# Effects of Saturated and Unsaturated Fatty Acids on Blood Platelet Aggregation *in vitro*.* (30702)

V. MAHADEVAN, H. SINGH AND W. O. LUNDBERG
*University of Minnesota, The Hormel Institute, Austin*

There is now much evidence that platelets are involved in intravascular clotting of blood. The "white head" of a thrombus is an aggregation of platelets that normally would not adhere to each other in the blood stream. Platelet adhesiveness and aggregation are therefore receiving increasing attention, and many studies have been conducted under *in vitro* conditions using a wide variety of agents (1-4).

Dietary fats have been implicated in the development of atherosclerosis and thrombosis in both animals and man. It has been well established that the composition and quantities of blood lipids in man are markedly influenced by dietary fat. Thus increasing attention is being given to relationships between dietary fats as well as blood lipids and such phenomena as platelet aggregation and thrombus formation. Among the plasma lipids that may be altered by changes in dietary fat are the unesterified fatty acids. Long chain fatty acids have been shown to promote thrombus formation in an *in vitro* system of flowing blood(5) and also when injected intravenously in dogs(6,7). It is important therefore to study the effects of various dietary fats and individual fatty acids on factors involved in platelet aggregation. Using a turbidimetric method, Haslam found *in vitro* that a saturated fatty acid, behenic, brought about rapid aggregation of washed human platelets under suitable conditions(8). Owren *et al*(9) have reported that the abnormal platelet adhesiveness in humans with known histories of cardiovascular disease is reduced to normal by oral administration of linseed oil or purified linolenic acid, and have presumed that the tendency toward thrombus formation in such subjects is thereby reduced also. In view of such findings, the effects of various saturated and unsaturated fatty acids, as well as their glyceryl esters, on platelet adhesiveness and aggregation in man and miniature pigs, is being studied in this laboratory. This paper presents the results of studies of the effect of sodium salts of short and long chain saturated fatty acids and of $C_{18}$ unsaturated fatty acids on the aggregation of platelets of pig blood, as measured turbidimetrically.

*Materials and methods.* The procedure involved a separation of the pig blood platelets from plasma. Siliconized apparatus was used during the collection of blood and in all steps of the preparation of the platelet concentrates. Nine parts of blood were mixed with one part of 3.8% (w/v) sodium citrate. The platelet buttons obtained by differential centrifugation were washed twice with saline in volumes equal to the plasma volume and then resuspended in the appropriate volume of saline to give an extinction of 1.00 at 620 m$\mu$ in a Bausch and Lomb spectronic 20 spectrophotometer. Aliquots of this suspension were used to determine the effect of various fatty acids on platelet aggregation. Suspensions of platelets thus prepared showed no spontaneous aggregation for more than 12 hours at room temperature. They remained unaffected for more than 1 hour after the addition of calcium chloride at the concentrations used in the experiments.

Saturated fatty acids, behenic, arachidic, stearic, palmitic, myristic, lauric, capric and caprylic and unsaturated fatty acids, oleic, linoleic and linolenic, all more than 99% pure, were obtained from stock supplies of The Hormel Institute fatty acid project and were converted to 4 mM dispersions of their sodium soaps by warming with 10% excess sodium hydroxide and adjusting the pH to 7.4 with 0.1 N HCl. Fine dispersions of the less soluble sodium soaps were obtained with the aid of a Branson sonifier operated for 10-15 minutes at 20 kc per second. Solutions of the soaps of unsaturated acids were made

* Supported by grants from U.S.P.H.S., Nat. Heart Inst. (HE 02772) and Oscar Mayer Foundation.

under an atmosphere of nitrogen with the aid of the sonifier.

Platelet aggregation was measured turbidimetrically using a modification of the method described by Born(10). To 3.6 ml of platelet suspension in an unsiliconized calibrated test tube was added 0.2 ml of 8 mM $CaCl_2$ solution and then 0.2 ml of the fatty acid suspension. The optical density of the suspension was then measured at regular intervals. During the measurements, the tubes were swirled for 10 sec at 2-minute intervals. It was observed that, in the absence of $CaCl_2$, the optical density of the platelet concentrates would remain constant for almost 1 hour, either with no shaking or with occasional gentle swirling of the tube by hand.

