TECHNICAL PAPERS

Physical State of Lipids and Foreign Substances Producing Atherosclerosis[1]

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In toxicopathological studies on polyvinyl alcohol, methyl cellulose, pure citrus pectin, and gum acacia, Hueper found that repeated intravenous injections of aqueous solutions of these substances resulted in their gradual accumulation in the arterial intima of experimental animals (dogs, rabbits, rats) (*2*). These lesions were of special significance because their morphological appearance and distribution were typically those of atherosclerosis, except that the deposits were of the injected foreign substance instead of lipid. Histological examination revealed 'foam cells' (phagocytes filled with the injected material), hyperplasia of cellular elements, etc. Deposits were also found in reticuloendothelial cells elsewhere. Hueper has expressed the opinion that these substances form a film at the plasma-endothelium interface, produce anoxia of the intima, and thereby cause these pathological changes in the intima. Because these substances are polymers of high molecular weight, he has referred to the lesions as experimental "macromolecular atherosclerosis." These foreign, colloidal compounds, which are stable and quite inert chemically, are of particular interest because of this atherosclerotic-like reaction of the arterial intima to their presence in the circulating blood and body fluids. In contrast, many other foreign substances (*i.e.* drugs) may be maintained at significant blood levels for prolonged periods without any deposition whatever in the arterial intima.

In a recent report (*3*) from this laboratory we presented observations on the physical state and particle size of the plasma lipids in sustained hyperlipemia of metabolic and experimental origin. It was shown that the lipid particles in the plasma in these known causative conditions of atherosclerosis are so large as to be readily visible in the dark-field microscope and are readily separated to the top in a definite lipid layer by moderate centrifugation, in contrast to normal plasma, wherein most of the lipid is stabilized in colloidal particles too small to be seen by dark-field illumination and not separated even by relatively high-speed centrifugation. Primarily, it was shown that the lipid particles (*chylomicrons*) appearing in normal plasma during alimentary hyperlipemia are also of large colloidal size, directly visible in the dark field and readily separated by centrifugation, and contain cholesterol, thus duplicating the coarse particulate distribution of lipids as found in sustained hyperlipemia. From these observations, coupled with clues from the characteristic histogenesis of the lesion, the theory was put forth that the cumulative effect of many fatty meals over a lifetime, by producing these transient showers of large lipid particles in the plasma, is the underlying cause of atherosclerosis in normal humans.

From the above considerations it seemed that similar observations on the particle size of these foreign substances which have been found to deposit in the arterial intima would be pertinent in the evaluation of this chylomicron theory of atherosclerosis. We therefore undertook the simple comparison of the physical state of these injected foreign substances with the lipids in sustained hyperlipemia, alimentary hyperlipemia, and normal blood.

(1) *Small (invisible) colloids or molecules*: (a) *Normal blood serum, fasting or after a nonfatty meal,* contains very few particles of sufficient size to be visible in the dark field. These appear as tiny, dancing specks and are for the most part at the extreme lower limits of detection by this method. High-speed centrifugation (22,500 × G for 1 hr) produced no observable separation of lipid particles to the top (in contrast to hyperlipemic serum). Ultracentrifuge studies on plasma proteins have indicated that with many times greater centrifugal forces much of the lipid in normal, fasting serum is sedimented to the bottom in protein-bound molecular complexes (*5*). The magnitude of the necessary centrifugal forces, the paucity of visible particles by dark field, and the clearly transparent appearance attest the high degree of dispersion and stabilization of the lipids intrinsic in normal plasma. We believe that deposition of lipid particles in the arterial intima does not occur under these conditions. (b) *Drugs, foreign substances* such as salicylates, barbiturates, sulfonamides, and others that form true solutions in water, are carried in molecular size far smaller than can be detected in the dark field, exhibit no demonstrable Tyndall effect, and are not separated from water or blood plasma by high-speed centrifugation. These molecularly dispersed foreign substances are not deposited in the arterial intima.

(2) *Large colloidal particles (colloidal suspensoids)*: (a) *Sustained hyperlipemia*: By dark-field illumination hundreds of large and easily visible particles are observed in each high-power field (*3*). This plasma is grossly milky or creamy by transmitted light and presents marked Tyndall effect by incident light. Centrifugation at 3,000–5,000 × G for 1 hr usually separates major portions of the lipid particles to the top. Centrifuging at 15,000–20,000 × G brings up all large lipid particles in a solid lipid layer

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on top of a now clear, *infra*natant plasma. Sustained hyperlipemia in man or experimental animals is known to result in the relatively rapid and severe development of lipid deposition in the arterial intima. (b) *Alimentary hyperlipemia*: The digestion and absorption of a fatty meal causes, for 3–5 hrs, the appearance of large and visible particles in the plasma of normal subjects. The plasma is grossly milky and exhibits marked Tyndall effect. This *hyper-* and *macrochylomicronemia* is indistinguishable from sustained hyperlipemia in dark-field appearance and in behavior in the high-speed centrifuge, and thus temporarily duplicates that physical state of the lipids which has been found to be characteristic of the known causative conditions of atherosclerosis (*3*). We contend that in normal humans deposition of lipid and atheromagenesis occur only during, and as a result of, the chylomicronemia produced by the ingestion of a fat-rich meal. (c) *Polyvinyl alcohol, methyl cellulose, pectin, gum acacia*: Dark-field study of each of these substances in aqueous solutions revealed that they are carried, not in 'true solution' or invisible colloidal dispersion, but in colloidal suspensions of many large and visible particles, the major portions of which are identical with chylomicrons in particle sizes. (Large particles were not as numerous in gum acacia solutions as in solutions of the other substances of the same concentration.) By stepwise increasing the centrifugal force in the high-speed centrifuge, it was grossly observed that the energies necessary to bring most of the lipid particles to the top in sustained or alimentary hyperlipemia were of comparable magnitude to energies necessary to sediment the greater part of these experimental substances to the bottom, providing further evidence of the similarity in particle size ranges (Stoke's law). The amount of sedimentation produced at different speeds was observed to be the same whether these substances were suspended in water or blood plasma, thus indicating that no change in physical state or particle size of these inert substances occurs as a result of admixture with plasma. From these observations it would seem justifiable to conclude that the large size of the colloidal particles in which these injected substances are carried is the cause of their deposition and retention from the plasma-derived nutrient lymph normally entering and passing through the arterial intima and is the stimulus to their phagocytosis and formation of the typical foam cells.

In a report concerning pellagrous South African natives, Gillman and co-workers (*1*) have related the large size of certain pathological iron-pigment molecules in the plasma with the cause of their deposition in the arterial intima and reticuloendothelial cells elsewhere (*cytosiderosis*). This observation adds support to the premise that the physical size of the colloids in plasma is the determining factor in their deposition in the arterial intima.

The above evidence is here put forth in confirmation of the *hyper-* and *macrochylomicronemia* theory of atherosclerosis (*3*). It is apparent that those large lipid particles which pass with the lymph into the intima incite the foreign-body response which is a characteristic histological feature of the origin and development of atherosclerosis. Triglycerides and fatty acids are rapidly resorbed from the depositing mixed-lipid particles. Cholesterol, which is difficult to resorb and remove, remains and accumulates as the predominant residue in the slowly developing lesion (*3, 4*). Lipid particles which contain larger percentages of cholesterol, as in sustained hyperlipemia (hypercholesterolemia), leave a greater residue and build up lesions more rapidly than lipid particles which contain smaller percentages of cholesterol (*i.e.* chylomicrons) (*4*). When this residue of inert and difficult-to-resorb cholesterol is thus trapped between the high-pressure blood column on one side and the internal elastic membrane on the other, it is withheld and prevented from being dispatched to the liver (and possibly other sites), where theoretically it could be relatively easily degraded or eliminated by cells that are specialized in such metabolic functions. Thus, the accumulation of lipids in the arterial intima seems to be a local, mechanical problem, and unrelated to general body balance or over-all metabolism of these substances.

It appears that the primary factor and *sine qua non* in atherosclerosis is the presence in the circulating blood of coarsely suspended colloidal particles considerably larger than those found in normal plasma and composed of, or containing, a substance relatively resistant to the resorptive and removal mechanisms of the arterial intima (macrophages and tissue fluid enzymes). It would seem that local intimal permeability and intravascular pressure are secondary factors that determine the local influx of plasma-derived nutrient lymph and thus determine the local distribution of the lesions in the vascular tree. Increased local permeability (rheumatic and syphilitic arteritis, etc.), or hypertension, may produce increased deposition, but only during periods when *hyper-* and *macrochylomicronemia* exists. The postprandial period following a fat-rich meal is the only time the plasma of normal individuals carries large amounts of coarsely particulate matter. Atherosclerosis, then, is the result of the fact that (a) oil and water do not mix, and therefore fats and oils in blood plasma must be carried in molecular complexes or stabilized colloidal particles; (b) fats entering the circulation from the intestine are absorbed and carried, not in the same finely divided state as the intrinsic lipids of the plasma, but in much larger particles of varying sizes; (c) the primitive scavenger and phagocytic function of reticuloendothelial cells in the arterial intima, plus the barrier action of the internal elastic membrane, are the responsible functional components of the mechanism of deposition and actual retention of those large particles that happen to be forced into the intimal tissue spaces with the plasma-derived nutrient lymph. Molecular substances and the finely dispersed normal plasma lipids and proteins pass through the arterial intima without being retained and deposited therein. In contrast, the large lipid particles in sustained or alimentary hyperlipemia, and the coarsely suspended foreign substances above described, are treated as particulate foreign bodies and are arrested, phagocytosed, and entrapped in the arterial intima. In conclusion, present evidence indicates that the ingestion of fat-rich meals,

by producing the temporary appearance of large lipid particles in the blood, causes the normal defense mechanisms of the intima to retain some of these particles and thus gradually and infinitesimally to build up the full picture of stenotic and occlusive arterial disease.

The pathogenic mechanism and etiology of atherosclerosis will be considered in greater detail in other communications from this laboratory.

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The Production of Mushroom Mycelium (*Agaricus campestris*) in Submerged Culture

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Commercial mushroom production in the United States is accomplished exclusively by growing the mushroom, *Agaricus campestris*, on composts, usually prepared from horse manure. The purpose of this paper is to present the first instance in which the mycelium of this mushroom has been grown on liquid medium under submerged fermentation.

Since the mycelium produced by this technique was obtained in good yield and was found to have the characteristic mushroom flavor, it seems possible that production in submerged culture will have considerable application in the manufacture of mushroom soups, gravies, and flavorings and in the production of spawn for seeding commercial mushroom beds.

For the preliminary tests, a strain of *A. campestris* was isolated from a white mushroom of this species taken from a commercial mushroom bed. A good growth of mycelium was obtained on wort agar. The mycelial mat was broken up in a sterile Waring blendor, and a sterile culture medium was inoculated with the suspension. The inoculated medium was then transferred to the fermentor and incubated at 25° C until a heavy growth of finely divided hyphae had been obtained. For tests on a small scale the fermentor equipped for rapid agitation during aeration and described by Feustel and Humfeld (*1*) was used, while for production in larger amounts the fermentor designed to draw in air by suction during agitation as described by Humfeld (*2*) was employed.

Good growth of mushroom mycelium was obtained on media containing either asparagus butt juice or press juice from pear waste as the main substrate. The addition of inorganic salts was essential in the pear juice medium. It was also found that fair yields could be obtained in media consisting of monosodium glutamate, dextrose, and inorganic salts.

After the culture was harvested, the mycelium was separated from the culture liquor by centrifuging. The mycelium was resuspended in water and recentrifuged. The samples made up in the preliminary work were dried from the frozen state.

Yields of mycelium up to 60% by weight of the sugar consumed have been obtained. The growth from a comparatively small inoculum is rather slow as compared to that of yeast; however, a continuous-fermentation experiment indicated that, once the maximum cell volume consistent with the composition of the medium has been attained, from one-half to three-quarters of the culture medium may be harvested and replaced with fresh sterile medium every 6–12 hrs of operation. Successful operation by this method has been accomplished over a period of 6 consecutive days. For instance, in one experiment, 3.75 liters of culture medium, which contained 20 gm of mycelium on the dry basis, was built up in 33 hrs to a volume of 16 liters with a dry weight of mycelium of 421 gm. At this stage 8 liters of the culture was harvested and 8 liters of fresh medium added to the 8 liters remaining in the fermentor. Maximum cell volume was attained in 12 hrs. This procedure was repeated, and there was no indication that such operation could not be continued indefinitely. This and other experiments indicate that no serious difficulties should be encountered in adapting the process to economic commercial production of mushroom mycelium.

The chemical compositions of commercial mushrooms and the mushroom mycelium produced by submerged fermentation were found to be very similar. McConnell and Esselen (*3*) report that *A. campestris* mushroom contains protein (N × 6.25), 35.6%; fat (ether-soluble material), 2.3%; and ash, 10.2%, calculated to a moisture-free basis. The mushroom mycelium produced by submerged fermentation, calculated to the same basis, contained protein, 49.1%; fat, 3.1%; and ash, 8.1%. It may be noted that the mycelium was somewhat higher in protein and fat content, and lower in ash. Other constituents such as carbohydrates and fiber have not been determined.

The adaptation of the submerged-culture process to the production of mycelium of the higher fungi would seem to offer the possibility of industrial-scale application to an extensive group of organisms. Such application may include the production of enzymes such as cellulase, solvents, antibiotic agents, and substances of pharmaceutical significance. Each species would probably need considerable investigation to determine optimal conditions for its propagation and for the production of such desired substances.

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