OBSERVATIONS ON SOME CAUSES OF GALL STONE FORMATION.

II. On Certain Special Nuclei of Deposition in Experimental Cholelithiasis.

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Plates 16 to 19.

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In a previous paper we have described gall stones developing in dogs under conditions so controlled that the complicating influences of infection, stasis, gall bladder activity, and inflammation of the bile passage may be ruled from account. The present communication embodies the results of a study of the early history of the stones with special reference to the rôle of preformed nuclei in their development.

Potential Centers of Stone Formation.

The calculi which form out of the liver bile of dogs permanently intubated for collection of the secretion consist almost wholly of two calcium salts—carbonate and bilirubinate[1]—in addition to the inevitable organic scaffolding or shadow. The conditions are relatively favorable to carbonate deposition. Only very occasionally does this appear to take place in the absence of a special center of deposition.[1] Sometimes a particle of talc from the wall of the rubber collecting tube serves the purpose of a nucleus, but more often the calculus forms in the midst of small masses of organic débris, or deposition of the salt occurs upon the surface of bilirubinate calculi. The formation of these latter is, by contrast, independent of organic débris, but often they contain minute, pigmented bodies, discrete

[1] Rous, P., McMaster, P. D., and Drury, D. R., *J. Exp. Med.*, 1924, xxxix, 77.

nuclei round which the stone seems to have been built up; while similar nuclei can be discovered not infrequently at the center of carbonate stones. As a whole, the findings indicate that the great majority of the calculi have their beginnings in preformed particles of one sort or another. The source and nature of such of these particles as have origin within the body will now be considered.

The importance of special centers of deposition for lithiasis has long been recognized; and many bizarre, and perhaps for that reason convincing, instances have been described. There is no need to enumerate the kinds of foreign bodies, including bacteria, reported in association with stones. Studies of the "strawberry gall bladder" have brought out the fact that small, rounded portions of the wall laden with cholesterol may be cast off into the bile; and it is supposed that upon these stone formation sometimes takes place.[2,3] Aufrecht[4] has advanced the opinion that pigmented particles, the product of disordered liver cells, may be set free into the bile and serve as the basis for stones; and Naunyn has suggested a rôle of "bile thrombi" in such relation.[5] From pathological gall bladders a variety of sediments may be obtained; and many believe that these have some connection with the development of stones. References to such sediments there have been from the time of Charcot, but no comprehensive study of them. Naunyn's brief surveys,[5,6] morphologic in their general character, and that of Lichtwitz[2] are perhaps the best on record. Aschoff[7] dismisses the subject with the statement that one can recover from the normal gall bladder all of the sediments (*Niederschläge*) which have been described as centers of crystallization. We have been unable to find the paper on the sediments by Torinoumi, which is mentioned by him.

Organic Débris in Dog Bile.

For observations upon the particulate matter in dog bile we have employed twelve dogs intubated for the purpose. The method and conditions of intubation have already been described.[8] A portion of the bile removed each day from the collecting balloon connected

[2] Lichtwitz, L., *Ergebn. inn. Med.*, 1914, xiii, 1.

[3] Boyd, W., *Brit. J. Surg.*, 1923, x, 337.

[4] Aufrecht, M., *Deutsch. Arch. klin. Med.*, 1919, cxxviii, 242.

[5] Naunyn, B., *Mitt. Grenzgeb. Med. u. Chir.*, 1921, xxxiii, 1.

[6] Naunyn, B., A treatise on cholelithiasis, Sydenham Society translation, London, 1896.

[7] Aschoff, L., *Klin. Woch.*, 1923, ii, 957.

[8] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 11.

with the common duct has been centrifuged immediately and the sediment examined with the microscope and, when advisable, in divers other ways.

The balloons, like the remainder of the collecting system, were cleansed of soluble matter prior to use by boiling them in a weak solution of sodium bicarbonate, soaking in 2 to 5 per cent hydrochloric acid, and rinsing thoroughly. They were autoclaved twice in a large excess of distilled water. Often they were kept in the flasks of sterilization for some weeks prior to use. The bile which gradually assembled in them in the course of each 24 hours was protected from the air but had cooled somewhat, owing to its situation within a basket bound against the skin surface. The cooling was undoubtedly responsible for the major share of the precipitation observed, but the fact that one similar in kind went on within the animal was sufficiently shown by the character of the deposit upon the walls of the tubing inside of the body. To facilitate deposition in the tube system, bulbs about 1 cm. in diameter had been blown in the shanks of some of the cannulas (Fig. 17) and in some of the short glass tubes connecting the arms of the rubber U lying inside the abdominal cavity.

A heavy bile sediment was frequently obtained from animals with damaged livers. Afanassiew,[9] Brauer,[10] and others[11] have drawn attention to the presence in the secretion under such circumstances of coarse cylinders and of cells from the hepatic parenchyma. We have observed these after sublethal injury by chloroform, in company with red cells, leucocytes, and intact circlets or rings of desquamated epithelium from the ducts. Under such circumstances the bile may be red with blood and yet the animal be lively, have a good appetite, and in other ways appear healthy. Bile from normal livers, which had become infected during the course of the day-to-day collection, often contained numerous small mucous spheres, light green, and loosely grouped in wreaths or in bunches like grapes (Fig. 7). These were presumably derived from an abnormal duct mucosa, though no evidence of derangement was to be seen later in microscopic sections of the duct wall. Very occasionally the grouped spheres were present in sterile bile. In the secretion from an infected biliary tract, large numbers of small cylinders were sometimes to be found

[9] Afanassiew, M., *Z. klin. Med.*, 1883, vi, 281.

[10] Brauer, L., *Z. physiol. Chem.*, 1903–4, xl, 182.

[11] Haupt, H. G., *Arch. internat. pharmacod.*, 1903, xi, 155. Pilzecker, A., *Z. physiol. Chem.*, 1904, xli, 157.

(Figs. 5 and 6) which much resembled urinary casts both in size and appearance. Some consisted of brown, granular mucus, others were colorless and appeared homogeneous, while yet others were made up almost wholly of bacteria. They corresponded to the smaller type of cylinder described by Brauer. As a rule, no cells whatever were present in association with these elements which latter were obviously the result of a duct irritation less in degree than that causing desquamation. Much shorter casts, like small brown mucous plugs, were found on one occasion in the uninfected, deeply pigmented bile secreted after intravenous injections of dog hemoglobin.

Hemoglobin injections caused a thickening of the bile with mucus, as many previous workers have noted. In the sediment this had the appearance of brown filaments or ill defined, granular clumps. Small, flat, shreddy masses or sheets of mucus were not infrequent in "normal" biles, when the cannula had caused local irritation of the duct by pressure.

The sterile bile from healthy ducts was often almost water-thin and contained, even on cooling, practically no mucus that could be thrown down with the centrifuge,—far less than the ordinary nubecula of urine. But a well defined sediment there usually was, one of more or less pigmented and imperfect calcium carbonate crystals. And we have repeatedly observed in the sediment early stages in the formation of carbonate stones identical in type with others, often much larger, recovered at autopsy from the tubing within the animal. They tended to develop about nuclei of special constitution. But before the nature of these latter is considered the fact should be emphasized that none of the organic débris just described was ever observed to act as a center for stone formation. In a previous paper[1] the frequent situation of carbonate stones within the layer of organic material sometimes coating the tube system has been emphasized, and the opinion has been expressed that the association is such as to indicate that some secondary change in the material is responsible for the inception of the stones. The fact that the material, when fresh, as in the bile sediment, does not induce carbonate deposition despite a tendency for the salt to come out of solution adds further support to this view.

Nuclei of Deposition.

The bile of the first 24 or 48 hours after the operation whereby intubation is effected, though often greatly concentrated, at least in some substances,[12] yields as a rule no sediment other than mucus, which for the time being is increased in amount. That of the 2nd or 3rd day contains a slight brown, or greenish brown, dust-like powder, less than $\frac{1}{100}$ cc. to a 15 cc. bile specimen. Under the microscope this dust has the appearance of minute, highly refractile spheres (Figs. 9 and 10), some separate, some partially fused into diplococcoid (Fig. 8) or dumb-bell forms, or into irregular concretions or even into elongate "worms." These nuclei, as we shall term them, are much heavier than water and bear washing with it in the centrifuge. They are brilliant with brown or yellow-brown pigment but can be decolorized by a few hours' incubation with commercial hydrogen peroxide that has been rendered neutral with sodium hydrate; and they are then seen to be doubly refractile. They may persist in the bile, though in diminished quantity, until about the 6th day after operation, but usually are not met thereafter unless the liver is injured in some way, when they may again appear for a greater or less period. They serve as centers for calcium carbonate deposition (Figs. 8, 11, 12, and 16), and in some animals only those first found after intubation are free from crystals of the salt or a concentric layering with it. The contrast between the well-nigh colorless carbonate and the deeply pigmented nucleus upon which it has come down is often a striking one.

Needless to say, the collecting balloon was emptied as completely as possible each day, of sediment as well as of bile. Thus stone formation within it was periodically interrupted, and only microscopic calculi could be expected in the sediment.

Before discussing the nuclei in detail, it will be well to meet the question whether they and the minute stones recovered from the bile of the balloon had any real connection with the calculi recovered from the tubes within the animal. Proof of the development of such nuclei *in vivo* was forthcoming in the instance of an animal the sterile bile from which contained mucous casts obviously derived from small ducts, with brown nuclei along their central axis identical with those we have just described. Other animals yielding nuclei into the balloon showed them at autopsy lodged here and there on the walls of the tube system. Often when stone formation had gone on for a considerable time, the large calculi sessile on the walls of the cannula and of the few centimeters of tubing nearest the bile source showed nuclei at their center (Figs. 13 and 18); while, further down, small nucleated stones were met, identical with those sometimes present in a 24 hour specimen from the balloon; and lower yet, nuclei were recovered in the free state from the tube wall. Free nuclei were indeed frequently present on the tubing of animals which failed to develop stones, whence it follows that they did not inevitably induce lithiasis.

Nature of the Nuclei.

What was the character and derivation of the nuclei?

They failed to take the stains for neutral fats (Sudan III, Scharlach R), but with Nile blue sulfate were colored a deep blue like the fatty acids. They did not consist of myelin, since they proved insoluble in alcohol; nor did they contain cholesterol as was sufficiently shown by their resistance to ether, to acetone, and to boiling chloroform, and by the further fact that chloroform extracts failed to give the Liebermann-Burchard reaction. They did not stain by Gram. Heat fixed them on the slide but they retained shape and remained anisotropic despite it. Dilute hydrochloric acid (10 per cent) in water deprived them of all refractility; they became somewhat smaller and the irregular forms became spherical. Sometimes they effervesced on such treatment and again not, but regularly they changed color, turning gradually green-black or deep blue. It was this deep color and the persistence of the forms as well-defined shadows which enabled us to distinguish them at the centers of large calculi softened with hydrochloric acid and flattened by pressure.

From the reactions as given, it is evident that calcium carbonate must have been one constituent of the nuclei. Their doubly refractile character may well have been referable to its presence. Yet they cannot have consisted only of carbonate tinted with bilirubin. For much of their substance survived the treatment with acid. The pigment did not come away from them in chloroform even on boiling, though after subjection to acid it passed into this solvent of bilirubin and then gave the Gmelin reaction. Calcium bilirubinate, the substance concerned in the more darkly colored calculi within the tube system,[1] behaves in this way. Stones composed in great part of it appear soft in comparison with carbonate stones, fracturing upon pressure into rosettes of fragments with rounded angles.[1] This was the case too with the nuclei (Fig. 10). A well defined shadow or scaffolding of elastic mucus was brought out by treatment of them with acid alcohol, in which their other constituents dissolved.

Altogether, it would seem that two substances besides the organic shadow were the main ingredients of the nuclei, calcium carbonate and calcium bilirubinate, this latter being the more important as distinguishing them from ordinary forms of carbonate precipitate in the bile. Their appearance and fracture pattern suggest attempts at crystallization.

The nuclei, as already stated, were recovered from the bile of healthy animals only during a brief period after the operation to insert the collecting apparatus. But they were frequently met with at other times when the liver was purposely damaged.

Thus, for example, they were seen in association with delayed chloroform poisoning, as also when metatoluylenediamine had been given by mouth. Concurrent observations on the incidence of nuclei and the pH of the bile were frequently made with the potentiometer, but no relationship between the two could be made out. Nor was the total calcium content of the bile, as obtained by ashing, different when they were present than when they were not. Ether anesthesia of several hours' duration did not cause them to appear. Intravenous injections of a concentrated calcium chloride solution were sometimes followed by a "shower" of them, and again not; and they appeared sometimes immediately after the relief of total obstruction of 24 to 48 hours' duration. Increased concentration of the bile consequent on prolonged fasting[12] did not cause their occurrence even when the change was great, as in one instance in which the secretion diminished from more than 150 cc. in a 24 hour period to between 11 and 12 cc. on each of 2 successive days with a reciprocal increase in the concentration of bilirubin.

Carbonate Deposition.

The stones forming out of calcium bilirubinate in the intubated animals were always restricted to the few centimeters of tubing nearest the bile source.[1] From this it would appear that there was relatively little of the material available for calculi. The state of affairs as concerns calcium carbonate was wholly different. Crystals of the salt (Fig. 14), not infrequently a copious sediment of them (Fig. 15), were in most instances present in the balloon specimens from a day or so after operation until the observations were terminated, weeks

[12] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.

or months later. The deposition of carbonate on the walls of the intraperitoneal tubing during the same period was comparatively slight, and took the form, not of a sediment but of discrete calculi. It follows that the precipitation in the balloon must be ascribed for the most part to extraneous factors. There can be no doubt that cooling was chief amongst these. Chilling the fluid in the ice box frequently brought on additional precipitation.

The crystals were sometimes perfect and almost colorless, and again, when they had come down out of a dark bile, deep yellow and poorly formed. Often their angles were blunted, or, when they were present in aggregates, these had undergone a rounding, evidently because a portion of the material had dissolved again. In this way spherical nuclei of the sort we have just described were sometimes simulated. But the substance of such pseudonuclei showed planes of cleavage instead of being homogeneous, and on treatment with acid they effervesced and vanished wholly save for a lightly tinted shadow. Occasionally forms were noted which can only be considered as intermediate between true nuclei and carbonate crystals. From a study of them one gains the impression that they arose through the combination in a different proportion of the same materials found in the true nuclei, that is to say crystalline carbonate and brown, amorphous bilirubinate. Many of the stones described in our previous paper[1] were so constituted.

Concentric deposition is a marked characteristic of the carbonate stones of human beings. The minute stones recovered from the balloon specimens of our dogs usually exhibited this characteristic pronouncedly (Fig. 19). Most of them were slightly pigmented, appearing of a brilliant green or yellow under the microscope.

The Rôle of Bile Thrombi.

When the liver had been damaged by methods that caused jaundice, elements closely resembling the so called bile thrombi that are present in the hepatic tissue under such circumstances appeared in the bile itself (Figs. 1, 2, 3, and 4). The question arose of their identity as also that of whether the nuclei just considered might not, though differing in aspect from bile thrombi, be composed of the same materials.

Heinrichsdorff[13] has employed microchemical methods to study the bile thrombi in formalin-fixed or frozen sections of the human liver. He bleached them with hydrogen peroxide and found that they were not anisotropic, though highly refractile. They exhibited the various forms which arise by the partial merging of a greater or less number of spheres, and they were almost completely destroyed by hydrochloric acid, suggesting, as the author remarks, that they contained calcium, though he was unable to demonstrate its presence. They were Gram-positive and became deep blue when submitted to Nile blue sulfate, but were only dubiously tinted by Sudan III. They gave some of the reactions for lipoids. Those from a case of hepatic carcinomatosis differed in minor details from such as were the result of ordinary biliary obstruction.

We have compared the thrombi of icteric dog livers with elements morphologically similar recovered from the bile of the same animals, and with the little nuclei occurring in the bile after the intubation of healthy dogs.

Icterus was produced by the oral administration of toluylenediamine or by gradual biliary obstruction or prolonged chloroform anesthesia. The thrombi were freed from the liver tissue by scraping the cut surface with a knife wet with water or salt solution, suspending the pulp thus obtained in one of these fluids, and filtering through several layers of gauze. There resulted a suspension of individual cells and thrombi. The latter could now be examined directly, or they could be rid of their association with tissue elements through the action of trypsin in 2.5 per cent solution rendered slightly alkaline with sodium bicarbonate. The thrombi alone survived the tryptic digestion and after a few hours of it at 37°C., could be recovered in enormous number by centrifugation. In one exceptional instance of a few scattered thrombi recovered from a liver shortly after chloroform poisoning, these were destroyed at the same time as the cells.

The thrombi were found to answer closely the description of those studied by Heinrichsdorff. The small ones were spherical or oblong, and the larger ones irregular, or even twig-like, and occasionally forked. They were obviously formed by the more or less perfect fusion of small spheres from within the liver cells, which spheres survived the treatment with trypsin as well as did the thrombi. The material composing them was homogeneous in appearance, highly refractile, and ranged from yellow to dark brown. They were readily bleached with peroxide and were not anisotropic, thus differing essentially from the nuclei of the bile sediment. They were much lighter than the nuclei, having approximately the same specific gravity as the liver tissue. They were Gram-positive, stained faintly to darkly blue with Nile blue sulfate, but were not colored at all with the

[13] Heinrichsdorff, *Berl. klin. Woch.*, 1920, lvii, 1217; *Centr. allg. Path. u. path. Anat.*, 1922, xxxii, 314.

stains for neutral fats; and they were unaffected by water, glycerol, ether, ethyl alcohol, chloroform, and acetone. They could be fixed on the slide by heat without loss of shape. They gave Gmelin's reaction. On standing in water in an open vessel, they gradually turned from brown or yellow to green, as would have been the case had the pigment associated with them been bilirubin as such. The pigment of the nuclei in the bile sediment remained, by contrast, unchanged even after several days in water, an additional reason beyond those already supplied for supposing it to exist in combined form (calcium bilirubinate). Dilute hydrochloric acid caused the thrombi usually, though not always, to lose their refractility together with most of their substance, a pigmented shadow alone remaining.

From this description it will be evident that in many of their characters the thrombi of jaundiced livers resemble the nuclei found in bile sediment, but that in others, and these essential ones, they differ therefrom. The elements in the bile from damaged livers which looked like liver thrombi gave identical reactions with those for these latter, as just enumerated. There is good reason to suppose them to be composed of the same materials. But that they came down as such through the narrow and tortuous bile radicals from an original situation within the lobule is open to doubt. No deposition of carbonate upon them was ever observed.

Nuclei in Human Bile.

In continuation of the work, it has seemed worth while to examine the sediment of human bile, and we have been enabled so to do through the generous cooperation of Doctor Allen Whipple of the College of Physicians and Surgeons of Columbia University. Several fistula specimens were procured from patients recently relieved of obstruction and with rapidly diminishing jaundice. All appeared highly dilute, in keeping with what has been observed of dog bile under like conditions of readjustment.[12] None yielded sediment other than an occasional cell. The nine consecutive cases furnishing gall bladder specimens were a diverse lot, comprising two instances of common duct obstruction by carcinoma of the pancreas, one of a definite cholecystitis without stones, two of dubious cholecystitis, and four with stones and a chronic mild inflammation of the gall bladder. No significant sediments were encountered except in this last group, only flakes of more or less stained mucus and a few desquamtaed cells. But in the very dark, thick biles of three of the four cases with stones, there was present in addition to these latter a sediment consisting of mucous flakes and particles, desquamated cells, cholesterol plates, and minute concentric concretions, or spheroliths, consisting of calcium carbonate (Figs. 20, 21, and 22); while, in one of the three, early stages in the development of cholesterol stones about such concretions were also found (Figs. 23 and 24) and in two of them the concretions were demonstrable within well formed calculi (Figs. 25 and 26). In the third case they were not sought there. Both through their character and relationship to stone formation as thus attested, the little spheroliths bore a striking resemblance to the nuclei we have encountered in dogs.

The carbonate concretions were as a rule spherical or boat-shaped (Figs. 21 and 22), more rarely diplococcoid or lemon-shaped, or with rays like a blunt star (Fig. 20), or even worm-shaped through the partial fusion of several spheres. They were yellow, green, brown, or colorless, and showed sharp-cut concentric zones, often further demarcated by pigment variations. Most, but not all, were brilliantly anisotropic. They resisted acetone, failed to stain with Scharlach R, but were rendered light to dark blue by Nile blue sulfate. They could be fixed on the slide by heat which, for the rest, seemed not to change them. Under pressure they broke along both radial and concentric lines. They effervesced actively when treated, after washing in water, with a dilute watery solution of hydrochloric acid applied with a micro-pipette, and the greater part of their substance rapidly dissolved, leaving a more or less pigmented shadow having the original shape (Fig. 26). That these carbonate spheroliths were not an *in vitro* product was sufficiently proven by their presence deep within some of the smaller calculi associated with them (Fig. 26). The primary stones were not examined, unfortunately, but in both the instances in which secondary stones were studied with reference to the point such inclusions were found. The stones consisted predominantly of cholesterol in these cases but differed in that those of one dissolved in acetone completely save for a thin mucous shadow and the contained spheroliths, whereas those of the other underwent only a surface disintegration in this solvent but when subjected to weak hydrochloric acid effervesced and fell apart into a multitude of cholesterol plates (Fig. 26). Evidently these latter had been held together in a matrix of calcium carbonate. Fragments of a large radial cholesterol calculus were present in the bile of the case first mentioned; and even before acetone treatment it was evident that these contained carbonate spheroliths. Afterwards, in the mucous shadow of them, the spheroliths stood forth strikingly, often several to a stone fragment. Curiously enough, they interfered almost not at all with the radial structure. The radial lines parted about them and joined again like threads of silk about bullets placed within the skein. Obviously the spheroliths were only accidental inclusions. Far different was the condition of affairs within certain other small calculi from the same animal. These showed a concentric structure, with sometimes a pigmented, earthy looking core (Fig. 25), though cholesterol predominated in them; and near their center one or more spheroliths were usually to be found. It cannot be said on such evidence that the stones had been built up about the spheroliths. But among the free bodies of this latter sort were to be noted some that had a thick surface layer of cholesterol plates, or of stained organic material (Figs. 23 and 24). Such beginnings of stone formation were not encountered in another bladder specimen searched for them; the stones, though small, were all fully formed. But within them after acid treatment the discrete shadows of spheroliths could be discerned. The bile of both cases was sterile on culture (aerobic, anaerobic), and microscopically as well. In a third case, in which the spheroliths existed as such, the secretion proved to be infected.

The spheroliths are probably the same that Naunyn terms calcium bodies and describes as highly refractile, smooth and spherical, rugose, or occasionally shaped like "morning stars." We have gone into details concerning them because they have received but scant notice in the past; while furthermore their relationship to the genesis of secondary stones seems not to have been noted. It is our impression from the few instances studied, as from the literature on the sources of calcium in human bile, that, unlike the nuclei we have recovered from dogs, they do not arise out of liver bile as such but from the contents of abnormal gall bladders. A large material should be studied with relation to their presence and their rôle in stone formation.

DISCUSSION.

There has been much debate among students of cholelithiasis over the share therein of organic débris. Naunyn[5] describes soft organic lumps upon which he believes that calculi are built up; and Bacmeister[14] holds that the common faceted stones, which are, as he remarks, the great majority of gall stones, form about desquamated epithelial cells, cell nuclei, bacteria, or mucus, by a deposition of calcium as carbonate or in organic combination. These are but two

[14] Bacmeister, A., *Beitr. path. Anat. u. allg. Path.*, 1908, xliv, 528.

among many opinions. Our observations make clear the fact that, in the dog at least, organic débris of the sorts mentioned is frequently voided in quantity under circumstances of liver or duct derangement, but that no deposition from the bile takes place thereon despite a well defined tendency for the calcium carbonate in the fluid —the principal constituent of calculi—to come out of solution. The deposits of calcium carbonate in spherolith form, and of cholesterol crystals and amorphous calcium bilirubinate, which we have encountered in the bile from human instances of cholelithiasis, have likewise proved to be independent of the numerous flakes and particles of organic débris also present in the sediment. On the other hand, calcium carbonate is, in the dog, frequently laid down within old accumulations of such débris presumably as a consequence of secondary changes or conditions therein;[1] while in man carbonate calculi have a notable association with severe inflammation of the biliary tract such as would favor accumulations of the sort. The distinction thus brought out is an important one. Organic material cannot accumulate in the bile channels except when they are for some reason poorly evacuated. Lessened motility of the ducts combined with an unusual supply of such material will of course have such result. And it may be significant that many of the successful attempts to induce stone formation in animals have involved procedures whereby both of these conditions are fulfilled (Gilbert and Fournier,[15] Mignot,[16] Ehret and Stolz,[17] and others). True, the authors of the experiments have considered infection to be the prime agent in the cholelithiasis; and though they lay emphasis on the importance of lessened motility, it is as furthering infection. But our work leaves no doubt that in the dog, at least, carbonate stones will form out of bile in the absence of infection; while that the substance can on occasion come out of sterile human bile in spherolith form is sufficiently shown by the findings in the few instances of cholelithiasis we have here described. Bacmeister[18] has noted the precipitation of small amounts of calcium carbonate out of sterilized human bile incubated

[15] Gilbert, A., and Fournier, L., *Compt. rend. Soc. biol.*, 1897, iv, 936.
[16] Mignot, R., *Arch. gén. méd.*, 1898, ii, 129.
[17] Ehret, H., and Stolz, A., *Berl. klin. Woch.*, 1902, xxxix, 13.
[18] Bacmeister, A., *Münch. med. Woch.*, 1908, lv, 211.

in vitro, and both he and Aschoff have questioned whether carbonate stones may not sometimes form in the absence of infection. Proof in the matter has heretofore been lacking.

In a previous paper we have advanced the view that the infection so often encountered in association with human stones consisting of calcium carbonate may act to cause them not by effecting changes in the bile favorable to precipitation,[19] as some have supposed, nor by eliciting though inflammation an exudate rich in calcium, as others have thought, but merely by setting up and maintaining such a pathological condition as would tend to further the accumulation of organic débris and its retention long enough for certain changes to take place which are a preliminary to carbonate deposition. The physicochemical circumstances determining the calcification itself may well be akin to those through which calcium salts come to be laid down in necrotic foci elsewhere in the body, though with this difference, of course, as concerns the biliary calculi of the dog, that no phosphate is deposited.

The activities of the gall bladder to alter and concentrate the bile and to elaborate calcium carbonate have not thus far been referred to because carbonate stones can form without their aid, while a recognition of them in our discussion greatly complicates the issue. It stands to reason that gall bladder conditions and activities must be of the greatest importance for certain clinical instances of lithiasis. They may well have been a prime factor in the causation of the calcium spheroliths we have described, which latter were recovered from thick, dark, bile specimens that had obviously undergone concentration and an addition of mucus.

Naunyn's query[5] as to whether "bile thrombi" coming down out of the liver may act as nuclei for calculus formation can be answered in the negative as concerns the experimental lithiasis with which we have had to do. The particles of what would appear to be thrombus material present in the bile from damaged livers were never observed to function as centers of deposition, unlike the minute pigmented bodies, or nuclei, consisting of calcium bilirubinate and carbonate that were often associated with them. These latter were a frequent precursor of lithiasis. That they were not preformed in

[19] Galippe, V., *Compt. rend. Soc. biol.*, 1886, xxxvii, 116.

the liver seems certain; for they were never to be seen in scrapings of the tissue. Their appearance suggests that they represent attempts at crystallization out of an impure solution. Bacmeister[18] has pictured spherical bodies not unlike them in appearance, though formed of cholesterol, which sometimes come down out of human bile long incubated *in vitro* under sterile conditions; and Bolt and Heeres[20] have reported that spheres of cholesterol come down out of the "bile" elaborated by frog livers perfused with salt solution. Our observations link such findings with actual body processes. The sequence of events as witnessed in the intubated dog is somewhat as follows: Upon damage to the liver, an abnormal bile is elaborated out of which the calcium nuclei fall, and some of them lodge on the walls of the tube system there serving as centers of deposition of calcium salts from the later, more normal, secretion. That the nuclei actually induce deposition from bile out of which it might not otherwise occur remains to be proven. In some instances, certainly, they persist for a long while in the tube system and no stones develop. But in the great majority of instances their presence is soon followed by lithiasis. The stones of such of our animals as were subjected to the single disturbance involved in intubation and were sacrificed early differed from those of others killed later only in that the calculous deposit about the nuclei was in general less considerable. Both sets of stones appeared to date back to the same period of inception, namely to the days immediately after operation when nuclei came down out of the bile. The occasional presence of several crops or generations of stones can be accounted for by the occurrence at subsequent times of liver damage as result of which showers of nuclei appeared.

The frequent presence in the human gall bladder of large numbers of calculi of practically identical size and character has led to a general recognition of the importance in such instances of critical periods of stone inception and formation. And the existence of nuclei of special composition at the center of the great majority of human stones has been frequently commented upon. Lichtwitz[2] has emphasized the point that the stone nucleus is the same or nearly the same in all human calculi, while asking whether this nucleus

[20] Bolt, N. A., and Heeres, P. A., *Arch. ges. Physiol.*, 1922, cxciii, 449.

forms on an inflammatory or non-inflammatory basis. It is possible that as in the dog, showers of nuclei sometimes come down out of the bile of pathological conditions. Our few observations on the occurrence of carbonate spheroliths in the human gall bladder and their relationship to the genesis of secondary stones are merely suggestive in this connection.

The relation of the calcium spheroliths of human and canine bile to the calcospherites sometimes encountered in the tissues is a matter upon which one can at the moment merely speculate.

The fact has already been stressed that stone formation is no inevitable consequence of the presence in dog bile of nuclei of the sort just discussed. They were sometimes recovered in quantity as such from the tubing of animals the bile of which had yielded them only once, and this weeks previously, in the postoperative period. There were exceptional animals which secreted bile that never throughout a long period of observation gave a precipitate even after 24 hours in the ice box. The food, treatment, and general condition of these dogs did not differ from that of the generality, bile from which gave day after day a marked carbonate deposit. One dog of a previous series had at autopsy good sized carbonate stones that had formed in the absence both of nuclei and of cellular débris.[1] All of which is to say that there are peculiarities of the individual which make decisively for or against carbonate lithiasis.

In our previous paper the question was raised why the stones of intubated dogs are never found in the ducts but are always limited to the foreign tubing; and a provisional answer was offered that duct motility, with the cleansing and possibly antagonistic action of the secretion from the mucosa, must be responsible. The question comes up more insistently in connection with the normal accumulation of bile in the gall bladder. Were the viscus a mere collecting bag for the highly unstable secretion as it comes from the liver, cholelithiasis could not but be one of the commonest of canine afflictions, especially since the bladder often contains a layer of old organic débris such as conduces to carbonate deposition. That stones are almost never met with in the unintubated dog argues for some special physiological safeguard against their formation. The aspect thus presented of the problem of cholelithiasis will be dealt with in a subsequent communication.

SUMMARY.

A day-to-day study has been made of the sediment in the sterile liver bile of intubated dogs. There exists a marked tendency for calcium carbonate to be deposited therefrom. After hepatic or duct injury a great deal of organic débris of various sorts may be present in the bile, but it never causes deposition out of the fluid save when it accumulates in quantity on the tube wall. Particles of the material of which "bile thrombi" are composed may be found in the bile when the liver has been appropriately damaged, but these fail to act as centers of stone formation. On the other hand, there are to be found in the secretion after many sorts of hepatic injury little nuclei which undoubtedly serve in this way. These nuclei consist of a mixture of calcium bilirubinate and carbonate with an organic shadow or scaffolding. They do not occur in normal bile, but, coming down on special occasions, prove favorable to deposition out of the secretion elaborated at later periods. We have found nuclei strikingly similar in their relationship to stone formation, but consisting almost wholly of carbonate, in the sterile bile from human gall bladders which contained large calculi. Many of them were encountered in a free state, and others with layers of cholesterol and organic matter upon their surface, while others yet were recognizable deep within matured stones.

The factors concerned in the genesis of gall stones consisting of calcium carbonate have been reviewed at some length in this paper and the preceding one. The evidence we have collected supports the view that the development of carbonate stones in human beings as well as in the dog may be a consequence, not of changes in the bile brought about by microorganisms, nor of the elaboration of an inflammatory exduate rich in calcium salts, but merely of inflammation such as leads to lessened motility of the duct system with the accumulation of organic débris. The fact that infection is almost the sole agent whereby such inflammation is set up and maintained in clinical instances had led too often to the conclusion that it serves as the essential agent in the process of calcification.

The present findings taken with those described in a preceding paper suffice for an understanding of the immediate history of the gall stones which develop in intubated dogs. But the factor of in-

dividual differences, as yet undefined in nature, bulks large in the problem of the lithiasis, as does that of the local physiological safeguards against it.

EXPLANATION OF PLATES.

PLATE 16.

FIGS. 1 and 2. Bile thrombi from a liver that had become icteric as the result of obstruction of the common duct. These were photographed in fresh preparations made by scraping the cut surface of the liver, suspending the pulp in water, and centrifuging. × 730.

FIGS. 3 and 4. Thrombus-like forms obtained from the bile of an intubated dog on the 6th day of chloroform poisoning. The animal was jaundiced. The bile was sterile. × 730.

FIG. 5. A brown, mucinous cast from the infected bile of an intubated dog. × 730.

FIG. 6. A bacterial cast from the same specimen. × 730.

FIG. 7. Mucous globules in wreath form from an infected bile. They were bright green. × 730.

FIG. 8. Secondary deposition of an almost colorless layer of calcium carbonate on a diplococcoid calcium nucleus of yellow hue. The layer shows some radial striæ. The specimen was obtained on the 6th day after operation, from the same animal that yielded the material of Figs. 6, 7 and 8 (*q.v.*). × 730.

PLATE 17.

FIG. 9. Deep brown, calcium nuclei recovered from bile secreted on the day after intubation of the animal. