Pathogenesis of Calcium Bilirubinate Gallstone:

Role of E. Coli, β-Glucuronidase and Coagulation by Inorganic Ions, Polyelectrolytes and Agitation

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FROM the practical viewpoint, especially in Japan, gallstones can be classified into three main types, that is gallstones of cholesterol line, those of bilirubin line and rare ones including inorganic stones (Table 1).

Gallstones of bilirubin line are characterized by their relatively high bilirubin content and low cholesterol content and are divided into two subtypes; one is the so-called calcium bilirubinate stone or pigmented calcium stone and the other is usually referred to as the pure pigment stone.[10, 39]

Calcium bilirubinate stones are prevalent in Asia and are very rare in Europe and the United States. In Japan, however, the incidence of these stones seems to have decreased in recent years.[12] Today, the incidence in Japan is estimated to be about 30 or 40 per cent of all gallstones, significantly lower than the 70 or 80 per cent in the period during and shortly after the Second World War. The typical calcium bilirubinate stones (Fig. 1)are dark or reddish brown and usually very fragile. The cut surface is sometimes amorphous and sometimes stratified in structure. The stones are formed not only in the gallbladder but also fairly commonly in every portion of the biliary system, including the intrahepatic biliary tree. The number and size of these stones differ considerably from case to case. Besides concrements of definite shapes, that is gallstones, bile often contains a muddy substance consisting chiefly of numerous fine particles of calcium bilirubinate which can be identified microscopically. According to our investigations,[34] the bilirubin content of these calcium bilirubinate stones varied from 40.2 to 57.1 per cent including ammonia-extractable fractions and the cholesterol content from 2.9 to 25.6 per cent. In contrast, analysis of gallstones of cholesterol line [28] showed the cholesterol content to be 96 per cent or more in pure cholesterol stones and over 71.3 per cent even in mixed stones. Bilirubin content was only 0.02 to 5.0 per cent in gallstones of this kind. Thus, calcium bilirubinate stones differ from cholesterol stones not only in clinical manifestations but also in composition, implying a difference in pathogenesis. Miyake and Ishiyama [24] proposed that the peculiarities of gallstones in the Japanese might be based on the difference of food and race between the Japanese and the Western people. At any rate, the difference in the varieties of cholelithiasis between Asian and Western countries—in other words the reason for a higher incidence of

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TABLE 1. *Classification of Gallstones*

I. Gallstones of cholesterol line
  1. pure cholesterol stones
  2. combination stones
  3. mixed stones

II. Gallstones of bilirubin line
  1. calcium bilirubinate stones or pigmented calcium stones
  2. so-called pure pigment stones

III. Rare gallstones

calcium bilirubinate stones in Asia [5, 12, 23]—is a matter of great interest for medical scientists of Asian countries.

Despite many theories concerning development of gallstones, little is known about the separation mechanism of stone constituents from bile. No theory systematically elucidates how the separated gallstone constituents coagulate and solidify in bile to form a solid concrement, although some speculations such as theory of Liesegang phenomenon [11] and coacervation theory [25, 42] have been presented. Recently, we studied characteristics of calcium bilirubinate stones, both clinically and biochemically, to clarify the mechanism of separation of calcium bilirubinate in bile and the mechanism of coagulation of the separated calcium bilirubinate, two major steps involved in pathogenesis of calcium bilirubinate stones.

So-called pure pigment stones are different from calcium bilirubinate stones in many aspects. They are occasionally encountered in conditions without any biliary infection, especially in hemolytic jaundice in which bile is abnormally concentrated and in other conditions with metabolic imbalances. The site of production of these gallstones is usually the gallbladder. They are usually greenish dark or almost black appearance and chemically are compounds of bilirubin or its derivatives with calcium and other inorganic elements. Further discussions on the so-called pure pigment stones will be made elsewhere.

