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Free Fatty Acid-Induced Platelet Aggregation: Studies with Solubilized and Nonsolubilized Fatty Acids

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Abstract □ The aggregation response of washed porcine platelets to the sodium salts of stearic, oleic, palmitic, and myristic acids was analyzed turbidometrically. The fatty acids were prepared as aqueous suspensions and as taurocholate- or albumin-solubilized systems. The final concentration of fatty acid in the platelet preparation varied between 70 and 600 μM. This range was within or below the normal physiological limits of 300–1200 μM. Platelet aggregation was observed with both the suspended and taurocholate-solubilized fatty acids. The extent of platelet aggregate formation increased with the fatty acid concentration and chain length. With the exception of stearate, the taurocholate-solubilized fatty acids were more active than the suspensions. Albumin-solubilized fatty acids were devoid of platelet aggregating activity. Particle-size analysis of the solubilized fatty acids indicated that fatty acid precipitation had occurred subsequent to the addition of taurocholate-solubilized fatty acids to the platelets. This precipitation did not occur with the albumin-solubilized systems, suggesting that the fatty acids must assume a particulate physical state to induce aggregation. Platelet aggregation induced by fatty acids was not inhibited by 80 nM epoprostenol, 75 μM alprostadil, or 150 μM indomethacin. This finding indicated that the fatty acid-induced platelet aggregation was independent of cyclic AMP-related calcium shift, cyclooxygenase–arachidonate, or granular nucleotide release mechanisms.

Keyphrases □ Fatty acids—induced platelet aggregation, solubilized and nonsolubilized fatty acids □ Platelets—free fatty acid-induced aggregation, solubilized and nonsolubilized fatty acids □ Coagulation—free fatty acid-induced platelet aggregation, solubilized and nonsolubilized fatty acids

Platelets are capable of several reactions necessary for participation in the hemostatic response. They "stick" to exposed subendothelial components and artificial surfaces (adhesion), as well as to one another (aggregation), in response to seemingly diverse stimuli. In addition, platelets release endogenous, pharmacologically active substances that accentuate adhesion, aggregation, and coagulation reactions. This reaction is referred to as platelet release. Thus, platelets can initiate and stimulate several phases of the overall coagulation process, although the aggregation reaction alone results in effective hemostatic blockage in small blood vessels and capillaries (1).

BACKGROUND

Thrombin, collagen, adenosine diphosphate (ADP), epinephrine, and long chain ($>C_{14}$) unesterified fatty acids are compounds encountered *in vivo* that have been shown to initiate platelet aggregation (1–10). Platelets normally remain unaggregated in most disease-free individuals. That they aggregate in response to various threats to hemostasis suggests a delicate balance between circulating platelets and the aggregation-initiating substance(s). Disruption of this balance may result in either control or exacerbation of disease. For example, the normal response to vascular injury involves the production of thrombin and subsequent formation of a fibrin network (11). In this case, platelet aggregation induced by thrombin would be a disease controlling reaction through augmentation of thrombus formation to control bleeding. In contrast, disease states that result in elevated blood fatty acid levels are accompanied by an increased incidence of thromboembolism and platelet hyperaggregability in the absence of vascular injury (12–17). Here the platelet aggregation response exacerbates the disease condition.

The delicate balance between platelet aggregation or nonaggregation in response to a stimulus is exemplified by the platelet–fatty acid interaction. Numerous studies (6–10) have demonstrated hypercoagulability and platelet aggregation both *in vivo* and *in vitro* in response to administered fatty acid concentrations within the normal physiological range of 300–1200 μM. The fact that most individuals are free from platelet aggregation abnormalities when *in situ* fatty acid levels range from 300 to 1200 μM suggests that the physical state of the administered free fatty acids is a critical determinant of aggregating activity.

It has been argued that the platelet aggregating activity of free fatty acids is related to micellar aggregates formed in aqueous solution (8–10, 18). Since the critical micellar temperature is >37° for most poorly soluble long chain fatty acids (19), a suspension state, rather than a micellar state, is anticipated. Inspection of data (7, 8, 10, 18) correlating platelet aggregating activity with free fatty acid chain length leads to the generalization that aggregating activity is negligible below C_{14}, with activity increasing markedly with chain lengths up to C_{20}; *i.e.*, the fatty acids least likely to form micelles exhibit the greatest platelet aggregating activity. This trend holds for both saturated and unsaturated fatty acids when the aggregation of washed platelet preparations *in vitro* is examined (9, 10, 20).

In the present study, the aggregation of washed porcine platelets was investigated as a function of the physical state of various fatty acid preparations. Final washed platelet fatty acid concentrations (70–600 μM) within normal physiological limits were examined (21, 22). The sodium salts of stearic, palmitic, myristic, and oleic acids were prepared

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as suspensions in buffered isotonic saline or as solubilized aqueous systems with either sodium taurocholate or albumin as the solubilizing agent. The effects of the nonspecific platelet aggregation inhibitors alprostadil, epoprostenol, and indomethacin (23) serve as a basis for mechanistic interpretations of the fatty acid-induced platelet aggregation reaction.

EXPERIMENTAL

Slaughterhouse porcine blood was collected into polyethylene vessels containing 7.5 ml of 0.077 *M* edetate disodium[1] and 0.71 *M* anhydrous α-D-glucose[1]/92.5 ml of whole blood as an anticoagulant. The pH of the anticoagulant solution was adjusted to 7.4 with sodium hydroxide. Collected blood was placed into an ice bath until processed further.

Aqueous suspensions of oleic[1], palmitic[1], or myristic[1] acid were gently heated with a slight molar excess of aqueous sodium hydroxide to form the respective sodium salts. The pH values of these solutions were >10.0 after complete dissolution of the fatty acid. The solutions were cooled to room temperature; the insoluble fatty acid salt was collected and washed repeatedly with deionized water to remove excess sodium hydroxide. This product was vacuum dried at 30° for 24 hr. Sodium stearate[1] was used as received. All fatty acids were >99% pure as listed by the supplier.

