively rapid loss occurred in blood or resuspended cells (1–3) ($t_{1/2}$ = 6 min) and rat serum ($t_{1/2}$ = 20 min) (4) with a slower loss occurring in plasma, $t_{1/2}$ = 53 (1) or 175 min (5). The rate of loss in the presence of erythrocytes approaches the biological half-life of organic nitrate (1.9 min) (1). The interaction of nitroglycerin with blood cells, serum, or plasma has been assumed by other workers to be an enzymatic reaction. However, our earlier work using an assay with resuspended red blood cells that could detect the major nitroglycerin metabolites (1,2-dinitroglycerin and 1,3-dinitroglycerin) showed that there was no metabolite in the medium over the entire time course of the loss of nitroglycerin (3). This observation led to the conclusion that the loss of nitroglycerin may not be enzymatic in nature. In this paper further studies are reported which probe the mechanism of loss of nitroglycerin when the drug is incubated with red blood cells. Since the rate of loss of nitroglycerin is rapid, it would appear that a knowledge of the mechanism of its loss would have significant impact on an analysis of the disposition kinetics of the drug and possibly its physiological activity as well.

EXPERIMENTAL

Nitroglycerin stock solutions were prepared from an alcoholic extract of a 10% aqueous solution of lactose adsorbate[1]. The major metabolites of nitroglycerin, 1,2-dinitroglycerin and 1,3-dinitroglycerin, were prepared from 2,3-dibromopropanol[2] and 1,3-dibromopropane-2-ol[3] using the method of Dunstan *et al.* (6). The purity of the compounds was determined by high-performance liquid chromatography (HPLC) and TLC and the solutions standardized as reported by Yuen *et al.* (7). All other chemicals used were obtained commercially and were reagent grade or better.

An electron capture gas chromatograph[4] was used with a data processor[5]. The GLC assay for nitroglycerin was reported earlier (9% QF-1[6] on 60–80 mesh Supelcoport[7]) (3). The column was silanized glass 2 mm × 0.915 m and the temperatures of the injection port, the column, and the detector were 150, 125, and 200°, respectively. The carrier gas was 5% methane in argon used at a flow rate of 21 ml/min. Under these conditions the retention times were: mixture of 1,2-dinitroglycerin and 1,3-dinitroglycerin, 3 min; nitroglycerin, 6.3 min; and internal standard (1-fluoro-2,4-dinitrobenzene[8]) 7.57 min. The system does not separate the two isomeric metabolites but does optimize their combined detection.

For the assay of 1,2- and 1,3-dinitroglycerin a method was used that was a slight modification of our earlier method (8). The column (silanized glass, 2 mm × 50 cm) consisted of 3% Carbowax 20M-TPA[9] on 60–80 mesh Supelcoport[7]. The injection port, column, and detector were at 150, 135, and 200°, respectively and the flow rate of the carrier gas was 20 ml/min. Using these conditions the two metabolites could be separated from each other and from nitroglycerin. The approximate retention times in minutes were: nitroglycerin, 2.7; *o*-iodobenzyl alcohol[10] (internal standard), 3.4; 1,2-dinitroglycerin, 6.5; and 1,3-dinitroglycerin, 8.2.

The concentration of nitroglycerin or its metabolite in the various

[1] Nitroglycerin 10% (w/w) in lactose, lot K17-O-H, ICI Americas, Atlas Chemical Div, Wilmington, DE 19899.
[2] Eastman Kodak, lot A5A, Rochester, NY 14650.
[3] Eastman Kodak, lot B6C, Rochester, NY 14650.
[4] 3700 Series dual column gas chromatograph, Varian Instrument Div., Palo Alto, CA 94303.
[5] Shimadzu Recording Data Processor. Chromatopac C-RIA Shimadzu Scientific Instruments, Columbia, MD 21045.
[6] Analabs, No. Haven, CT 06473.
[7] Supelco, Bellefonte, PA 16823.
[8] Eastman Organic Chemicals, Distribution Products Ind. Rochester, NY 14650.
[9] Applied Science Labs, State College, PA 16801.
[10] Aldrich Chemical Co., Milwaukee, WI 53233.