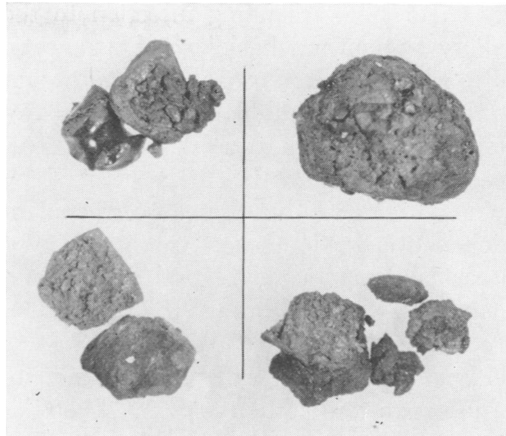

FIG. 1. Typical calcium bilirubinate stones taken from common ducts of four patients.

Inducement to Formation of Calcium Bilirubinate Stone

Although the incidence of calcium bilirubinate stone was quite high in the general population of Japan, farmers and fishermen of low income and poor living circumstances were more likely to be involved than better class patients.[12] Laboratory examinations revealed that the bile of patients with calcium bilirubinate stones had almost invariably been infected with *Escherichia coli.* Eggs or fragments of cuticles of *Ascaris lumbricoides* were also identified in 55 per cent of pigmented calcium stones collected from many hospitals at the time directly after the Second World War. Tung *et al.*[45] reported from Vietnam that they found roundworm elements in 70 per cent of examined gallstones as a kernel of gallstone formation. These facts indicate that contamination of the biliary tract may induce formation of this kind of gallstones. Accordingly, local factors such as intracholedochal migration of the roundworm or other parasites and duodenitis of unknown origin, which causes papillitis, might be responsible for the development of calcium bilirubinate stones. Should papillitis occur in consequence of these conditions, stagnation of bile, ascending infection to the biliary system and backflow of pancreatic juice would result in formation of gallstones in the stagnated and dilated bile ducts. Details of these facts were described previously.[12] Although it has been claimed by some authors[4, 29] that the cause of cholangiohepatitis in Southeast Asia is on most occasions *Escherichia coli* and these bacilli may reach the liver through the portal vein, we believe that "ascending infection" as outlined above is more likely as the route of infection to the biliary system. The relatively common atrophic gastritis which occurs in Japan and is associated with hypo- or achlorhydria[47] may facilitate bacterial growth in the duodenum and also promote ascending infection to the biliary infection. Stock and Tinckler[41] in Hong Kong presumed that clonorchiasis sinensis, another parasitic disease of the biliary tract, would be a common cause of the calcium bilirubinate stone in Southeast Asia where heavy infestation of the worm is not rare. In Japan, clonorchiasis is less commonly associated with gallstones than is invasion of *Ascaris lumbricoides* into the common bile duct. One reason for this may be that in Japan infestation of *Clonorchis sinensis* is usually not severe, and another may be the fact that *Clonorchis sinensis* migrates from the papilla of Vater into the intrahepatic ducts at the time of metacercaria which is too small to induce significant irritation to the papilla of Vater. A solid calcium bilirubinate stone was experimentally formed by Sato[33] in our laboratory by keeping an ascarid carcass for about 10 months in the gallbladder of a living dog. Following this experiment, Atsumi,[1] also of our department, demonstrated that calcium bilirubinate was precipitated easily in the form of sporadic dots on the eggshell of the roundworm within 4 to 5 days after introduction of a saline suspension of the eggs into the gallbladder of a dog. At any rate, the fact that the parasite gallstones are exclusively calcium bilirubinate stones is an enigma.

As to the biochemical aspect of bile in cholelithiasis, it was revealed that not only cholesterol but also bilirubin were unexpectedly lower in concentration in cases with calcium bilirubinate stones than in those with cholesterol stones, although the content in bile of bile acids generally decreased in cholelithiasis and showed no noteworthy difference according to the kind of gallstones. On the other hand, the fact that Japanese residing in Honolulu or in California, unlike those residing in Japan, rarely develop calcium bilirubinate stones[12] gives some interesting suggestions as to the mechanism of formation of these gallstones. The average daily per capita intake of fat and animal protein by Asians is generally lower than that by Europeans and Americans.[14] From laboratory and clinical data, it seems likely that food, which influences the metabolism of bilirubin, is also related to formation of calcium bilirubinate stones. The recent decrease in incidence of calcium bilirubinate stones in Japan is presumed due to both improvement of diet and a decrease of infestation by intestinal parasites.

Separation Mechanism of Calcium Bilirubinate in Bile

Hypothesis of Combination of Bilirubin with Calcium in Bile

With recent progress in assay methods in biochemistry, many research workers have dealt with chemical constitution of bile and gallstones, and the mechanism of precipitation of gallstone constituents from bile is more clear.[30] In 1954, Isaksson[7] reported on the dissolving power of cholesterol in human bile and advocated that a decrease in lecithin-bile salt complexes might promote separation of cholesterol in bile; this theory has been supported by others.[26] However, no theory has elucidated precipitation of calcium bilirubinate on the basis of chemical experiments.

The bilirubin moiety of the calcium bilirubinate stone has been identified to be free, non-esterified bilirubin since it is chloroform-extractable after treatment with dilute hydrochloric acid.[36] Therefore, bilirubin is presumed to be present in the stone in water-insoluble compounds with inorganic elements, mostly with calcium. In other words the main ingredient of so-called pigmented calcium stones is bilirubinate of calcium. Little has been known of the mechanism leading to the combination of bilirubin with calcium. In the last decade Billing and Lathe,[3] Schmid[38] and Talafant[43] reported that bilirubin in the circulating blood is esterified in the liver to form water-soluble glucuronide before it is excreted into bile. In further studies, including some in our department,[9, 27, 37, 40] it has been pointed out that pigments in bile are mostly glucuronides of bilirubin but approximately 20 per cent is non-glucuronide bilirubin. The latter fraction includes salts of bilirubin and pigments that, on electrophoresis, move with proteins, phospholipids or bile acids.

Bilirubin glucuronide is known to undergo hydrolysis into free bilirubin and glucuronic acid in the presence of the enzyme β-glucuronidase. The activity of β-glucuronidase in bile was studied primarily in a variety of biliary disorders.[15, 35] Normal bile was always free of β-glucuronidase activity, whereas bile infected with *E. coli*, especially specimens obtained from patients with calcium bilirubinate stones, exhibited intense activity of this enzyme. The activity was maximal at about pH 7.0 which was near the optimal pH for β-glucuronidase from *E. coli*. This indicates that the bile in the case of calcium bilirubinate stone is under the influence of β-glucuronidase of bacterial origin. According to Sasaki,[32] β-glucuronidase from a group of coli bacilli showed the most intense activity of various other bacteria tested. It is presumed that bilirubin glucuronide in bile is hydrolyzed into free bilirubin and glucuronic acid by enzymatic activity, and calcium, which increases in the biliary tract in inflammatory conditions,[6] combines with the liberated free bilirubin at its free carboxyl radical, yielding the water-insoluble calcium bilirubinate. This hypothesis is outlined in Table 2.

TABLE 2. *Formation of Calcium Bilirubinate*

Bilirubin glucuronide in bile + Intensified β-glucuronidase activity
↓
Hydrolysis
↓
Release of glucuronic acid and increase of free bilirubin
↓
Calcium bilirubinate formed by combination of calcium with carboxyl radical in free bilirubin

A precipitate of calcium bilirubinate was prepared when free bilirubin (Daiichi Chemical Co.) was dissolved in water in the form of sodium or potassium salts and calcium hydroxide added to the solution, or when calcium hydroxide was immediately added to a water suspension of the bilirubin preparation.[13]

In Vitro Precipitation of Calcium Bilirubinate

Miyake[22] and Westphal *et al.*[46] observed that fine concrements of bile pigments were produced in the gallbladder of dog by prolonged bile stasis combined with injection of *E. coli*. It has long been known that human bile sometimes produces precipitates when it is left standing in an upright vessel. Bacmeister[2] observed over 50 years ago that a sediment of cholesterol formed in bile from patients who had cholesterol stones. Recently, Tera[44] observed a similar phenomenon and advocated that precipitation of cholesterol from bile could occur without a simultaneous change in lecithin-

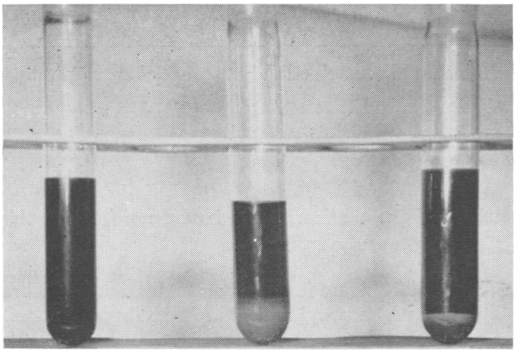

FIG. 2. Precipitate formation on incubation of human bile. See text for explanation.

bile salts concentration, contrary to the opinion of Isaksson.[7] Such precipitation in bile seems to represent an initial stage of gallstone formation but investigations have been limited to bile obtained from patients who had cholesterol stones. A similar experiment was undertaken by Saitoh[31] with bile obtained at laparotomy from various patients who had calcium bilirubinate stones, cholesterol stones and no biliary disorders. Collected samples were centrifuged and incubated at 38° C. for 48 hours and observed for precipitation. Bilirubin and glucuronic acid contents of the samples were determined before and after incubation, and the resulting precipitates were subjected to physical and chemical analyses.

Control bile samples developed no precipitation nor appreciable change in concentration of bilirubin or glucuronic acid. The bile from patients with cholesterol stones occasionally produced precipitates even in non-infected specimens; the precipitates showed about the same appearance as in Tera's experiment, i.e., a large quantity of cholesterol crystals. Bile from patients with calcium bilirubinate stones, mostly infected with *E. coli,* invariably yielded a precipitate of spherical particles which consisted mainly of calcium bilirubinate and resembled a suspension of powdered pigmented calcium stone. After incubation, the content of bilirubin decreased and that of free glucuronic acid increased in the supernatant, demonstrating that bilirubin glucuronide in bile was hydrolyzed by the enzymatic activity of β-glucuronidase of bacterial origin into free bilirubin and glucuronic acid, and the former combined with inorganic elements to form the spherical particles of calcium bilirubinate. Chemical and physical analyses, especially paper electrophoresis and infrared spectroscopy, of the precipitate showed the presence of calcium bilirubinate and substances such as phospholipids, polysaccharides, bile acids and insoluble proteins which are all common ingredients of calcium bilirubinate stones. Thus, such precipitation of infected bile is considered an initial stage of pigmented calcium stone formation. When a large amount (50 mg./ml.) of a bacterial preparation of β-glucuronidase (Sigma Chemical Co.) was added to normal bile, the incubation procedure resulted in formation of precipitates similar to those from infected specimens. Figure 2 shows three test tubes containing a control bile sample (left), a bile sample of a case of pigmented calcium stone (middle) and a normal bile sample supplemented with β-glucuronidase (right), respectively, after incubation. Precipitates are seen in the latter two samples but not in the first. It should be noted that precipitates of bile in patients with gallstones are quite different, not only in mechanism of formation but also in composition, between patients with cholesterol stones and those with pigmented calcium stones that are invariably infected with *E. coli.*

Substances Inhibiting the β-glucuronidase Activity

Although β-glucuronidase of bacterial origin plays an essential role in the development of calcium bilirubinate stones, biliary infection with *E. coli* does not always give rise to gallstones. It is presumed that

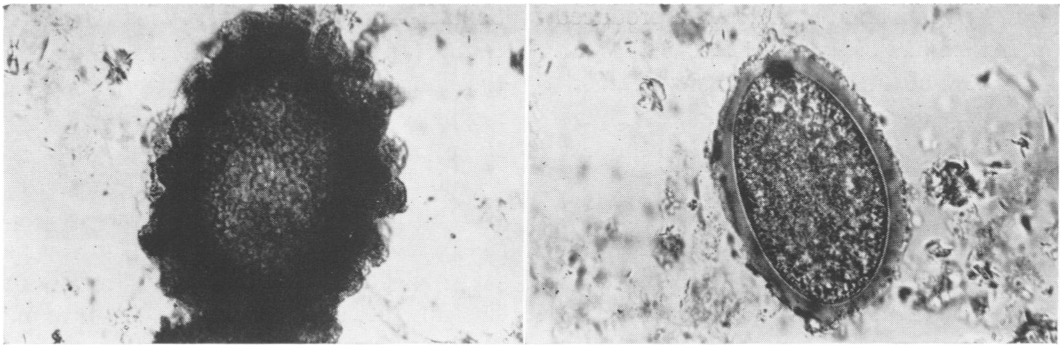

FIG. 3. Remarkable precipitation of calcium bilirubinate onto the surface of ascaris eggs that were incubated in human bile (left). The precipitation is apparently inhibited by addition of glucaro-1,4-lactone to bile, as indicated in the right picture.

bile contains substances that inhibit the β-glucuronidase activity. Matsushiro[18] demonstrated that normal bile considerably inhibited the activity of bacterial β-glucuronidase; glucuronic acid and copper ion among usual constituents of bile had some inhibitory effects. Effects were almost negligible at concentrations in the ranges found in bile, and the inhibitory effect of bile seemed not to be ascribable to these substances.[19] In 1963, Marsh[17] elucidated a pathway through which glucuronic acid is metabolized into glucaro-1,4-lactone, a powerful inhibitor of β-glucuronidase. This substance is partly excreted in urine as confirmed by Marsh and later by Ishidate.[8] Recently, Matsushiro[20] qualitatively identified this substance for the first time in bile by means of paper chromatography. A successful method was devised for quantitative estimation of glucaro-1,4-lactone (as glucaric acid) in bile by means of column chromatography.[48] Oral administration of this substance was followed by a marked reduction in the β-glucuronidase activity in bile. In *in vitro* experiments, precipitation of calcium bilirubinate from infected bile was prevented by adding glucaro-1,4-lactone to the specimen. Precipitation of calcium bilirubinate onto ascarid eggs in infected bile was prevented by application of glucaro-1,4-lactone (Fig. 3).[21] These facts indicate that inhibitors of β-glucuronidase in bile, especially glucaro-1,4-lactone, control separation of calcium bilirubinate in bile by depressing the activity of bacterial β-glucuronidase. According to determinations, the content in bile of inhibitory substances is subject to considerable individual variation. Although the reason for this is not known, we believe that diet may be a significant factor. It may be significant that the separation mechanism of ingredients of calcium bilirubinate stones has been clarified and that one can experimentally produce, or inhibit, precipitation of calcium bilirubinate from bile, unlike ingredients of cholesterol stones.

Coagulation Mechanism of Calcium Bilirubinate Particles Sedimented in Bile

The above-mentioned theory explains the separation mechanism of calcium bilirubinate as insoluble particles in bile. However the mechanism by which these particles coagulate and solidify to form a gallstone is not clear. Most previous work on gallstone formation, both cholesterol and bilirubin, have left this subject untouched. Recently, Yusa and Gaudin[49] succeeded in preparing pellet-like flocks from a suspension of kaolinate. The mechanism of coagulation of bile sediment has been investigated by Maki and Suzuki,[16] who produced a gallstone-like concrement *in vitro* from a suspension of human bile sediment consisting chiefly of calcium bilirubinate. Experimental results are described in the following.

Coagulation of a colloidal substance may first require elimination of hydrophil layers on colloidal particles to bring the particles close to each other. Adequate energy is necessary to coagulate particles firmly, as pointed out by Yusa and Gaudin.[49] These conditions can be brought about by the following procedures: 1) addition of electrolytes which approximate the colloidal particles by shrinking the ionic atmospheres around them, 2) addition of a high-molecular-weight substance that promotes aggregation of the particles by its bridging action, and 3) application of physical movement to supply kinetic energy required for consolidation of the aggregate. An outline of the experimental procedures is described below.

Coagulating Effects of Inorganic Substances

Bile was collected from patients with calcium bilirubinate stones through a T-tube inserted in the common bile duct, and the sediment of calcium bilirubinate was prepared by incubating it. To simplify the condition, the experiment was performed with a suspension of the sedimented calcium bilirubinate in distilled water. The ζ-potential of suspending particles of calcium bilirubinate measured approximately -20 mV in distilled water when determined with a microscopic electrophoresis apparatus. When inorganic cations, such as calcium ion, which are opposite in electric charge to the particles of bile sediment were added to the suspension, the absolute value of the ζ-potential was lowered and the suspending particles coagulated. The larger the valence of the cation, the more remarkable the coagulating effect, agreeing with Schultz-Hardy's law. Hydrogen ion concentration appeared to significantly influence the above mechanism, the coagulation being more remarkable at an acid pH than in the alkaline range.

The coagulate thus obtained by addition of an inorganic electrolyte was gel-like flocks which were hydrophilic and did not drain well. Such a hydrophilic nature of the flocks is due to a hydrated layer surrounding each particle, and this must be removed to transform the flocks into well drained coagulates. This was attained to some extent by adding calcium carbonate to the suspension. The dehydrating effect of calcium carbonate may be that fine particles of this compound adsorb colloid particles on their surfaces and thus disintegrate the gel structure of the flocks of the colloid particles.

Coagulating Effects of Organic Substances

A group of high-molecular-weight organic compounds are also effective as coagulating agents for colloidal substances and a study was made of their effects on the suspension of bile sediments. Konanfloc 1250 and Konanfloc 2000 (Konan Chemical Co.) are both linear polyelectrolytes of positive charge, exhibiting marked coagulating effects. It was found that the effect of such a polyelecetrolyte was enhanced by simultaneous addition of an inorganic electrolyte such as calcium chloride or aluminium chloride; on such occasions the coagulation of particles was more remarkable and the supernatant became more transparent than in the suspension coagulated with a polyelectrolyte alone.

The effect of linear high-molecular-weight organic compounds on coagulation of bile sediment is probably due to two factors. One is its remarkable adsorption characteristics and the other a bridging action by which molecules of a high-molecular-weight substance combine many colloid particles.

