quired for phosphorylation and activation of tension. Furthermore, by the use of ATP analog, ATPγS, we have shown that although Ca^{2+} is required for the initial thiophosphorylation of the 20,000-dalton light chain, thereafter Ca^{2+} is not required for the maintenance of either thiophosphorylation of the light chains or tension. This indicates that it is the phosphorylation of the light chain rather than the presence of Ca^{2+} which is required for the activation of tension in smooth muscle fiber preparations. This same conclusion was reached by Hartshorne and co-workers (*12*) for the soluble actomyosin Ca^{2+}-sensitive adenosinetriphosphatase from chicken gizzard when they used ATPγS.

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Selective Phospholipid Adsorption and Atherosclerosis

Abstract. *Disaturated (fully saturated) lecithins adsorb onto solid surfaces more readily than lecithins in which one or both fatty acids are unsaturated. If saturated lecithins adsorb to arterial walls as they do to glass and polystyrene surfaces, there may be increased probability of atherosclerosis when the disaturated lecithin content of plasma is elevated. Analyses of lecithins in plasma samples from patients with myocardial infarction, and from patients with premature atherosclerosis but with low concentrations of plasma cholesterol and triglycerides, are consistent with the hypothesis that a high concentration of disaturated lecithin in plasma may be a significant risk factor for atherosclerosis, independent of triglyceride and cholesterol concentrations.*

In order to supplement recent observations on the physical properties of lecithin and cholesterol (*1*), I measured the adsorption of phospholipids in aqueous dispersions on a variety of solid surfaces. Among synthetic lecithins containing different combinations of fatty acids a highly selective adsorption was found for those in which both fatty acids were saturated. These results suggested that if such phospholipids in plasma also adsorb on arterial walls, elevated plasma concentrations of disaturated (fully saturated) lecithins might be related to the presence of atherosclerosis. Assays of these lipids in plasma were therefore made. Results of the phospholipid adsorption studies and of the plasma measurements are reported here.

Dispersions of pure synthetic lecithins (*2*) in water were prepared by dialysis (*3*). As shown in Fig. 1, appreciable amounts of the disaturated dimyristoyl lecithin (DML) and dipalmitoyl lecithin (DPL) adsorbed on the surface of polystyrene beads (*4*). The amount adsorbed increased with increasing concentration of lipid in the dispersion and, at 20°C and high dispersion concentrations, reached more than twice the amount expected for condensed monolayer adsorption. The amount adsorbed decreased with increasing temperature. Under identical conditions more DPL was adsorbed than DML.

The data of Fig. 1 are equilibrium values, attained within 1 to 2 hours of mixing the beads with the dispersions, and were constant for at least 3 days. If the DPL system at 20°C was raised to a higher temperature, lipid desorbed from the beads until a new temperature equilibrium was reached. This desorption process at higher temperatures took several days instead of the 1 to 2 hours required to reach adsorption equilibrium.

In marked contrast to the behavior of the disaturated lecithins, DML and DPL, no adsorption to polystyrene beads occurred with dispersions of the unsaturated lecithins, dioleoyl lecithin (DOL) and 1-palmitoyl-2-palmitoleoyl-lecithin (PPoL). If either or both fatty acid moieties of the lecithin were unsaturated, adsorption was greatly reduced or abolished, even at temperatures as low as 5°C.

The intrinsic adsorption properties of DPL were not affected by the presence of large amounts of DOL in the dispersion. When DPL was codispersed with a fivefold excess of DOL at 50°C and the amount of DPL adsorbed plotted against the amount of DPL in the mixed dispersion, the points fell on the 50°C adsorption isotherm for pure DPL dispersions shown in Fig. 1.

The influence of the solid surface in the adsorption process was examined by using glass beads (*5*) and comparing the adsorption of DPL, DOL, and PPoL under conditions similar to those in the polystyrene bead experiment. As before, DPL was adsorbed while no adsorption occurred with DOL and PPoL. If the particle size of the two types of beads was taken into account, the amount of DPL adsorbed per unit surface area was approximately the same on both polystyrene and glass beads (Fig. 1). Thus both lecithin selectivity and extent of adsorption were independent of major differences in the chemical nature of the adsorptive surface, suggesting that the adsorption of disaturated lecithins can occur under a variety of conditions. A recent report (*6*) indicates that DML and DPL adsorb to membranes of Chinese hamster fibroblasts with temperature dependence similar to that in the polystyrene adsorption studies reported here (Fig. 1); in contrast, DOL, under similar conditions, is incorporated into the cell.

The unusual adsorption properties of the lecithin dispersions raised the possi-

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bility that these phospholipids in plasma might adsorb preferentially to arterial walls. If the adsorption behavior is similar, the amount of disaturated lecithin accumulated on the vessel wall would be proportional to the amount of this lipid in the circulating plasma. If plaque formation can be initiated by accumulation on the artery wall of a lipid such as DPL (and its homologs) or other phospholipids with similar adsorptive properties (*7*), the occurrence of atherosclerosis might be influenced by the concentration of these phospholipids in the plasma.

In order to test the adsorption hypothesis, the plasma concentration of disaturated lecithins was measured in two groups of subjects: a random sample of 21 nonfasting donors to the NIH Blood Bank, in whom neither hypertension nor angina was present, and a second group of six donors, each of whom had suffered a myocardial infarction 1 week to 2 years earlier and in whom atherosclerosis had been established clinically (*8*). Plasma from heparinized fresh whole blood was used in all procedures (*9*). Serum concentrations of cholesterol, total lecithin, and sphingomyelin for the two populations are given in Table 1. Figure 2 shows the concentration of disaturated lecithin in each of the samples as a function of donor age; this analysis required measuring the fraction of lecithin that is disaturated (principally DPL, 1-stearoyl-2-palmitoyl lecithin, and distearoyl lecithin) and was obtained by a combination of methods (*10*).

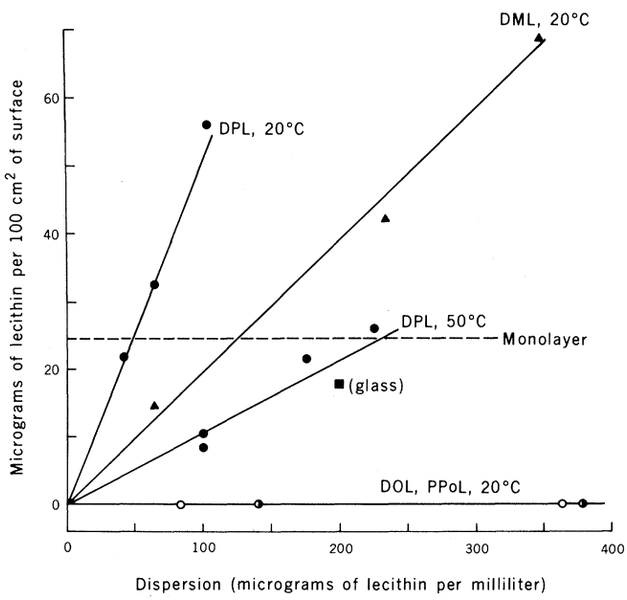
Table 1. Comparison of the plasma lipids from a random population of blood donors free of hypertension and angina (control) and from myocardial infarct survivors.

Lipid	Control (21)*	Myocardial infarct (6)*
Cholesterol (mg/100 ml)	231 ± 49 (131-326)	299 ± 56 (212-370)
Total lecithin (mg/100 ml)†	163 ± 47 (101-271)	194 ± 39 (140-250)
Sphingomyelin (mmole/liter)	0.5 ± 0.1 (0.3-0.7)	0.6 ± 0.1 (0.5-0.8)

*Number of samples used; mean values with standard deviations and with the range in parentheses are listed. The values of lecithin and sphingomyelin represent about 90 percent of the total phospholipid in these plasma samples. The remainder is composed principally of lysolecithin and phosphatidyl ethanolamine. †Calculated by assuming 775 is the average molecular weight of lecithin in plasma.

The average lipid concentrations given in Table 1 were consistently higher in the group with coronary artery disease than in the random population. As in many similar studies, there is considerable overlap of individual values; for example, some of the patients with myocardial infarcts had serum cholesterol values below 250 mg/100 ml. In contrast to the lipid values in Table 1, Fig. 2 shows that in every individual who had had a myocardial infarct the concentration of disaturated lecithin was higher than in any member of the population in whom there were no obvious symptoms of coronary artery disease. Thus there is a striking correlation between the plasma concentration of disaturated lecithin and the presence of atherosclerosis, as predicted by the adsorption hypothesis.

Drs. H. B. Brewer, Jr., K. Kent, and E. J. Schaefer of the National Heart, Lung, and Blood Institute (NHLBI) were consulted for suggestions of other possible studies which might offer a more critical test of the disaturated lecithin adsorption hypothesis. At their suggestion plasma lecithins were studied from patients who were known either to have or not to have premature coronary artery disease but who did not exhibit the major risk factors of elevated serum cholesterol, serum triglycerides, and hypertension. The criteria for patient selection were plasma cholesterol concentrations below 250 mg/100 ml, plasma triglyceride concentrations less than 200 mg/100 ml, and the absence of a history of hypertension. (Cigarette smoking was not included in the analysis because it is



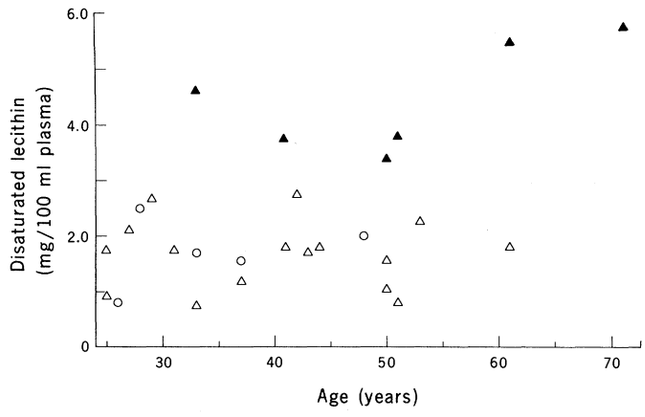


Fig. 1 (left). Adsorption isotherms of lecithins. Beads and dispersions were heated separately to the experimental temperature before mixing. Samples of the mixtures were removed immediately after mixing, and then after 1, 2, 24, 48, and 72 hours. The samples were centrifuged (Eppendorf 3200) for 3 minutes, and the supernatant was discarded. The beads were resuspended in water at the experimental temperature to wash out all lipid that had been trapped in the bead pellet that formed after centrifugation. This procedure was repeated six times, since control experiments indicated that further washing does not remove any more lecithin. When this washing procedure was not performed, highly irreproducible results were obtained. The beads were analyzed for phosphorus (*14*); to obtain the amount in the dispersion, the amount present on the beads was subtracted from the total amount present initially. Each data point represents the mean of three determinations, where the maximum deviation from the mean is ± 10 percent. A fresh dispersion was prepared to obtain each data point. Symbols: ▲, DML; ●, ■, DPL; ○, DOL; and ◑, PPoL. Fig. 2 (right). Concentration of disaturated lecithins (principally DPL, and 1-stearoyl-2-palmitoyl lecithin and distearoyl lecithin) as a function of donor age for the random population and for myocardial infarct survivors. Males are indicated by triangles and females by circles. Solid symbols denote donors with earlier documented myocardial infarction (all males). The maximum mean deviation is ± 10 percent, based on a minimum of three independent measurements of the concentration.

so widespread in the United States that the number of nonsmoking patients available for study would be severely limited.)

Patients with these characteristics were selected from an ongoing NHLBI program in which all the patients had symptoms of coronary disease (angina pectoris) and for whom coronary angiography was used to determine whether atherosclerosis was present. During the period of April through September 1977, only 15 donors with the required characteristics were obtained. Plasma samples, which had already been assayed for cholesterol and triglycerides, were generously provided by Drs. Brewer, Kent, and Schaefer. The diagnoses for these patients, obtained by coronary angiography, were not revealed to me until the lecithin analyses were completed.

The median value of 1.8 mg/100 ml for the random population data (Fig. 2) was chosen as the separation criterion between high and low plasma concentrations of disaturated lecithin. This choice was based on the possibility that among the random population sample there might be some individuals with atherosclerosis, and it is assumed that these individuals would have higher values of disaturated lecithin. It is recognized that this median value is arbitrary and must be established from a much larger sample size.

Of the 15 samples, 12 were from donors (42 to 68 years of age) with atherosclerosis. The disaturated lecithin content of these 12 plasma samples varied from 1.9 to 4.8 mg/100 ml, all above the median for the random population (Fig. 2). The mean of the disaturated lecithin concentrations for these 12 donors was 2.92 ± 0.25 ($\pm$ standard deviation); for the 21 donors of the random population the mean was 1.74 ± 0.13. The means are highly significantly different by Student's t-test ($P < .001$). The remaining three patients—two males, ages 23 and 28, and a woman, age 56—each of whom had angina pectoris but for whom there was no indication of atherosclerosis in the coronary angiograph, had concentrations of disaturated lecithin of 2.0 to 3.8 mg/100 ml, which was in the same range of concentrations that patients with atherosclerosis had. Thus, for all 15 donors, each of whom had angina pectoris, the disaturated lecithin was elevated above the median of the random population: in 12 of these patients atherosclerosis was diagnosed by coronary angiography. For these 12 patients, plasma concentrations of disaturated lecithin correlated more strongly with atherosclerosis than concentrations of either cholesterol or triglycerides, which were low for all the donors, even donors with atherosclerosis.

In view of the complicated and dynamic nature of the arterial wall (*11*), of the subtleties of the atherosclerotic process (*12*), the possible cooperative involvement of many classes of plasma lipids, and of the possibility that injury to the arterial wall may be a precondition for plaque formation (*13*), it may not be possible to implicate any lipid in atherosclerosis unequivocally except by analyses of samples far larger than were available to me. However, the facts (i) that there is a rational physicochemical mechanism by which plasma disaturated lecithins might contribute to atherosclerotic plaque formation, (ii) that disaturated lecithin titer is uniformly high in patients who have suffered heart attacks, and (iii) that the titer in patients with atherosclerosis equals or approaches the level for those who have had myocardial infarcts suggest that plasma disaturated lecithin titer deserves further, more intensive investigation as a possible independent risk factor in atherosclerosis.

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2. The synthetic lecithins used in this study were obtained from Applied Science Laboratories; impurities were less than 1 percent as determined by thin-layer chromatography and were therefore used without further purification.
3. Lipid dispersions were prepared by adding 1 ml of a methanol solution of each lipid to 25 ml of a solution of methanol and water (5 : 1), followed by dialysis against distilled water at 5°C for 3 days. Prior to use the dialysis tubing was rinsed in a solution of methanol and water (5 : 1). The equilibrium characteristics of dispersions prepared in this way are described elsewhere (*1*).
4. Polystyrene beads (diameter, $1.011 \pm .005$ μm; density, 1.05 g/ml) were obtained from the Dow Chemical Co.
5. Glass beads (diameter, 75 ± 13 μm; density, 2.5 g/ml) were obtained from Applied Science Laboratories.
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7. The composition of phospholipids in plasma lipoprotein is about 75 percent lecithin, 15 percent sphingomyelin, and the remainder principally phosphatidyl ethanolamine and lysolecithin [V. P. Skipski, in *Blood Lipids and Lipoproteins: Quantitation, Composition, and Metabolism*, G. J. Nelson, Ed. (Wiley-Interscience, New York, 1972), p. 471]. Preliminary adsorption studies on polystyrene beads with aqueous dispersions of synthetic phosphatidyl ethanolamines indicated similar behavior to that of lecithin, that is, the disaturated fatty acid compound was strongly adsorbed, but with unsaturated fatty acids on the molecule adsorption is virtually abolished. Adsorption studies with bovine sphingomyelin (synthetic material was not available) under the same conditions indicated a significant but low degree of adsorption, considerably smaller than for the disaturated lecithins and phosphatidyl ethanolamines. Since the phosphatidyl ethanolamine concentration is relatively low in plasma, and sphingomyelin is poorly adsorbed, the lecithins were the only phospholipids in plasma that were analyzed; they are assumed to be representative of all plasma phospholipids with similar adsorptive properties.
8. Five of the plasma samples were obtained from Drs. M. Edson and W. H. Glinsman; the sixth sample was obtained from an NIH colleague for whom atherosclerosis had been confirmed by angiography. Their cooperation is gratefully acknowledged.
9. Whole blood (5 to 10 ml) was centrifuged at 5°C for 30 minutes at 5000 rev/min. The plasma was removed from the packed cells and recentrifuged at 5°C for 15 minutes at 10,000 rev/min; clear plasma was removed and a sample was used for cholesterol analysis [C. C. Allain, L. S. Poon, C. S. G. Chan, W. Richmond, P. C. Fu, *Clin. Chem.* **20**, 470 (1974)]; 1-ml portions were used for phospholipid analyses. The remainder was frozen at −20°C for storage.
10. Lipids were extracted from freshly separated plasma by the method of Rose and Oklander [H. G. Rose and M. Oklander, *J. Lipid Res.* **6**, 428 (1965)], using 2-propanol and chloroform in successive extractions. Thin-layer chromatography was used to separate the lecithin and sphingomyelin components of the plasma sample, a mixture of chloroform, methanol, and water (65:25:4) being used as the solvent system. From the chloroform solution of the plasma lipid extract (final volume, 1.0 ml) 50 μl (in duplicate) was deposited, and the lecithin and sphingomyelin developed spots were carefully scraped into tubes for phosphorus analysis (*14*). Another 400-μl sample was deposited plus 50 μl of the internal standard DML solution (1.0 mg/ml). The lecithin spot was heated under nitrogen overnight at 120°C in a mixture of acetic anhydride and acetic acid (3 : 2) containing 0.1 percent butylated hydroxy toluene. At the completion of the reaction 1 ml of water was added, followed by extraction of the diglyceride acetates with a mixture of chloroform and methanol (2:1) as the solvent system (three extractions were sufficient); the dried extract was dissolved in ethyl acetate. The acetolysis procedure for preparing diglyceride acetates is quantitative for those lecithins that contain only saturated or monounsaturated fatty acids. If polyunsaturated fatty acids are present, these compounds do not give quantitative yields of diglyceride acetates. The overall yield of diglyceride acetates thus prepared from plasma lecithin is between 80 and 85 percent. Argentation thin-layer chromatography [M. Kito, M. Ishinaga, M. Nishihara, M. Kato, S. Sawada, T. Hata, *Eur. J. Biochem.* **54**, 55 (1975); A. Kuksis and L. Marai, *Lipids* **2**, 217 (1967)] yielded only qualitative separations of disaturated lecithins and therefore a gas chromatographic procedure was utilized for analysis of diglyceride acetates. A 3-foot (2-mm internal diameter) glass column was filled with 1 percent OV-17 on 100/120 Supelcoport and was conditioned for 24 hours at 350°C. Temperature-programmed runs at 4° per minute between 235° and 300°C separated all the diglyceride acetates according to fatty acid chain length, but not according to degree of fatty acid unsaturation. To separate the disaturated species, some of the diglyceride acetate solution was subjected to ozonolysis at Dry Ice–acetone temperatures for 2 to 3 minutes (Micro-ozonizer, Supelco). Ozonolysis cleaves all carbon-carbon double bonds yielding lower molecular weight compounds [M. Beroza and B. A. Bierl, *Anal. Chem.* **39**, 1131 (1967)]. Thus, by running consecutively the complete mixture of diglyceride acetates followed by the same solution subjected to ozonolysis, the amount of disaturated compounds is readily calculated. The 1-aceto-2,3-dimyristin was used as the internal standard; some of the breakdown products of the ozonolysis reaction appear with a similar retention time as the internal standard, but the peak for the latter is readily extracted from the chromatogram. A more complete description of these methods will be presented elsewhere.
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