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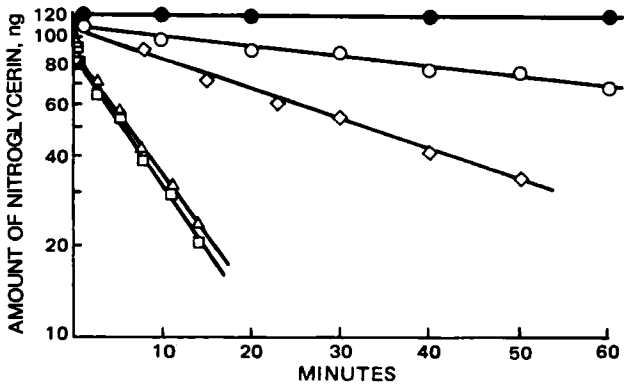

Figure 1—*Relationship between the logarithm of the amount of nitroglycerin (ng) extracted from 2 ml of several preparations initially at 60 ng/ml and the time in minutes. The preparations used at 37° together with their interpolated half-lives (in parentheses) were: (●) normal saline and protein free plasma (∞); (○) plasma (130); (◇) washed red cells diluted 1/3 of normal erythrocyte count (30); (△) red cells at normal erythrocyte count (7); (□) whole blood (6.7).*

samples used (described below) was determined relative to the internal standard (3). Using the QF-1 chromatographic system the precision was ~ ±8% at 1 ng/ml. The lower limit of nitroglycerin detection was ~50 pg/ml. The metabolites had a detector response of ~1/2 that of an equal amount of nitroglycerin, and the precision was ±10% at 3 ng/ml using the Carbowax 20 M-TPA system.

The loss of nitroglycerin or metabolite was followed in a number of different systems: whole blood, washed and resuspended red blood cells, plasma, protein-free plasma, and normal saline solution. The majority of the studies reported used resuspended cells, since the loss of nitroglycerin is the same in these systems as in whole blood (1, 3), and the resuspended cells represent more clearly defined systems that yield trouble-free reference blanks.

The various systems used in the several studies were obtained using approximately 100-ml portions of heparinized whole blood obtained from normal subjects. The blood was transferred to centrifuge tubes, centrifuged at 1200 rpm at 5° for 15 min, and then the plasma was separated from the cells. A portion of this plasma was used for a set of experiments. The remainder of the plasma was rendered protein free by ultrafiltration at 30 psi using a suitable membrane[11]. The cells obtained by centrifugation were washed three times with saline solution and reconstituted after centrifugation to 100 ml with saline solution. The cell count in such preparations was measured[12] and adjusted if necessary to a normal erythrocyte count (~5 × 10^6 cells/μl).

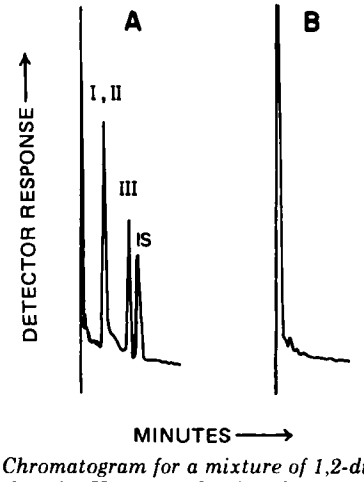

Figure 2—*(A) Chromatogram for a mixture of 1,2-dinitroglycerin, I, and 1,3-dinitroglycerin, II, 1 ng each; nitroglycerin, III (1 ng) and 1-fluoro-2,4-dinitrobenzene, IS, (internal standard) using a 9% QF-1 column. The retention times found in minutes were: I and II = 3; III = 6.3; IS = 7.6. (B) Chromatogram for red cell sample without drug.*

[11] Model 12 Ultrafiltration Cell with PM10 Membrane, Amicon Corp., Lexington, MA 02173.

[12] Micro-Computerized Elzone, Model APC-80XY, Particle Data, Inc., Elmhurst, IL 60126. Dilution procedure for red cell count accompanies the instrument manual.