Cholic acid[1] was recrystallized repeatedly from 95% ethanol. Purity was checked with UV spectroscopy and TLC, using isoamyl acetate[2]–propionic acid[2]–*n*-propanol[2]–water (40:30:20:10) as the developer. Taurine[1] was used as received. Sodium taurocholate was synthesized according to Lack *et al.* (24). The synthesized sodium taurocholate was found to be pure by TLC.

Epoprostenol[3] (PGI_2), alprostadil[3] (PGE_1), indomethacin[1], disodium adenosine diphosphate[1], fibrinogen[4] (porcine, fraction I), and albumin[1] (porcine, fraction V) were used as received. Reagents[1,2] in the phosphate, tromethamine, Tyrodes, and imidazole buffers were reagent grade and used as received.

Washed porcine platelets were prepared by Haslam's method (25) at 5°. Polycarbonate centrifuge tubes were used for all separations. Final suspension of platelets was in a 9:1 solution of 0.15 *M* NaCl and 0.15 *M* tromethamine buffer, pH 7.4. Platelets that did not resuspend immediately with gentle agitation were discarded. Platelets were counted and examined under phase contrast light microscopy[5] at 600× in a hemocytometer. The platelets were seen as individual entities without pseudopod formation, an observation that was confirmed by scanning electron microscopy[6] at 2K magnification. On this basis, the platelets were judged to be of normal morphology and in the unaggregated state. Platelet counts were adjusted to ~700,000/mm^3 for all aggregation experiments. Platelets prepared in this manner, and subsequently stored in an ice bath, remained viable for >6 hr without spontaneous aggregation.

The platelet aggregation reaction was followed turbidimetrically (26) with a commercially available aggregometer[7]. Aggregation was recorded as an increase in the monochromatic (600 nm) light transmittance of a washed platelet suspension with time. Results were relative to blanks containing all reagents except platelets. The 100% transmittance baseline was set with the blanks. Thus, the magnitude of the light transmittance was directly proportional to the extent of platelet aggregation.

For the aggregation experiments, 500 μl of washed porcine platelet suspension was added to a siliconized aggregation cell. Then 20 μl of 0.34 m*M* $CaCl_2$ and 40 μl of 0.11 mg of fibrinogen/ml were added. The system was equilibrated to 37° (~3 min) before proceeding. Under these conditions, the platelets did not aggregate spontaneously during 15 min of observation.

Aggregation was induced with either disodium adenosine diphosphate or fatty acid. Disodium adenosine diphosphate was prepared at a concentration of 2.4 m*M* in an isotonic imidazole–hydrochloric acid buffer, pH 6.8. The fatty acids were prepared at a concentration of 4 m*M* in isotonic phosphate buffer, pH 7.4, with and without 47 m*M* sodium taurocholate or 26 g % albumin as a solubilizing agent. Addition of 80 μl of the disodium adenosine diphosphate solution or 10–100 μl of the various fatty acid preparations to the calcium and fibrinogen equilibrated platelets marked the starting point of the aggregation reaction. Final reagent concentrations were 1.03 m*M* Ca^{2+}, 16 μg of fibrinogen/ml, 0.29 m*M* disodium adenosine diphosphate, 70–600 μ*M* fatty acid, 0.83–7.12 m*M* sodium taurocholate, and 4.0 g % albumin.

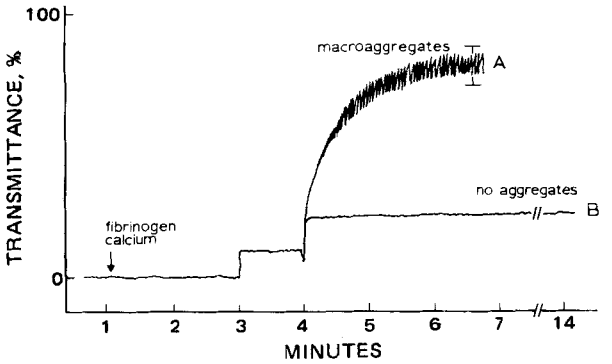

Figure 1—*ADP-induced platelet aggregation. Reagent concentrations were: A, 0.29 m*M *ADP; and B, 0.29 m*M *ADP with 75 μ*M *alprostadil or 80 n*M *epoprostenol. Each reagent was administered at 3 min.*

The effects of epoprostenol, alprostadil, and indomethacin on fatty acid-induced platelet aggregation were investigated. The prostaglandins were prepared in buffered solution at the respective pH values of maximum stability[3] immediately prior to addition to the platelets to minimize aqueous degradative effects. Indomethacin was freshly prepared each day. Epoprostenol was prepared at a concentration of 57.0 μ*M* in isotonic tromethamine buffer, pH 8.7. Alprostadil was prepared at a concentration of 0.5 m*M* in isotonic phosphate buffer, pH 7.4. Indomethacin was prepared at a concentration of 1.0 m*M* in Tyrodes' buffer, pH 7.4. The final concentrations in the platelet media were 80 n*M* epoprostenol, 75 μ*M* alprostadil, and 150 μ*M* indomethacin. Additions of these inhibitors to the platelets were made ~1 min prior to the fatty acid or disodium adenosine diphosphate additions.

Behavior of the solubilized fatty acid preparations in the aggregation cell was investigated under simulated conditions. The sodium taurocholate or albumin-solubilized fatty acid preparations were diluted to the same extent as in the aggregation cell. Isotonic phosphate buffer, pH 7.4, filtered three times through 0.22-μm microporous filters[8] was used as the diluent for all procedures. These samples were analyzed as a function of time and particle size with an electronic particle analysis instrument[9] equipped with a 30-μm aperture tube and a 100-μl manometer. The instrument was calibrated with 2.2-μm latex beads. The minimum detection limit was ~0.45 μm (diameter) under these conditions. Analysis of particle size with suspended fatty acid (nonsolubilized) preparation could not be completed due to the blockage of both the 30- and 100-μm aperture tubes. This finding indicated the presence of at least a small percentage of very large particles in these suspensions.