*Results.* Fig. 1 shows the effects of saturated fatty acids on platelet aggregation. Behenic, arachidic and stearic were all effective in promoting rapid and complete platelet aggregation. In 30 minutes the optical densities decreased from 1.00 to 0.1, 0.18 and 0.2, respectively. Platelet clumps became discernible during these changes. Palmitic, myristic and lauric acids manifested activity after 30 minutes but the aggregation was far from complete even after 45 minutes. The short chain fatty acids, caprylic and capric, were quite inactive in bringing about platelet aggregation in 45 minutes. Thus, under these conditions, the chain lengths of the fatty acids have a direct bearing on platelet aggregation.

Figure 2 shows the influence of the $C_{18}$ unsaturated fatty acids, oleic, linoleic and linolenic, on platelet aggregation. Oleic was almost as effective as stearic but linoleic and linolenic resembled the short chain fatty acids in their ineffectiveness.

To determine whether higher concentrations of $CaCl_2$ would influence the rate of aggregation, the experiments with the long chain saturated fatty acids and linolenic acid were repeated using an approximately threefold increase in $CaCl_2$ concentration. Fig. 3 indicates that with behenic and stearic acids, complete aggregation was attained more rapidly (15 and 20 minutes, respectively), whereas with linolenic acid no change was observed.

Because of the report that both purified linolenic acid and linseed oil caused a lowering of platelet adhesiveness in cardiovascular patients(9), an examination of the effect of linolenic acid on platelet aggregation initiated by behenic and stearic acids was made. The results of 2 experiments with behenic acid are shown in Fig. 4. Curve A shows that linolenic acid inhibited the effect of behenic acid completely when both were added at the same time in equimolar concentrations. When linolenic acid was added 10 minutes after addition of behenic acid (curve B), the aggregation process was stopped, but the aggregation that had already occurred was not reversed.

Following addition of linolenic acid, there was an immediate slight increase in optical density from 0.64 to 0.75 and a return to 0.64 in 3 minutes, after which the optical density remained constant. The increase therefore could not be attributed to a reversal of platelet aggregation, but instead represented a contribution to the optical density by the linolenic acid dispersion itself. The results shown in curves A and B were essentially duplicated when corresponding combinations of linolenic and stearic acids were employed.

Because the short chain saturated acids, capric and caprylic, were similar to linolenic acid in failing to initiate platelet aggregation when tested independently, it became of interest to determine if they could also inhibit the effect of behenic acid. Curve C in Fig. 4 shows that unlike linolenic acid, capric (caprylic) acid was ineffective in arresting aggregation in a platelet suspension to which behenic acid and $CaCl_2$ had been added.

*Discussion.* Platelet aggregation appears to form the basis for both the physiologic process of hemostasis and the pathologic process of white thrombus formation. Several studies have indicated that a relationship exists between lipids, platelet aggregation and thrombosis(5,6,11,12).

Haslam(8) reported that long chain saturated fatty acids caused rapid aggregation of washed human platelets in the presence of $CaCl_2$ when suspended in tris-buffer. In the present investigation, the effects of both