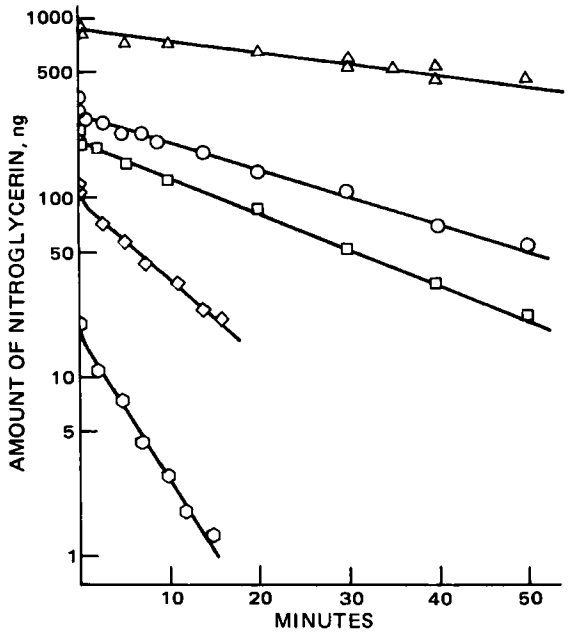

Figure 3—*Relationship between the logarithm of the amount of nitroglycerin (ng) extracted from 2 ml of washed red blood cells in normal saline and the time in minutes. The suspensions at normal erythrocyte count and at 37° were incubated with varying initial concentrations of nitroglycerin. The concentrations used and the interpolated half-lives were: (△) 480 ng/ml, 52 min; (○) 180 ng/ml, 20 min; (□) 120 ng/ml, 16 min; (◇) 60 ng/ml, 7 min; (○) 10 ng/ml, 4 min.*

In general, 2 ml of a particular preparation (whole blood, resuspended cells, plasma, protein-free plasma, or saline) was transferred to each of a series of 12-ml silanized tubes and equilibrated at 37° (~20 min). An aliquot (~50 μl) of a solution of nitroglycerin metabolite in normal saline was added to each tube to give a final concentration in each set of tubes of 10, 20, 60, 120, 180, or 480 ng/ml. The tubes were gently stirred using a rotator submerged in a water bath at 37°. Periodically over a given time interval, a tube was removed, the internal standard was added (if necessary), and the preparation was immediately extracted twice with 3 ml of ethyl acetate[13]. Both extracts were combined and concentrated or diluted for GC analysis. A portion of this solution (5 μl) was injected into the gas chromatograph for determination of the nitroglycerin and/or metabolite concentration.

The extraction efficiencies of nitroglycerin, 1,2-dinitroglycerin, and 1,3-dinitroglycerin in the red cell suspensions were previously reported: 84, 84, and 75%, respectively (3). The extraction efficiency for the metabolites from plasma was 81% (9).

RESULTS AND DISCUSSION

When nitroglycerin is incubated with whole blood or with red blood cells that are washed and suspended at a normal erythrocyte level, the loss of nitroglycerin in either preparation is the same, and after an apparent initial rapid step, it follows an apparent first-order process. Figure 1 shows the loss of nitroglycerin initially at 60 ng/ml in both whole blood and washed red cells. The half lives for the first-order loss are 7 min in whole blood and 6.7 min in red blood cells. These results are similar to those found by Armstrong *et al.* (1): 6.2 and 6.6 min, respectively, for the whole blood and suspended cell preparations at an initial nitroglycerin level of 50 ng/ml at 37°. The rate of reaction is dependent on the number of cells in the system at a constant concentration. If the initial concentration is held at 60 ng/ml and the concentration of cells is reduced threefold, the first-order half-life increases to 30 min (Fig. 1).

In whole blood and resuspended cell systems no metabolite (or hydrolysis product) accumulates in the medium. The dinitro compounds are the principal hydrolysis or metabolic products of nitroglycerin. This observation supports our earlier conclusion (3) that loss of nitroglycerin from whole blood and red cell systems may not be enzymatic, as assumed by others (1, 2). Figure 2A shows a chromatogram of a mixture of 1,2-

[13] Baker Resi-Analyzed for pesticide analysis, J. T. Baker Chem. Phillipsburg, NJ 08865.

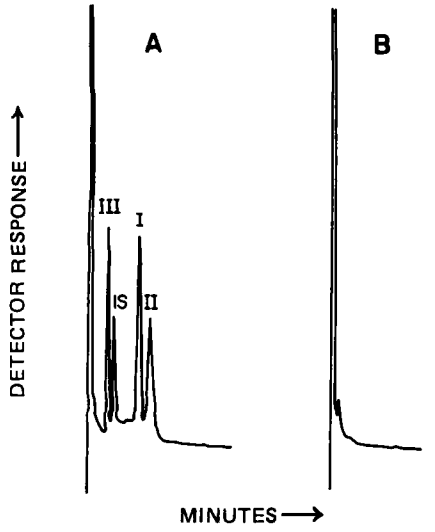

Figure 4—*(A) Chromatogram for a mixture of 2 ng of 1,2-dinitroglycerin (I), 1.6 ng of 1,3-dinitroglycerin (II), 1 ng of nitroglycerin (III), and o-iodobenzy alcohol (IS) using a 3% Carbowax 20M-TPA column. The retention times found in minutes were: I = 6.5; II = 8.2; III = 2.7; IS = 3.4. (B) Chromatogram for red cell sample without drug.*

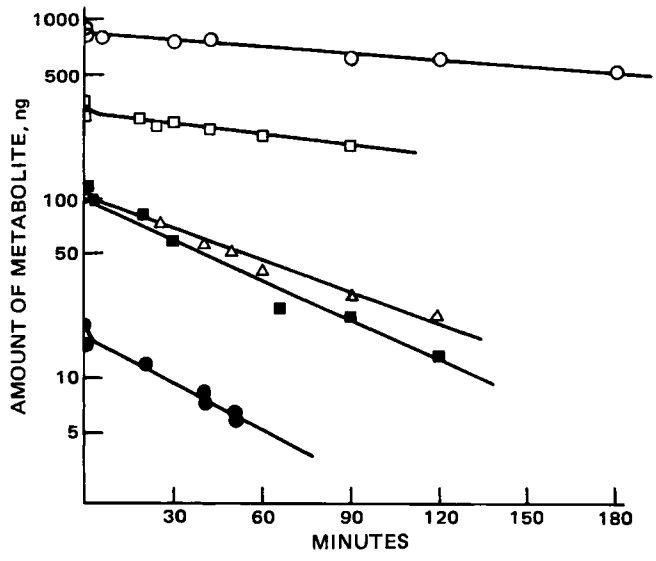

Figure 5—*Relationship between the logarithm of the amount of nitroglycerin metabolite extracted from 2 ml of red cell suspensions at normal erythrocyte count and time in minutes. The suspensions at 37° were incubated with varying initial concentrations of metabolite. The concentrations of the metabolite (I = 1,2-dinitroglycerin; II = 1,3-dinitroglycerin) used, and the interpolated half-lives were: (○) II, 480 ng/ml, 228 min; (□) II, 180 ng/ml, 141 min.; (△) I, 60 ng/ml, 51 min; (■) II, 60 ng/ml, 40 min; (●) II, 10 ng/ml, 33 min.*

dinitroglycerin (1 ng), 1,3-dinitroglycerin (1 ng), nitroglycerin (1 ng), and internal standard. The base line for a red cell blank containing no drug or metabolite is given in Fig. 2B and clearly shows no interfering materials. If metabolites were present in the blood or red cell systems, as presented in Fig. 1, the assay would have detected them.

The loss of nitroglycerin in human plasma at the same initial concentration as in whole blood or in red cell suspension follows an apparent first-order process (Fig. 1) without a detectable rapid initial phase. The reaction occurs at a much slower rate in comparison with blood studies: half-life 130 min at an initial concentration of 50 ng/ml at 37°. This slower rate in plasma was also observed by Armstrong *et al.* (1) who reported a half-life of 53 min and by Maier *et al.* (5) who reported a half-life of 175 min. The latter group used a slightly diluted sample. The decomposition of nitroglycerin in plasma may be associated with its interaction with protein, since protein-free plasma and normal saline solution containing the same initial concentration of nitroglycerin show no drug loss (Fig. 1). It is not possible to unequivocally state that the loss of nitroglycerin in plasma does not result in dinitroglycerin formation. The levels of 1,2-dinitroglycerin or 1,3-dinitroglycerin that would be expected over a 1-hr period would not be detected with any certainty. The reason for this is that the detector sensitivity for the dinitro compounds is about one-half that of nitroglycerin (8). The interaction of nitroglycerin with sulfhydryl groups (e.g., on protein) *via* a nonenzymatic process would generate denitrated compounds. This is a possibility since the nitroxyl groups of nitroglycerin should be good leaving groups. In support of this possible reactivity, Needleman reported that the interaction of nitroglycerin with sulfhydryl groups leads to the same products in the presence or absence of enzyme (10).

The effect of various initial concentrations of nitroglycerin on drug loss following its incubation with a constant level of red blood cells at 37° is given in Fig. 3. The loss of nitroglycerin initially at 10, 60, 120, 180, and 480 ng/ml follows a terminal first-order process at all concentrations. At concentrations ≤180 ng/ml an initial fast reaction phase is suggested. The first-order half-life increases as the initial concentration increases. The apparent half-lives as determined by graphical interpolation are: 4 min at 10 ng/ml, 7 min at 60 ng/ml, 16 min at 120 ng/ml, 20 min at 180 ng/ml, and 52 min at 480 ng/ml. Therapeutic levels of nitroglycerin are believed to be in the range of 1.2–11.1 ng/ml (11), and thus the interaction of the drug with red blood cells should probably be a factor to be considered in an analysis of nitroglycerin pharmacokinetics or pharmacodynamics.

At each of the several concentrations of nitroglycerin used in the studies summarized in Fig. 3, no metabolite or hydrolysis product of nitroglycerin accumulated in the medium. This led to the question of whether or not any denitrated products formed and, having reacted very rapidly with cells, did not accumulate in the medium. To get an answer to this question, the kinetics of the loss of the dinitro compounds to red blood cells was undertaken. The assay method utilizing a 3% Carbowax 20M-TPA column and an ethyl acetate extraction provided a method for accurate separation and determination of both dinitro isomers. Figure 4A shows the chromatogram for a mixture of nitroglycerin, *o*-iodobenzyl alcohol (internal standard), and the two isomeric dinitroglycerins. The amounts of components injected were: 1 ng nitroglycerin, 2 ng 1,2-dinitroglycerin, and 1.6 ng 1,3-dinitroglycerin. Figure 4B showing the chromatogram for a red cell system with no drug or metabolite added indicates that no interfering materials are extracted. The amount of metabolite extracted from samples incubated at a constant level of red cells in normal saline at 37° at various times is presented in Fig. 5. After a possible initial phase, the loss of metabolite followed an apparent first-order process at each level of 1,3-dinitroglycerin studied and the single level of 1,2-dinitroglycerin used. The half-lives for the 1,3-isomer interpolated from the plots increased as the initial concentration increased: at 10 ng/ml, 33 min; at 60 ng/ml, 40 min; at 180 ng/ml, 141 min; and at 480 ng/ml, 228 min. Although the same trend is found for the dependence of half-life on initial concentration as for nitroglycerin, the reaction at comparable concentrations is much slower for the dinitro compound. This suggests that if nitroglycerin loss results in the formation of metabolites released to the medium, the metabolites should have been detected. Figure 5 also shows that the first-order rate of loss of the 1,2-isomer is about the same as the 1,3-isomer. When both are initially present at 60 ng/ml, the half-life interpolated for 1,2-dinitroglycerin is 51 min and that for 1,3-dinitroglycerin is 40 min. Therefore, even if one isomer were preferred in the denitration of nitroglycerin, it would have been detected in the loss of the drug incubated with red blood cells. It would also seem that whatever the mechanism of loss of nitroglycerin to red blood cell is, the loss of metabolite may follow a similar mechanism since the same general kinetic behavior is found for it.

Although the mechanism of loss of nitroglycerin incubated with erythrocytes is not known, based on the observations presented here and elsewhere (3, 12) it appears that the loss of nitroglycerin to red blood cells need not be enzymatic in nature, although the possibility is not entirely eliminated. This evidence is primarily the absence of metabolite in the medium following the loss of nitroglycerin, and the inactivity toward nitroglycerin of the glutathione transferase isolated from erythrocytes as reported by Marcus *et al.* (12). The erythrocyte enzyme designated as the ρ form is quite different from the most active δ form found in the liver or indeed different from any of the liver forms, α–ϵ (12). The unusual rate dependence (Fig. 3) and the absence of metabolite or hydrolysis product suggest that the loss could be physical in nature. It would appear that a mixed kinetic process or processes occur when intervening steps involve slow and fast kinetic states. The mechanism (or mechanisms) of nitroglycerin loss in blood should be established since its elucidation will not only impinge on valid pharmacodynamic analysis but even impinge on factors such as obtaining meaningful blood level determinations. Studies are currently being undertaken to explore an adsorption/ab-

sorption mechanism. No particular mechanism is concluded, nor has an enzymatic mechanism been conclusively eliminated. If the interaction with red cells is physical in nature, it is possible that interaction with other biotissues can be quite significant and that what has been found with red blood cells is only one of many important interactive phenomena. Indeed, McNiff *et al.* have recently suggested that the target tissue (blood vessels) for nitroglycerin might have a higher concentration of drug than found in plasma (13). Furthermore, Armstrong *et al.* conclude that the intact *nitroglycerin molecule is essential* for initiation of relaxation based on dose-response curves of nitroglycerin effects on phenylephrine-contracted canine dorsal pedal arteries and medial saphenous veins (14). These workers found that relaxation occurred without the release of detectable amounts of metabolites into the incubation medium.

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Liquid Crystal Solubilization of Cholesterol: Potential Method for Gallstone Dissolution

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Abstract □ Solubilization rate and phase equilibrium studies were conducted for cholesterol in aqueous sodium oleate solutions. The components interacted to form a lamellar liquid crystalline phase, and this phenomenon was investigated as a potential method for cholesterol gallstone dissolution. Phase equilibria data for cholesterol–sodium oleate–water showed that the mesophase contained approximately equimolar amounts of cholesterol and oleate with large amounts of water. The cholesterol solubilization rate from a static pellet in sodium oleate solutions was much faster than dissolution in sodium cholate solutions and was independent of oleate concentration from 2.5 to 10%. In these experiments, the medium became a cloudy disperson of liquid crystalline phase in the micellar solutions. The rate-limiting step in the solubilization process appears to be dispersion of fragments from the liquid crystalline layer on the cholesterol surface. This hypothesis was consistent with the kinetic effects of viscosity, stirring rate, and oleate concentration. By converting cholesterol to a liquid crystalline phase, the solubilization process avoids the limitations of micellar solubility and interfacial resistance which control cholesterol dissolution in bile salt-containing media.

Keyphrases □ Cholesterol—solubilization in fatty acid salt solutions, dissolution in bile salt solutions, potential method for gallstone dissolution □ Solubilization—cholesterol in fatty acid salt solutions, bile salt solutions, potential method for gallstone dissolution

Cholecystectomy is the primary method for elimination of gallstones, which are composed primarily of cholesterol. In ~5% of cases, because of size or location, some stones in the ductal system cannot be removed using physical extraction techniques and are retained (1). A number of approaches have been tried for *in situ* dissolution of common bile duct stones using solvents such as ether and chloroform and solutions of bile salts or heparin (2). Recent clinical studies have shown that infusion of monooctanoin (glyceryl monooctanoate), an excellent solvent for cholesterol *in vitro* (3), dissolves common duct stones in 50–70% of patients, although treatment for 2–3 weeks is required (4, 5). The solution is infused into the bile duct *via* a T-tube drain in postcholecystectomy patients or through a nasobiliary tube inserted using a duodenoscope.

This paper reports initial investigations toward development of liquid crystal solubilization as a method for cholesterol gallstone dissolution. Ekwall *et al.* (6, 7) reported the formation of liquid crystalline droplets on the surface of cholesterol monohydrate crystals suspended in fatty acid salt solutions. Longer chain-length salts interacted more strongly with cholesterol than did shorter ones. In the present study, the rate of solubilization of cholesterol was much faster than simple dissolution in sodium cholate solutions. The cholesterol was present in the medium primarily in the form of droplets of liquid crystalline phase dispersed in the micellar solution.

EXPERIMENTAL

Phase Equilibria—Mixtures of cholesterol (1.6–11.8%), sodium oleate (1.2–14.8%), and water were weighed into glass ampules. The ampules were flushed with nitrogen, sealed, warmed to 80° in a water bath, cooled to room temperatures (22–24°), and then allowed to stand for 3 weeks with periodic shaking. A sample of the contents of each ampule was ob-

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