RESULTS

The aggregation response to 0.29 m*M* ADP was followed turbidimetrically as shown in curve A of Fig. 1. This tracing was the average platelet aggregation response of five animals. The aggregation was immediate and monophasic, leading to macroscopic aggregates (macroaggregates) visible to the unaided eye. Higher concentrations of ADP did not result in further increases in light transmittance. Curve B shows that the addition of either 75 μ*M* alprostadil or 80 n*M* epoprostenol completely inhibited ADP-induced platelet aggregation. The stepwise increases in light transmittance at 3 and 4 min reflected the dilution of the washed platelet preparations by the reagent additions and did not represent aggregation. The imidazole–hydrochloric acid buffer system showed no intrinsic platelet aggregating activity.

The relative platelet aggregating activities of various fatty acid suspensions are compared in Fig. 2. There were no distinguishable differences in the average aggregation patterns elicited by the saturated (stearate) or monounsaturated (oleate) C_{18} fatty acids at a final concentration of 600 μ*M*. Aggregation proceeded rapidly with production of large macroaggregates in a clear medium. The response to 600 μ*M* palmitate (C_{16}) differed from the C_{18} fatty acids in the time course to macroaggregate formation. Though immediate in onset, the palmitate-induced aggregation proceeded somewhat slower than the stearate or

[1] Sigma Chemical Co., St. Louis, MO 63178.
[2] J. T. Baker Chemical Co., Phillipsburg, NJ 08865.
[3] Courtesy of The Upjohn Co., Kalamazoo, MI 49001.
[4] Nutritional Biochemicals Corp., Cleveland, OH 44128.
[5] Nikon Biophot, Nippon Kogaku Inc., Garden City, NY 11530.
[6] Cambridge Mark II A SEM, Cambridge, England.
[7] Model 335, Chronolog Corp., Havertown, PA 19083.
[8] Millipore Corp., Bedford, MA 01730.
[9] Coulter Counter model ZBI, Coulter Electronics, Hialeah, Fla.

Table I—Final Sodium Taurocholate Concentrations in Relation to Solubilized Fatty Acid Concentrations

Final Solubilized Fatty Acid Concentrations, μM	Final Sodium Taurocholate Concentrations, mM
600	7.12
320	3.85
170	2.01
70	0.83

oleate reactions. Myristate (C_{14}) was significantly less active than either the C_{16} or C_{18} fatty acid suspension. The aggregation response to myristate was minimal and did not result in visible aggregate formation. Thus, a general trend of aggregating activity increasing with chain length was observed when fatty acid suspensions were added to washed porcine platelets.

The data presented in Fig. 3 show the general relationship between fatty acid concentration and platelet aggregation. The speed and extent of palmitate-induced aggregation increased with fatty acid concentration. The 320 and 600 μM additions resulted in macroaggregate formation. Generally, this trend was also followed by oleate and stearate. However, as anticipated from Fig. 2, the C_{18} fatty acids were more active than equivalent amounts of palmitate, with macroaggregates formed at all concentrations examined (70–600 μM).

The platelet aggregating activities of equal concentrations of fatty acids in suspended and solubilized physical states were compared (Fig. 4). Sodium taurocholate was the solubilizing agent. The final taurocholate concentrations were related to the final fatty acid concentrations (Table I). Figure 4*a* shows that platelet macroaggregates formed more rapidly with taurocholate-solubilized 70 μM oleate than with suspended 70 μM oleate. Taurocholate-solubilized palmitate and myristate systems also were more active than the suspended counterparts at equivalent fatty acid concentrations (Figs. 4*b* and 4*c*). The 170 μM palmitate and 600 μM myristate suspensions elicited a weak platelet aggregation reaction. However, taurocholate-solubilized 170 μM palmitate and 600 μM myristate elicited platelet aggregation responses that led to macroaggregate formation. Comparisons between solubilized and suspended oleate, palmitate, and myristate at concentrations not shown in Fig. 4 followed this same trend; taurocholate-solubilized fatty acids were more active inducers of aggregation than the respective suspension counterparts. Sodium taurocholate alone was devoid of activity at all concentrations examined (Table I).

The suspended 70 μM stearate system was more active than the taurocholate-solubilized 70 μM stearate (Fig. 4*d*). At higher stearate concentrations, this same pattern was noted; however, macroaggregates were produced with both the taurocholate-solubilized and suspended forms. Thus, a reversal in the trend observed with the other fatty acids was noted with stearate.

The aggregating activities of taurocholate-solubilized oleate, stearate, palmitate, and myristate are compared in Fig. 5. Oleate (70 μM) elicited a rapid platelet aggregation response with macroaggregate production. Stearate, palmitate, and myristate were completely devoid of aggregating activity at this concentration.

The aggregation response to albumin-solubilized fatty acids is shown in Fig. 6. Turbidimetric tracings and light microscopy both indicated that platelet aggregation had not occurred in response to either albumin-solubilized 600 μM oleate or palmitate. In the suspended state, these

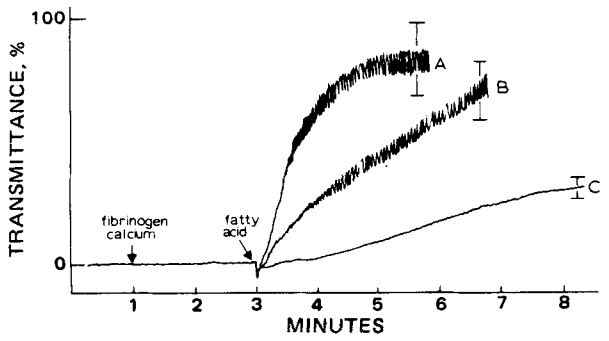

Figure 2—*Platelet aggregation response to 600 μM fatty acid suspensions as a function of chain length. Key: curve A, oleate or stearate (C_{18}); curve B, palmitate (C_{16}); and curve C, myristate (C_{14}).*

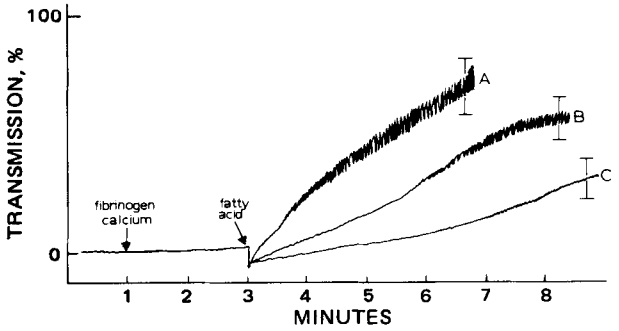

Figure 3—*Platelet aggregation as a function of the final suspended fatty acid concentration. Key: curve A, 600 μM palmitate; curve B, 320 μM palmitate; and curve C, 170 μM palmitate.*

concentrations of oleate and palmitate both elicited an immediate platelet aggregation response leading to macroaggregate formation (Fig. 2).

With macroaggregate production, the formation of a fibrous mucoid network subsequent to aggregate appearance was noted and was postulated to be fibrin. In platelet-free blanks, this network did not form. To

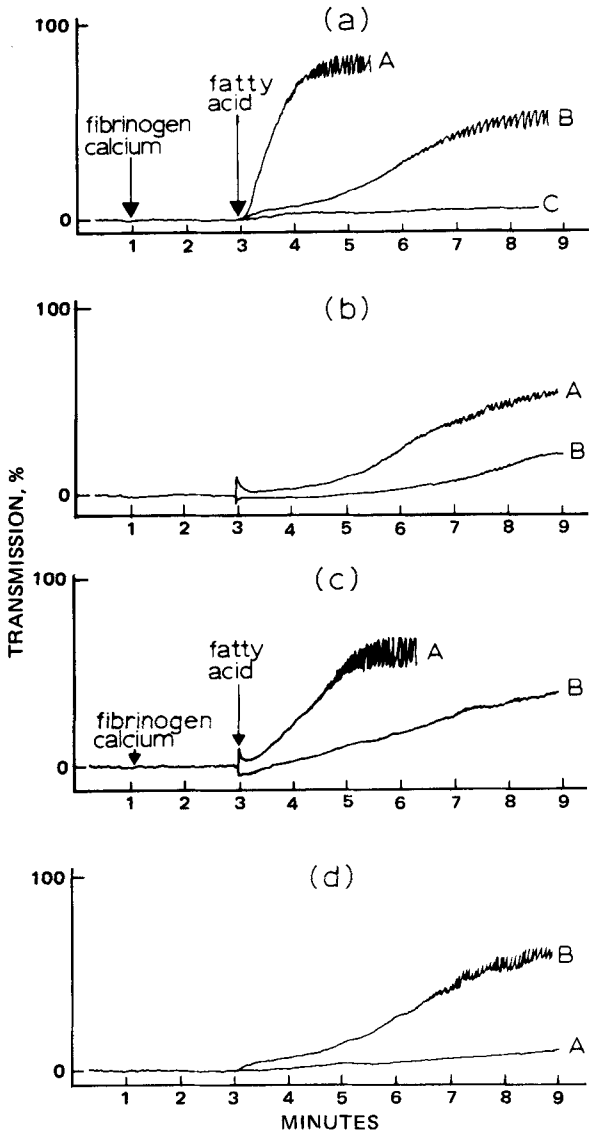

Figure 4—*Platelet aggregation as a function of the physical state of 70 μM oleate (a), 170 μM palmitate (b), 600 μM myristate (c), and 70 μM stearate (d). Key: curves A, sodium taurocholate-solubilized fatty acids; curves B, suspended fatty acids; and curve C, sodium taurocholate without fatty acid.*

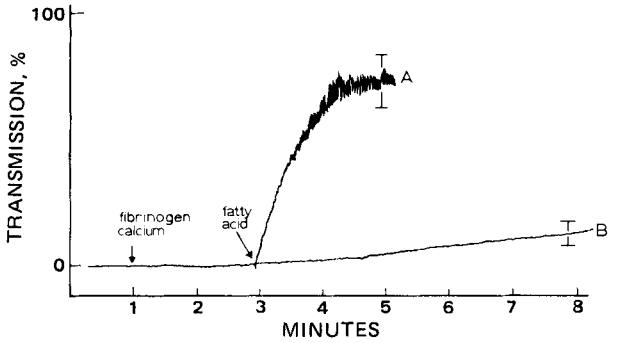

Figure 5—*Platelet aggregating activities of taurocholate-solubilized 70 μM fatty acids. Key: curve A, oleate; and curve B, stearate, palmitate, or myristate.*

determine its significance in relation to platelet aggregation, the calcium and fibrinogen requirements of washed porcine platelet aggregation were studied. Aggregation was induced with taurocholate-solubilized 170 *μM* oleate. Curve A in Fig. 7 shows that macroaggregate formation did not depend on fibrinogen. Without fibrinogen, the aggregation pattern was essentially identical to that with fibrinogen, although the fibrous mucoid network did not form in the absence of fibrinogen. Curve B of Fig. 7 reveals that calcium was required for aggregation. Without calcium, neither platelet aggregates nor the fibrous mucoid network formed. Although fibrinogen was not a requirement for aggregation, both fibrinogen and calcium were included in all trials to facilitate comparison with the predominant methodology in the literature.

The effects of alprostadil, epoprostenol, and indomethacin on platelet aggregation induced by suspended fatty acids are given in Fig. 8. A monophasic aggregation pattern leading to macroaggregate formation proceeded with or without the inhibitors. The time courses and extents of aggregation were not altered or inhibited by these agents.

The observed platelet aggregation patterns elicited by suspended or taurocholate-solubilized stearate are given in Fig. 9. Inhibitors were present as indicated. Aggregation activity increased with the taurocholate-solubilized stearate concentration up to 320 *μM*. The solubilized 600 *μM* system was somewhat less active than the solubilized 170 and 320 *μM* systems. However, macroaggregates formed with all but the taurocholate-solubilized 70 *μM* stearate system within the 10-min observation period. The platelet aggregation response to the taurocholate-solubilized stearate systems was unaltered by the inhibitors. The activity of the suspended stearate systems increased with concentration. Platelet aggregation induced by suspended stearate was more rapid than with taurocholate-solubilized stearate at all concentrations. The platelet response to suspended stearate was unaltered by the inhibitors; the possible exception was the aggregation induced by suspended 70 *μM* stearate. In this case, a decreased aggregation response was observed with both alprostadil and indomethacin. However, aggregate formation still was extensive.

Platelet aggregation patterns observed subsequent to additions of taurocholate-solubilized oleate are given in Fig. 10. Inhibitors were present as indicated. The 70 and 170 *μM* solubilized systems (Fig. 10*a*) induced a monophasic macroaggregate reaction. The response to the 320 and 600 *μM* solubilized oleate was anomalous. The 320 *μM* addition (Fig. 10*b*, curve A) elicited a biphasic response. Small aggregates appeared

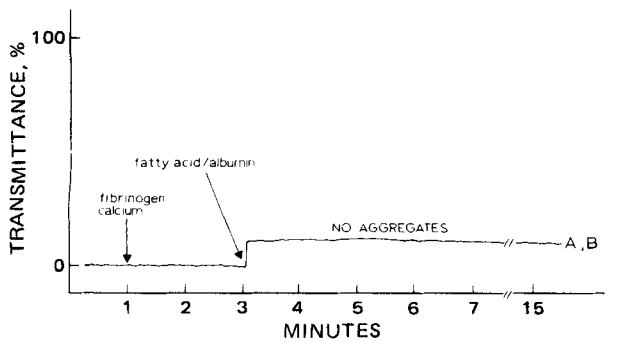

Figure 6—*Platelet aggregation response to albumin-solubilized fatty acids. Final fatty acid concentrations were: A, 600 μM oleate; and B, 600 μM palmitate. The final albumin concentration was 4 g %.*

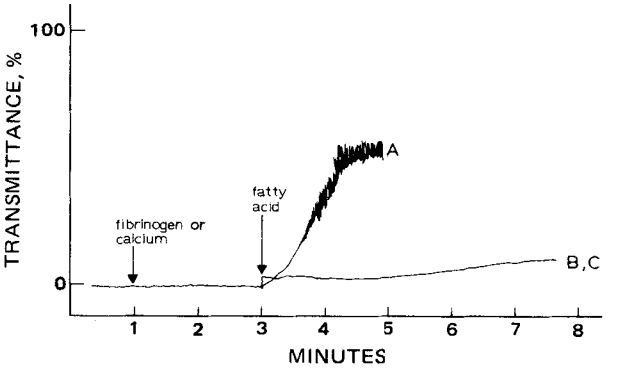

Figure 7—*Calcium and fibrinogen requirements of washed porcine platelet aggregation. Aggregation was induced with taurocholate-solubilized 170 μM oleate under the following conditions: curve A, 1 mM calcium without fibrinogen; curve B, 16 μg of fibrinogen/ml without calcium; and curve C, no calcium or fibrinogen.*

during the first phase and then disappeared during the second phase, leaving a clear solution devoid of visible aggregates. The 600 *μM* system elicited a monophasic response that also resulted in a clear, aggregate-free, solution.

Platelets were not visible in either of these clear solutions under 600× phase contrast light microscopy. Scanning electron micrographs at 2200× revealed unidentifiable debris. This finding was viewed as evidence for platelet lysis in response to the taurocholate-solubilized 320 and 600 *μM* oleate systems. This behavior was not observed with taurocholate alone or with any other taurocholate-solubilized fatty acid system examined. The aggregation inhibitors did not alter the observed responses to taurocholate-solubilized oleate except at the 320 *μM* concentration. Curve B of Fig. 10*b* reveals that epoprostenol actually enhanced the aggregate production. In the presence of epoprostenol, a monophasic response resulting in stable macroaggregate formation was observed.

The behavior of the taurocholate-solubilized oleate and stearate solutions was studied. Samples were diluted to the same concentrations used in the aggregation experiments and were examined with an electronic particle-size analyzer (Figs. 11 and 12). In all cases, the number of particles above 0.72 μm was negligible over the 10-min observation period. No particles were detected in the 4 m*M* fatty acid–47 m*M* sodium taurocholate stock solutions prior to dilution.

Dilution of a taurocholate-solubilized stock solution of oleate to 70 and 170 *μM* oleate resulted in rapid particle formation in the 0.45–0.72-μm diameter range. The taurocholate-solubilized 320 and 600 *μM* oleate systems precipitated much less. Particles were not detected in a 7.12 m*M* taurocholate system containing no fatty acid.

The behavior of taurocholate-solubilized stearate contrasted with oleate in that precipitation increased as the stearate concentration increased. However, in all cases, the extent of the stearate precipitation was less than with the taurocholate-solubilized 70 and 170 *μM* oleate systems.

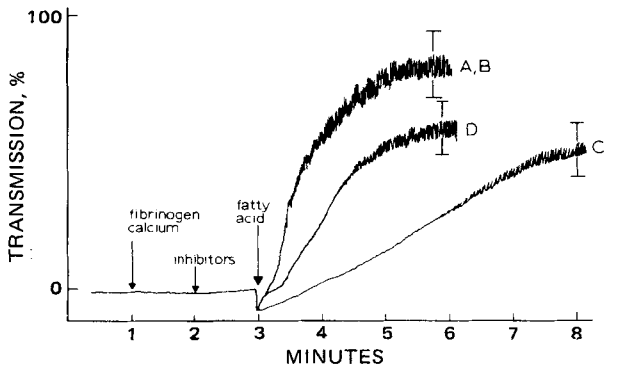

Figure 8—*Effects of alprostadil, epoprostenol, and indomethacin on platelet aggregation induced by suspended fatty acids. Key: curve A, 600 μM stearate with and without 75 μM alprostadil, 80 nM epoprostenol, or 150 μM indomethacin; curve B, 600 μM oleate with and without 150 μM indomethacin; curve C, 320 μM palmitate with and without 80 nM epoprostenol; and curve D, 170 μM oleate with and without 80 nM epoprostenol, 75 μM alprostadil, or 150 μM indomethacin.*

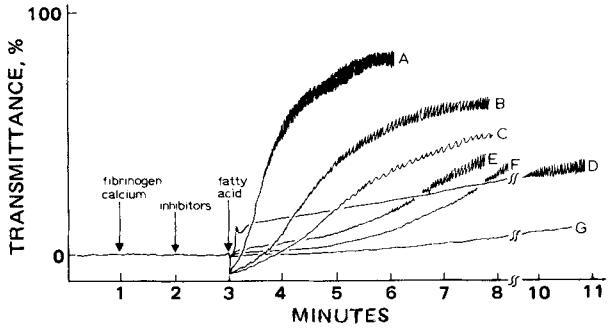

Figure 9—*Platelet aggregation as induced by taurocholate-solubilized and suspended stearate. Key (suspended stearate): curve A, 600 μM stearate with and without 75 μM alprostadil, 80 nM epoprostenol, or 150 μM indomethacin; curve B, 70 μM stearate; and curve C, 70 μM stearate with 75 μM alprostadil or 150 μM indomethacin. Key (taurocholate-solubilized stearate): curve D, 600 μM stearate with and without 80 nM epoprostenol; curve E, 320 μM stearate with and without 75 μM alprostadil, 80 nM epoprostenol, or 150 μM indomethacin; curve F, 170 μM stearate with and without 80 nM epoprostenol; and curve G, 70 μM stearate.*

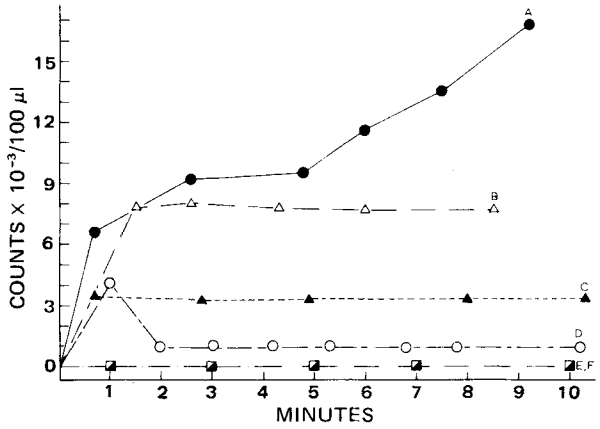

Figure 11—*Particle-size analysis of 4 mM oleate solubilized with either 47 mM sodium taurocholate or 26 g % albumin subsequent to dilution to 70 μM oleate/0.83 mM taurocholate (curve A), 170 μM oleate/2.01 mM taurocholate (curve B), 320 μM oleate/3.85 mM taurocholate (curve C), 600 μM oleate/7.12 mM taurocholate (curve D), 7.12 mM taurocholate (curve E), and 600 μM oleate/4 g % albumin (curve F). Size range was 0.45–0.72 μm in diameter.*

Prevention of fatty acid precipitation from the solubilized systems was possible if the final sodium taurocholate concentration was 47 m*M*. However, this concentration resulted in immediate and complete lysis of platelets. To alleviate this problem, it was necessary to replace taurocholate with another solubilizing agent, albumin, that would solubilize the fatty acids without lysing platelets. Particle-size analysis of albumin-solubilized oleate and palmitate (Figs. 11 and 12) indicated that no particle formation occurred above the 0.42-μm limit of detection. Light microscopy confirmed that the platelets were not lysed or visibly damaged by the albumin concentration employed.

DISCUSSION

Porcine platelets resemble human platelets in several key areas (27): (*a*) membrane proteins; (*b*) monophasic aggregation induced by high concentrations of ADP; (*c*) potent inhibition of ADP, thrombin, and collagen-induced aggregation by alprostadil and inhibition of ADP by AMP; and (*d*) similar nucleotide content in the platelet storage granules. The present report indicates that the washed porcine platelet system can be used to distinguish the platelet aggregating activities of ADP and various fatty acids in suspended or solubilized physical states. Therefore, the porcine platelets were believed to be a reasonable *in vitro* model for the simulated study of human platelet behavior.

The platelet aggregation reaction is complex. To correlate the platelet responses to the many and varied aggregation inducing and inhibiting agents, a basic platelet reaction hypothesis has been proposed (23). Under this system, induction of platelet aggregation occurs with the interaction of inducing agents with receptor sites located on the platelet surface; changes thus induced lead to liberation of a transmitter substance [believed to be calcium ions (23, 28–30)] into the cytosol. The transmitter stimulates ATP-dependent contractile processes leading to aggregation.

Alprostadil and epoprostenol stimulate adenylate cyclase (1, 3, 31–33), which leads to increased production of cyclic adenosine monophosphate (AMP) in platelets. Elevated cyclic AMP results in platelet vesicular uptake of calcium ions from the cytosol (28, 30), which is accompanied by a reduction in platelet aggregation. Other work has shown that alprostadil can effectively inhibit platelet aggregation independent of cyclic AMP (34). The inhibitory effects of prostaglandins on platelet aggregation are thought to occur at the intermediate transmission step in the basic platelet reaction hypothesis and are, therefore, believed to be independent of the type of inducer (23). Literature data support this belief (1, 23, 29–31, 33) and suggest that epoprostenol is 30–40 times more potent than alprostadil (31).

Included in the basic platelet reaction hypothesis is the release of arachidonic acid from platelet membrane phospholipids through the action of phospholipase A_2 (31). The arachidonic acid then is converted into the cyclic endoperoxide prostaglandins G_2 and H_2 by cyclooxygenase. The endoperoxides react by alternate pathways to form thromboxane A_2 and epoprostenol. The endoperoxides and thromboxane A_2 are potent inducers of platelet aggregation while epoprostenol is a potent inhibitor (31, 35). It has been proposed that the balance among these compounds of common origin determines whether aggregation occurs *in vivo* (31). Indomethacin inhibits cyclooxygenase (36–38), thus preventing platelet aggregation associated with endoperoxide formation.

The aggregation response of the washed porcine platelets to 0.29 m*M* ADP was classically monophasic (Fig. 1), proceeding rapidly to macroaggregate production. The complete inhibition of this reaction by 75 μ*M* alprostadil or 80 n*M* epoprostenol agreed qualitatively with the literature (27, 39). However, on a quantitative basis, the ADP concentration was 10–20 times higher than normally used in *in vitro* aggregation studies (1, 3, 23, 29, 31, 34). This finding suggests that ADP-related aggregation was effectively eliminated by the prostaglandins.

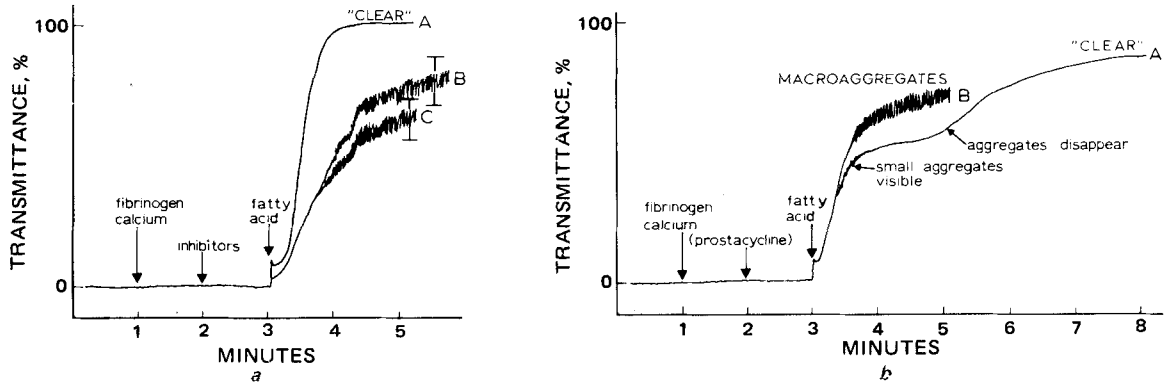

Figure 10—*Platelet aggregation as induced by taurocholate-solubilized oleate. Key for* a*: curve A, 600 μM oleate with and without 75 μM alprostadil; curve B, 170 μM oleate with and without 750 μM alprostadil, 80 nM epoprostenol, or 150 μM indomethacin; and curve C, 70 μM oleate with and without 75 μM alprostadil or 150 μM indomethacin. Key for* b*: curve A, 320 μM oleate; and curve B, 320 μM oleate with 80 nM epoprostenol.*

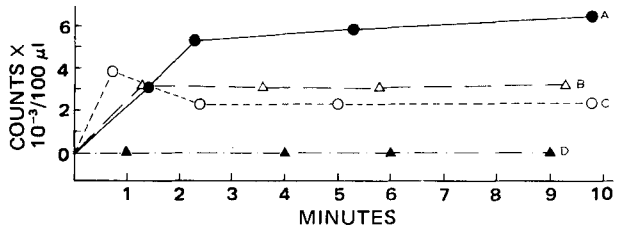

Figure 12—*Particle-size analysis of 4 mM stearate solubilized with 47 mM sodium taurocholate and 4 mM palmitate solubilized with 26 g % albumin subsequent to dilution to 600 µM stearate/7.12 mM taurocholate (curve A), 320 µM stearate/3.85 mM taurocholate (curve B), 70 µM stearate/0.83 mM taurocholate (curve C), and 600 µM palmitate/4 g % albumin (curve D). Size range was 0.45–0.72 µm in diameter.*

The platelet aggregating activity of the suspended fatty acid systems increased with both the fatty acid chain length and concentration (Figs. 2 and 3), in agreement with previous work (7, 8, 10, 18). Unsaturation of the hydrocarbon portion of the fatty acid molecule appeared to have no discernible effect on the platelet aggregation activity. This observation was in general agreement with other work where washed platelet aggregation was induced by fatty acids in suspension (9, 10, 20).

The effects of alprostadil, epoprostenol, and indomethacin on suspended fatty acid-induced platelet aggregation were examined (Figs. 8 and 9). With the possible exception of 70 µM stearate, these agents did not appear to alter the fatty acid-induced aggregation reaction, although they have been reported to inhibit collagen, thrombin, serotonin, epinephrine, vasopressin, ADP, and divalent cation ionophore-induced platelet aggregation (1, 23, 29–31, 34, 40, 41).

Simple correlation of platelet aggregating activity with the fatty acid chain length and concentration was not possible with the taurocholate-solubilized systems. Complex behavior was noted (Figs. 9 and 10), which differed markedly for the oleate as compared to the stearate systems.

A correlation of taurocholate-solubilized oleate-induced platelet aggregation with precipitate formation was found. The taurocholate-solubilized 70 and 170 µM oleate systems elicited immediate platelet aggregation reactions leading to platelet macroaggregates (Fig. 10). Immediate, extensive precipitate formation also was observed with these preparations (Fig. 11). The anomalous platelet response to taurocholate-solubilized 320 and 600 µM oleate was accompanied by a marked reduction in precipitate formation. Apparently, these latter systems remained solubilized to a greater extent, which may be attributed to the higher associated concentrations of taurocholate (Table I).

However, the platelet response to these preparations did not correlate strictly with taurocholate concentration. Taurocholate-solubilized 600 µM stearate (Fig. 9), myristate (Fig. 4), or palmitate or 7.12 mM taurocholate alone did not lyse platelets, although the final taurocholate concentration was equivalent to the solubilized oleate systems that lysed platelets. Thus, the platelet aggregating activity of the taurocholate-solubilized oleate systems appeared to depend on the formation of large numbers of particles subsequent to the addition to the washed platelet preparations. The biphasic response of the platelets to taurocholate-solubilized 320 µM oleate appeared to begin with platelet aggregation, ultimately ending in lysis (Fig. 10). This biphasic transition from the monophasic platelet aggregation response, elicited by the lower oleate concentrations, to the monophasic lysis of platelets by the higher oleate concentrations was a property unique to the taurocholate-solubilized oleate systems and corresponded directly with the extent of precipitation (Fig. 11).

All concentrations of taurocholate-solubilized stearate were less active than taurocholate-solubilized 70 and 170 µM oleate in the induction of platelet aggregation (Fig. 9). Precipitation from the stearate systems (Fig. 12) also was less extensive than from the oleate systems. Thus, although the taurocholate-solubilized oleate and stearate systems had differences in precipitation patterns and platelet aggregating activity, the development of a particulate physical state subsequent to taurocholate–fatty acid additions to washed platelets appeared to be important.

To investigate the dependence of fatty acid-induced platelet aggregation on a particulate physical state, oleate and palmitate were solubilized with albumin. The final albumin concentration subsequent to the addition to the washed platelets was 4 g %, which was sufficient to prevent precipitation of both 600 µM oleate and palmitate (Figs. 11 and 12). Under these conditions, the fatty acids were devoid of aggregating activity (Fig. 6) and did not lyse platelets, suggesting a particulate form of the free fatty acid was necessary to elicit the aggregation response.

The effects of alprostadil, epoprostenol, and indomethacin on the platelet response to taurocholate-solubilized oleate and stearate were examined (Figs. 9 and 10). These agents had no discernible effect on the platelet aggregation reaction except where induced by taurocholate-solubilized 320 µM oleate. In this case, epoprostenol appeared to enhance aggregation by preventing the second phase lysis (Fig. 10*b*). This increased resistance to cell damage associated with prostaglandin exposure was previously noted with red blood cells and gastric mucosal cells (42–45).

The ineffectiveness of alprostadil, epoprostenol, and indomethacin at inhibition of solubilized or suspended fatty acid-induced platelet aggregation suggested that the aggregation was independent of nucleotide release from endogenous storage granules within the platelets, cyclic AMP-related cytosol calcium shifts, and endoperoxide–thromboxane A_2 mechanisms. A particulate physical state for the fatty acids appeared to be prerequisite for platelet aggregating activity. The greater platelet aggregating activity, in general, of the taurocholate-solubilized systems (Fig. 4) suggests that specific particle-size and number requirements exist for optimal interaction, which are best achieved as the fatty acids precipitate out of the solubilized state. Based on normal particle growth kinetics, it is expected that the average particle size of these precipitates present during the aggregation process would be considerably smaller than the average particle size of the nonsolubilized suspensions.

Thus, fatty acid-induced platelet aggregation did not fit the scheme developed in the basic platelet reaction hypothesis (23). Rather, a direct physical interaction of fatty acid particles with platelets, leading to aggregation, appeared to be consistent with the observations. Although the precise role of calcium in these reactions remains unknown, the formation of calcium bridges between negatively charged fatty acid particles and negatively charged platelets (46) would account for both the particulate fatty acid physical state requirements and the ineffectiveness of the aggregation inhibiting agents.

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Pharmacokinetics of Sulfisoxazole Compared in Humans and Two Monogastric Animal Species

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Abstract □ The pharmacokinetic profile of sulfisoxazole was studied and compared in dogs, swine, and humans. The trial was conducted over a 72-hr period after intravenous administration and a 96-hr period after oral administration in dogs and swine. In humans, the trial was conducted over an 8-hr period after oral administration. A two-compartment model system was used to define the pharmacokinetic profile. The mean half-lives for the distribution phase were 4.08, 1.30, and 0.56 hr in dogs, swine, and humans, respectively. For the elimination phase, the mean half-lives were 33.74, 46.39, and 7.40 hr in dogs, swine, and humans, respectively. The mean volume of the central compartment was approximately the same in dogs and swine, 10.6 and 10.5 liters, respectively. Humans had a smaller volume of distribution, 7.7 liters. The steady-state volumes of distribution were 17.2, 30.3, and 16.2 liters in dogs, swine, and humans, respectively. Dogs and swine excreted 42.2 and 30.7%, respectively, of the intravenous dose and 29.4 and 18.3%, respectively, of the oral dose. The bioavailability was 69.8% in dogs and 100.0% in swine. The fraction of drug bound ranged from 30 to 50% in dogs, 40 to 60% in swine, and 25 to 40% in humans.

Keyphrases □ Sulfisoxazole—pharmacokinetics in humans, dogs, and swine □ Pharmacokinetics—sulfisoxazole, comparison in humans, dogs, and swine □ Antibacterials—sulfisoxazole, pharmacokinetics in humans, dogs, and swine

Sulfisoxazole is an effective antibacterial agent often used in the treatment of urinary tract infections. Previous studies in dogs, swine, and cattle only measured blood levels of sulfisoxazole and sulfisoxazole acetyl following intravenous (1), subcutaneous (1), oral (2), and peritoneal (1) administrations. No detailed pharmacokinetic analyses were undertaken to characterize the absorption, distribution, metabolism, and excretion of sulfisoxazole in these animals. Likewise, human studies (3–5) of sulfisoxazole after single oral ingestion were limited to the measurement of blood concentrations of the parent drug and the acetyl metabolite. In one study (2), the steady-state blood level was measured following multiple-dose administration.

The first complete pharmacokinetic study in humans was conducted by Kaplan *et al.* (6) in which sulfisoxazole was administered intravenously, intramuscularly, and orally. Pharmacokinetic parameters were determined and availability was assessed. The present study compared sulfisozaxole pharmacokinetics in dogs, swine, and humans.

EXPERIMENTAL

Materials—A 12.5% sulfisoxazole[1] solution was prepared with lithium hydroxide. The solution was filtered and placed in sterile 50-ml ampuls before use. Sulfisoxazole acetyl[2] was used as a reference standard for serum and urinary metabolite assay. All chemicals and solvents used in the high-performance liquid chromatographic (HPLC) assay were high purity solvents[3] and were filtered before use.

Experimental Model—The trial consisted of three female dogs (~2

[1] Hoffmann–La Roche, Nutley, N.J.
[2] Acetyl-N^4-sulfisoxazole, Hoffmann–La Roche, Nutley, N.J.
[3] Burdick & Jackson solvents, Bodman Chemical Co., Doraville, Ga.

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