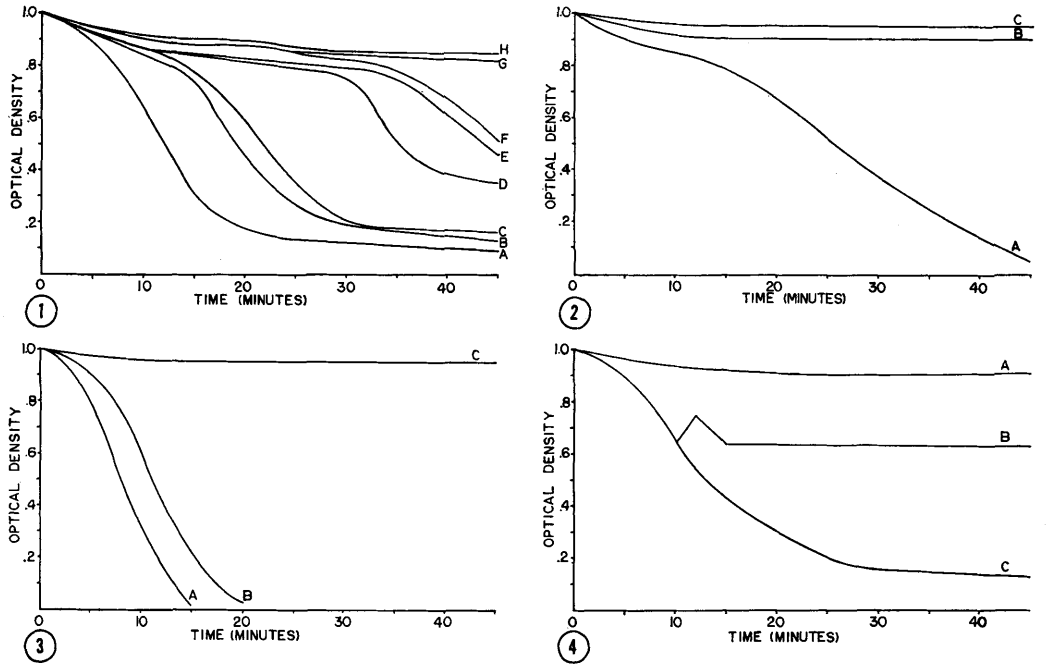


FIG. 1. Effect of saturated fatty acids on aggregation of washed pig platelets. To samples of platelets in saline were added 0.2 ml 8 mM $CaCl_2$ and the fatty acids in 200 $\mu$M final concentration. A to H are behenic, arachidic, stearic, palmitic, myristic, lauric, caprylic and capric. Final volume 4 ml in each case.

FIG. 2. Effect of unsaturated fatty acids on aggregation of washed pig platelets. To samples of platelets in saline were added 0.2 ml 8 mM $CaCl_2$ and the unsaturated fatty acid in 200 $\mu$M final concentration. A, oleic; B, linoleic; and C, linolenic. Final volume 4 ml in each case.

FIG. 3. Effect of high concentration of $CaCl_2$ on the rate aggregation of washed pig platelets brought about by behenic, stearic and linolenic acids. To samples of platelets in saline were added 0.2 ml 0.025 M $CaCl_2$ and the fatty acids in 200 $\mu$M final concentration. A, behenic; B, stearic; and C, linolenic. Final volume 4 ml in each case.

FIG. 4. Effect of linolenic and capric acids on aggregation of washed pig platelets initiated by behenic acid. To samples of platelets in saline were added 0.2 ml of 8 mM $CaCl_2$ and the fatty acids in 200 $\mu$M final concentration. A, behenic and linolenic added simultaneously; B, linolenic added 10 min after addition of behenic; and C, behenic and capric added simultaneously. Final volume 4 ml in each case.

saturated and unsaturated fatty acids on washed pig platelets were studied in saline suspension because tris-buffer caused aggregation of the platelets in a few minutes. No aggregation was observed when the fatty acids were added in the absence of calcium ions.

The rather high melting points of the long chain saturated fatty acids and their poor solubility in aqueous medium are the main difficulties in designing experiments to study their effects on platelet aggregation. These difficulties were overcome by the use of a sonicator to obtain homogeneous and stable dispersions of the fatty acids. The size of the fatty acid micelles is an important factor in comparing the relative activities of the individual fatty acids. However, in aqueous solutions, the minimum and maximum sizes of the micelles of ionic soaps reportedly do not differ greatly and concentration does not markedly affect micellar size(13). Whether or not the micellar size is affected by concentration, a comparison of the relative activities of the individual fatty acids seems justified in this study since equimolar dispersions of the sodium soaps of the fatty acids were used.

Aggregation is probably the result of a series of biophysical and biochemical reactions, possibly involving more than one pathway. Shore and Alpers(14) found that

stearate and longer chain saturated fatty acids caused clumping of platelets with release of serotonin and histamine in rabbit platelet-rich plasma. Release of ADP by fatty acids by direct damage to the platelet membrane, as well as activation of adsorbed Hageman factor, has been postulated to cause platelet aggregation(8). The differences in behavior observed in this study between long chain saturated fatty acids on the one hand, and the short chain fatty acids and the unsaturated fatty acids, linoleic and linolenic, on the other, in their effects on platelet aggregation need further explanation. Owren *et al*(9) found that an increased thrombotic tendency in patients is associated with an increased activity of the anti-Willebrand factor and factor VIII. It may be that the unsaturated fatty acids prevent platelet aggregation by lowering the activity of the anti-Willebrand factor. Whereas Owren *et al*(9) reported that only linolenic acid was effective in reducing platelet adhesiveness in humans with cardiovascular disease, our *in vitro* studies with pig platelets have shown that linoleic is also capable of retarding platelet aggregation.

Although long chain fatty acids injected into dogs were thrombogenic and fatal according to some investigations, they were reported to be inactive when incubated with albumin(6). This suggests that under certain conditions plasma-free fatty acids may not be fully or effectively bound by albumin. In the present study the fatty acid dispersions were made in saline. Whether solubilization of the fatty acids using albumin and other solubilizers will affect their observed effects on platelet aggregation needs further study.

Mechanisms by which linoleic and linolenic acids may bring about inhibition of platelet aggregation alone or in the presence of long chain saturated fatty acids might be either by preventing damage to the platelet membrane, or by lowering the activity of a factor or factors which promote aggregation.

1. Shermer, R. W., Mason, R. G., Wagner, R. H., Brinkhous, K. M., J. Exp. Med., 1961, v114, 905.
2. Mitchell, J. R. A., Sharp, A. A., Brit. J. Haematol., 1964, v10, 78.
3. O'Brien, J. R., J. Clin. Path., 1962, v15, 446.
4. Gaarder, A., Jonsen, J., Laland, S., Hellem, A., Owren, R. A., Nature, 1961, v192, 531.
5. Connor, W. E., Poole, J. C. F., Quart. J. Exp. Physiol., 1961, v46, 1.
6. Connor, W. E., Hoak, J. C., Warner, E. D., J. Clin. Invest., 1963, v42, 860.
7. Day, H. J., Soloff, L. A., Clin. Res., 1963, v11, 192.
8. Haslam, R. J., Nature, 1964, v202, 765.
9. Owren, P. A., Hellem, A. J., Ödegaard, A., Lancet, 1964, ii, 975.
10. Born, G. V. R., Cross, M. J., J. Physiol., 1963, v168, 178.
11. Mustard, J. F., Murphy, E. A., Roswell, H. C., Downie, H. G., Am. J. Med. Sci., 1962, v33, 621.
12. Soloff, L. A., Wideman, M. P., Nature, 1963, v199, 695.
13. Stigter, D., Overbeek, J. T. G., quoted by Reerink, H., J. Colloid Sci., 1965, v20, 217.
14. Shore, P. A., Alpers, H. S., Fed. Proc., 1963, v22, 504.

Received September 9, 1965. P.S.E.B.M., 1966, v121.

## Free Plasma Estrogens in the Channel Catfish.*† (30703)

B. E. ELEFTHERIOU, K. W. BOEHLKE AND O. W. TIEMEIER
(Introduced by M. X. Zarrow)
*Department of Zoology, Kansas State University, Manhattan, Kan.*

Recent interest has been aroused in isolation, identification and measurement of estrogenic steroids in various fish. Estradiol-17$\beta$, estrone and estriol have been identified and measured in ovaries and blood of several spe-

* Contribution No. 353 from Agri. Exp. Station and No. 380 from Dept. of Zoology.
† Supported in part by grant 2R01-HD-00013 from Nat. Advisory Child Health and Human Development Council, Nat. Inst. Health.