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MACROMOLECULAR MATERIAL IN BILE AND ITS RELATIONSHIP TO GALLSTONE FORMATION*

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The use of the term 'macromolecule' to describe some of the constituents of human gallbladder and hepatic bile is made in the broadest sense, denoting molecules with molecular weights larger than a few thousands. The term will include both homogenous molecules such as proteins, and heterogenous polymolecular aggregates. It will not indicate their reactive groups or geometrical configurations.

The recent interest in the physical chemistry and biochemistry of bile has stemmed from the work of Verschure et al.1,2 who re-introduced the concept of certain macromolecules in human bile having a possible role in the pathogenesis of cholelithiasis. In the last decade there has been considerable expansion of knowledge in this field and the subject has recently been well reviewed.3,4 This paper will deal with 3 classes of macromolecule in human bile which are of particular interest; proteins, mucous substances and micelles.

Proteins
There is now good reason to believe that proteins immunologically identical with the plasma proteins are present in human bile. Verschuer and Hoefsmit5 performed paper electrophoresis on both gallbladder and hepatic bile, identifying 4 protein fractions—P1, which moved most rapidly towards the anode, and which they believed to be a lipoprotein; P2, which had an electrophoretic mobility equal to serum albumin; P3, which was a mixture of alpha and beta globulins; and P4, which showed no mobility. It is now accepted that P4 represents mucous substances (i.e. 'mucoprotein').6 Immunoelectrophoresis and gel precipitation studies indicate that albumin and globulins, antigenically similar to the serum proteins, can be found in bile from both normal and diseased gallbladders as well as from the common bile duct,7 although Hardwicke et al.8 have stated that non-serum proteins may appear in bile. There is considerable disagreement over the nature of the rapidly-migrating, pre-albumin fraction P1. Verschure9 originally suggested that it represented a lipoprotein and that it was important for the transport of lipids in bile, but the studies of Norman10,11 suggest that this is not so and that there is no lipoprotein. Other investigators have claimed that a small amount of protein (not specifically a lipoprotein) is bound to a complex of cholesterol, lecithin, bile salts and bile pigments.12,13 Rawson1 believed that this protein showed no antigenic determinants with the serum proteins, whereas Clauson et al.14 reported the rapidly-mobile protein fraction to contain 2 weak antigens. It has been suggested that albumin may absorb to a lipid complex,15 and in this connection it is of interest that the mobility of albumin on agar-gel electrophoresis, has been shown to be higher for bile than serum, this being a reversible physical phenomenon.16 On the other hand, Russell and Burnett17 have strongly denied the presence of any protein in the leading electrophoretic fraction, suggesting such an appearance to be an artefact of staining.

The total quantity of protein lost in bile is small (20-50 mg./ml.).18 There may be errors when the protein is measured, either by the biuret method19 or by the Lowry method20 and at present it seems that the most accurate method for measuring bile proteins is by quantitative immunological techniques.21

In assessing reports concerning the character of the bile proteins it is important to distinguish between studies on bile aspirated at the time of operation, and bile obtained at postmortem. Furthermore, the resolution of bile proteins by gel electrophoresis gives a pattern quite different from that given by paper electrophoresis. It still remains to be determined whether there is a 'bile lipoprotein'; whether proteins other than the serum proteins appear in bile; whether any change in the quantity or nature of the bile proteins accompanies gallbladder disease; and whether these proteins, normal or abnormal, play any role in the pathogenesis of gallstones.

Mucous Substances
The role of mucous substances in the pathogenesis of gallstones has been stressed by Womack et al.22 who believed that 'there may be some alteration in the nature of gallbladder mucus causing it to act in the induction and development of stones in the gallbladder'. The results of experimental cholelithiasis would tend to support this suggestion.

There has been much confusion in the nomenclature used to classify this group of macromolecules and the term 'mucus' is best avoided, implying viscous properties that only some of these compounds possess. The mucous sub-
stances are proteins containing carbohydrates or, more specifically, amino sugar-containing compounds. They may be separated into 2 main groups—acid mucopolysaccharides, containing hexuronic acids and loosely linked with the protein moiety and glycoproteins which do not contain hexuronic acid and in which the carbohydrate is firmly linked to the peptide. Both the acid mucopolysaccharides and the glycoproteins contain the 2-amino sugars, glucosamine and galactosamine (i.e. hexosamines), and the measurement of hexosamine may therefore be used as an index of the content of mucous substances.

In an attempt to study further the relationship between mucous substances and gallstones, we studied total hexosamine values in bile samples freshly aspirated from normal and pathological gallbladders and from the common bile duct. Pathological gallbladders were defined as those gallbladders containing gallstones. We found that the pathological gallbladder bile contained a greater quantity of mucous substances than bile from normal gallbladders, the difference being more apparent when the hexosamine content was expressed as a function of the total solids in bile. Thus the hexosamine concentration appeared to be a function of abnormality and was not simply the index of the degree to which the bile had been concentrated. Expressing the hexosamine concentrations in this manner showed a surprising elevation in the common bile duct samples (all of which had been obtained by T-tube drainage). Our results differed from a similar study by Giles et al. who found the hexosamine levels to be lower in bile from pathological gallbladders than normal gallbladders. However, the majority of their normal gallbladder samples were obtained at postmortem.

We measured the relative viscosity of bile and found that pathological gallbladder bile was more viscous than normal gallbladder bile, gallbladder bile being more viscous than hepatic bile. There was a wide variation in the viscosity of pathological gallbladder bile, confirming the clinical impression that bile from diseased gallbladders was either 'thick and sticky' or 'thin and watery'. No correlation existed between the hexosamine content and relative viscosity of normal gallbladder bile, whereas such a correlation was demonstrated in pathological gallbladder bile.

Mucous substances appear to be associated in some way with gallstones. These substances in excess, too, might account for the increased viscosity of bile from diseased gallbladders, which has been postulated to be important in the formation of stones. The fundamental question is, however, whether these changes precede or follow stone formation. An experimental study in hamsters suggested the former idea, and in this connection our finding of increased mucous substances in the T-tube drainage of patients who have had stones, may be of significance.

**Micelles**

The major components of gallstones include cholesterol and the bile pigments. An understanding of the manner whereby the non-polar, insoluble cholesterol is carried in bile is thus essential to the problem of gallstone formation. In bile, cholesterol must be solubilized either in the form of a lipoprotein, or as a micelle, and the evidence is overwhelming in favour of the latter phase.

The ability to form micelles is a property of association colloids such as detergents and the bile salts. In dilute solutions these compounds exist as unassociated molecules, but at higher concentrations they form polymolecular aggregates termed micelles, the concentration at which this occurs being known as the critical micellar concentration. Under physiological conditions the bile salts are always above their critical micellar concentration, i.e. in the micellar phase, and these micelles are in equilibrium with the unassociated molecules in solution. Compounds forming micelles have been called amphiphilic (possessing both feelings) for they possess both hydrophilic and hydrophobic regions. It is important to distinguish between micellar solutions, which can be thought of as true solutions, and emulsions which are unstable, have large particle size, scatter light strongly and usually require energy for their formation. The concept of a micelle is therefore an aggregate of molecules having their polar, hydrophilic groups oriented towards the aqueous phase, and the non-polar, hydrophobic hydrocarbon groups oriented towards the centre of the micelle. This centre can be regarded as a tiny droplet of solvent and indeed it is capable of dissolving lipids, the process being known as micellar solubilization.

Two types of micellar solutes may be distinguished—non-polar solutes which are thought to be dissolved in the centre of the micelle, and polar solutes which are believed to be dissolved with their polar groups between the ionized heads of the amphipathic molecules. The addition of the polar solutes apparently increases the solubilizing potential of the micelle with regard to the non-polar solutes. These concepts have been applied to bile, where it is believed that the bile salts act as detergents maintaining cholesterol (the non-polar solute) in solution. Phospholipids (the polar solute) aggregate within the bile salt-cholesterol micelle, this incorporation being of fundamental significance, for the mixed or expanded micelle which results, can solubilize more cholesterol than the original bile salt micelle.

A point worth stressing, for it explains many of the earlier studies on bile, is that as long as micellar solution is not excessively diluted, it behaves as a solution of any macromolecule. In 1908, Long and Geppart described the formation of a complex between bile salts and lecithin, since which time there have been a number of studies in 'macromolecular complexes' in bile. The subject has been well reviewed by Juniper. It is probable that what have been studied are the mixed micelles comprising bile salts, cholesterol and phospholipids. Because the micelles are in equilibrium with the unassociated molecules in solution, any technique which upset this equilibrium will distort or destroy the micelle. Techniques used for the study of true macromolecular colloids should be applied to bile with great caution. An understanding of some of the techniques used to study the macromolecular complexes, or micelles, in bile should help dispel some of the confusion in this field. Three in particular will be discussed: analytical ultracentrifugation, preparative ultracentrifugation and Sephadex gel filtration.

**Analytical Ultracentrifugation**

Verschure demonstrated the presence of a single macromolecular component when human bile was submitted to analytical ultracentrifugation. A mean extrapola-
of the mixed micelles in bile to hold onto bilirubin may be the key to the formation of stones albeit that the conjugated bilirubin present in bile is known to be water-soluble.

Although the analytical ultracentrifugation studies have provided much interesting and useful data, they must be interpreted with caution. The meaning of the sedimentation coefficients obtained in bile, is still a matter for debate. Even more treacherous are attempts to calculate molecular weights for the sedimenting material; bile samples are usually diluted 1:10 in saline before being studied, a manoeuvre which will disrupt the micelle and affect its apparent molecular weight. The inability to obtain an accurate value for the partial specific volume of the complex will result in serious inaccuracies in the calculation of the molecular weight.

Preparative Ultracentrifugation

Because of the inherent difficulties in the above technique we have recently used isopycnic gradient ultracentrifugation in an attempt to determine the molecular weight of the mixed micelles in human bile. Cesium chloride was used to form the gradient. The method is based on the principle that macromolecules will concentrate and float at a position when the densities of the macromolecules and solvent are identical. It has been shown that the density and distribution of the macromolecules in the gradient is a function of their molecular weights. When bile and cesium chloride were mixed in the appropriate volumes and densities and centrifuged at 40,000 r.p.m. for 60 hours, a well-marked band of pigment was obtained in the gradient of cesium chloride. The gradient was harvested and the distribution of the banded pigment determined. It was then possible to calculate the molecular weight of the material represented by the pigments by using the formula derived by Meselson et al. Both gallbladder and hepatic bile samples contained pigment-binding macromolecules with calculated molecular weights varying from 65,000 to 75,000 in normal gallbladder bile; from 35,000 to 75,000 in pathological gallbladder bile and from 11,000 to 20,000 in hepatic bile. A relation between the molecular weight of the complex and the concentration of the bile was demonstrated; the more concentrated the bile the greater the molecular weight.

This method is not beyond criticism. Preferential interactions between the molecules and either the solvent or the gradient material will cause uncertainty in the values obtained and this reservation must apply, particularly when as complex an aggregate as a mixed micelle is being studied. Furthermore, heterogeneity in density among the micelles can cause substantial errors in molecular weights calculated by this method.

Gel Filtration

Recently attempts have been made to study the size and composition of the macromolecular material in bile by Sephadex gel filtration. Sephadex is a cross-linked dextran gel which acts as a 'molecular sieve', separating molecules of different sizes, and it has been used particularly in the estimation of the molecular weights of proteins. Recently it has been used to study the dimensions of pure and mixed bile salt micelles. When human gallbladder bile is applied to a Sephadex gel column, 2 or 3 macromolecular fractions may be recovered. Nakayama and Miyake have also attempted to derive molecular weights for the different fractions, claiming that of the larger fraction to be in the order of a million and the smaller around 36,000. Our studies with Sephadex gels have also shown 2 pigment-binding macromolecular fractions, the smaller of which, both on the basis of its filtration characteristics and on isopycnic gradient ultracentrifugation, gave molecular weights varying from 13,000 to 27,000. However, isopycnic gradient ultracentrifugation of the bile applied to, and eluted off the columns, gave widely differing values for the calculated molecular weights of the macromolecules; the eluted aggregates were much smaller than those initially applied to the gel column. Further studies have shown that the greater the quantity of gel used the greater the reduction in the size of the eluted aggregate. Equilibration of the column with bile salts before the elution of the bile samples will affect the size of the eluted complexes. The presence or absence of 2 pigment-binding fractions could be varied by the height of the columns, the quantity of gel used and whether or not the columns had been pre-equilibrated with bile salts. Thus Sephadex gel filtration of bile was associated with significant alterations in the pigment-binding micelles, depending upon a variety of conditions governing the filtration process. The technique may be used to advantage to demonstrate the heterogeneity and instability of the micelles in bile, but attempts to define fractions of different composition and size should be interpreted with reserve.

Composition of the micelles in bile. In contrast to the uncertainties over the size of the mixed micelles in bile there is general agreement over their composition; cholesterol, phospholipids and bile salts. Verschuer believed that, of the total bile lipids, 79% of the cholesterol, most of the dihydroxy bile salts and an unspecified amount of the lecithin were held together in a complex. Similar values have been given by Thureborn, Nakamura and Tamesue. Our results, too, were of the same order; 66% of the cholesterol, 69% of the phospholipids and 65% of the total bile salts, in bile, were present in the form of polymolecular aggregates, presumably micelles. We also found that 66% of the bile pigments were related to the complex as well as 30% of the proteins in bile, the latter finding being of interest because of the present uncertainty as to the relationship of the bile proteins and the mixed lipid micelles.

The significance of the ratio of the phospholipids and bile salts to cholesterol, in bile, has been stressed by Isaksson who showed that a critical range of 11/1 to 12/1 (lecithin—bile salts/cholesterol) was the minimal amount of the system required to dissolve a given amount of cho-
HYPOCHROMIC ANAEMIA IN CHRONIC INFECTIONS

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Textbooks of haematology¹ and medicine,² a review of 378 patients in Oxford,³ and a recent annotation in The Lancet⁴ indicate that iron deficiency is usually considered to be by far the commonest cause of hypochromic anaemia. When there is a block in the incorporation of iron into haemoglobin, as in thalassaemia, pyridoxineresponsive anaemia and refractory sideroblastic anaemia, hypochromia may also occur. However, these conditions are apparently uncommon among the Bantu in South Africa.

Some years ago we were impressed by the frequency with which hypochromia was reported in patients admitted to this hospital suffering from a variety of chronic infections. We found this surprising in a hospital population composed largely of Bantu patients, in the majority of whom high tissue-iron deposits may be expected⁵ and in whom iron-deficiency anaemia should therefore be relatively infrequent.

We accordingly started a controlled investigation of the anaemia associated with long-standing infections. Our results in Bantu patients with amoebic liver abscess have already been published.⁶ We have since studied a further series of 34 patients selected on the grounds only that they suffered from chronic infection. Thirty-two were Bantu and 2 Indian, and all but 6 were male. Pulmonary tuberculosis was the commonest infection (23 cases). Six patients had pyogenic lung abscess, while the remainder suffered from bacterial endocarditis, polyarthritis, empyema, pleural effusion and tuberculous peritonitis.

For the purpose of control our results were compared with the haematological findings in 51 subjects, all of whom were in apparent good health (30 Bantu males, 8 White females and 13 White males).

METHODS

Morning specimens of blood were taken shortly after admission to hospital and before treatment had been instituted. Haemo-

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