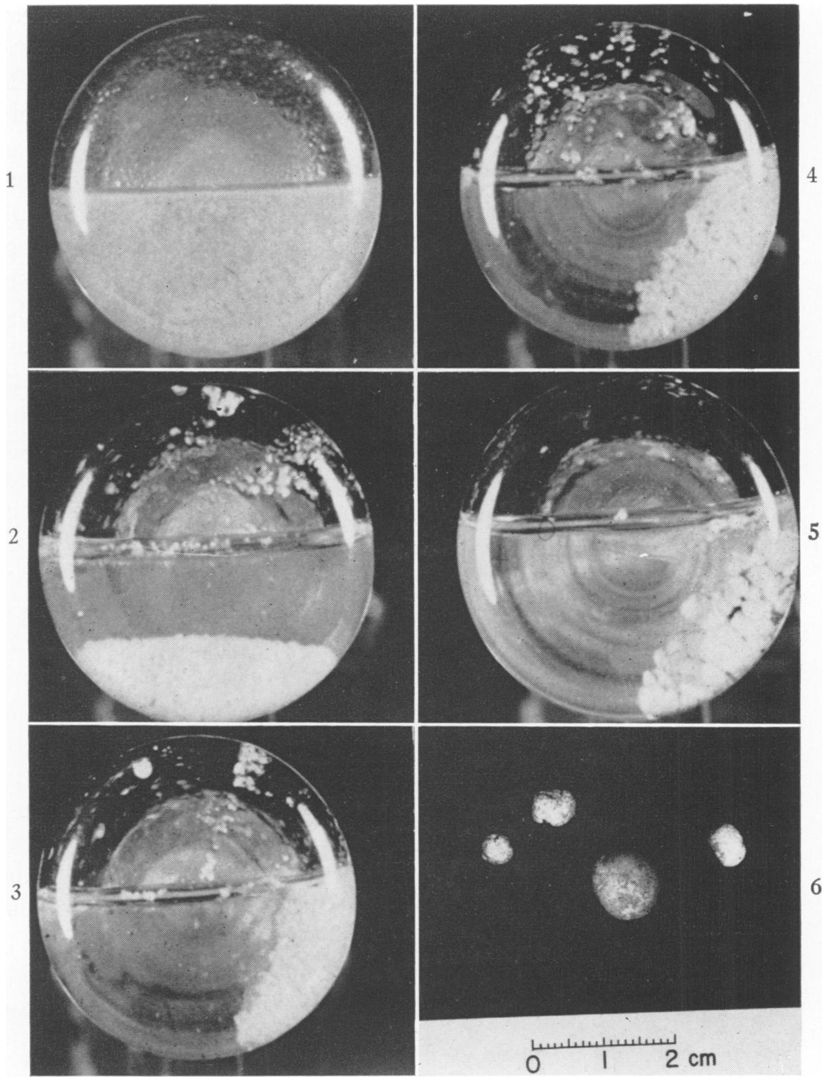

FIG. 4. Development in vitro of gallstone-like concrements as recorded by motion picture. In Frame 1, the flask contains a water suspension of bile sediment separated from a case with calcium bilirubinate stones. Frame 2, the same suspension after addition of calcium carbonate, Konanfloc 2000 and calcium chloride. Note that the suspending particles have been deposited as a flocky mass. Frames 3 to 5 represent the progress of coagulation and solidification of the deposit during rotation of the flask; the coagulates of the deposit grow larger and the supernatant becomes clearer with lapse of time since onset of rotation. Frame 6, the gallstone-like concrements yellowish brown in color produced by this procedure.

The bridging action is apparently due to the large number of function groups arranged around a linear skeleton in the molecule.

Application of Physical Movement to the Sample

On the basis of experiment by Yusa and Gaudin, the suspension of bile sediment was subjected to slow rotation with the inorganic and organic reagents that proved most effective for coagulation of the sample. In the course of rotation, visible coagulates were formed in the suspension, and the coagulates increased in size and finally formed solid concrements in about 30 minutes. The progress of change is shown in Figure 4 which consists of fragments of a movie film taken during the rotating movement of bile supplemented with calcium carbonate, calcium chloride and Konanfloc 2000. Although the role of rotation in the formation of gallstone-like concrements has not yet been established, a probable interpretation is that an adequate form of agitation must be supplied to over-

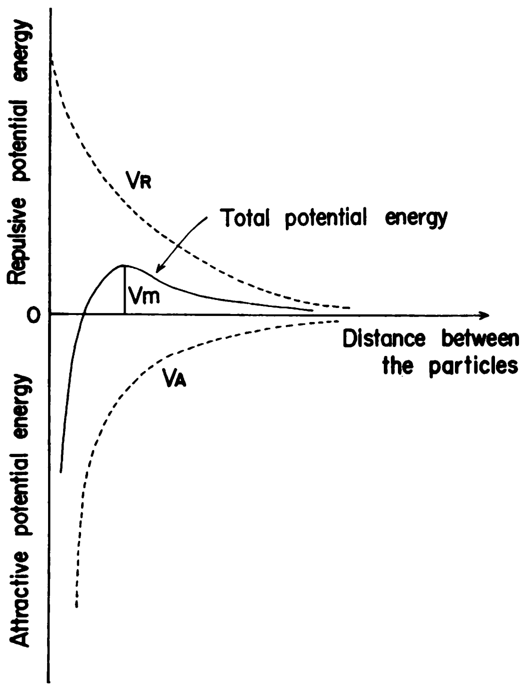

FIG. 5. Schematic explanation of potential energies existing between two colloid particles. V_m, the potential energy barrier, is the maximum value of the algebraic sum of repulsive and attractive energies and serves as a measure of the thermodynamic stability of the system. Two particles coagulate if V_m is smaller than kinetic energy of the particles and disperse in the opposite condition. In other words, kinetic energy of the system must exceed V_m for colloidal particles to coagulate together. V_R: Repulsive potential energy. V_A: Attractive or van der Waals potential energy. V_m: Potential energy barrier.

whelm the potential energy barrier (Fig. 5) that may increase with increase in the size of the particles as coagulation progresses.

These experiments may provide a model for study of the coagulation mechanism of calcium bilirubinate stones *in vivo.* In the human being, many inorganic ions such as Na, K, Ca, Fe, Cu, Mg, Al etc. are present in bile, and high-molecular-weight organic substances that act as bridging agents may be increased in inflammatory processes. But it is not yet clear which inorganic ions and high-molecular-weight substances are actually promoting coagulation of particles of calcium bilirubinate in the biliary tract. The physical energy required for the last step of consolidation may be supplied by motility of the gallbladder and by bile flow due to pressure of bile secretion or movement of the body as a whole. The capability of the biliary system to absorb water provides an additional favorable condition. The fact that solid concrements resembling genuine calcium bilirubinate stones were actually produced *in vitro* on the basis of our hypothesis would justify our view concerning the initiation of calcium bilirubinate stones which are in many instances amorphous. However the reason stratified structure is occasionally seen in gallstones of this type and the coagulation mechanism of cholesterol stones remain unsolved. The theory by Maki and Suzuki may also apply to coagulation mechanisms of other substances separated from a colloidal solution in the living organism.

In conclusion, the pathogenesis of gallstones should be discussed from three aspects, inducement, separation and precipitation of gallstone ingredients in bile and consolidation of these constituents. The problem has hitherto been dealt with vaguely without differentiating these three aspects.

Summary

Both stagnation and infection of bile, especially with *Escherichia coli,* together with dietary deficiencies, appear to induce formation of calcium bilirubinate stones which are common in the Asian area.

As to the mechanism by which calcium bilirubinate separates out of bile, the activity of β-glucuronidase of bacterial origin is presumed to play an essential role; the enzyme hydrolyzes bilirubin glucuronide into free bilirubin and glucuronic acid, and calcium in bile combines at the carboxyl radical of liberated bilirubin to form calcium bilirubinate. On the other hand, bile usually has an inhibitory effect on the β-

glucuronidase activity. This is mainly due to glucaro-1,4-lactone which is present in bile and known to be the most powerful inhibitor of this enzyme. Glucaro-1,4-lactone in bile was identified both qualitatively and quantitatively as glucaric acid.

A new hypothesis has been presented on the mechanism of consolidation of calcium bilirubinate stones: Particles separated from bile, consisting mainly of calcium bilirubinate, are negatively charged and undergo loose coagulation as a result of electrostatic effect of inorganic ions and bridging action of high-molecular-weight organic substances. The coagulate then consolidates more tightly under influence of physical movement. In support of this hypothesis, solid stone-like concrements which resembled the amorphous pigmented calcium stones in man were produced in a model experiment.

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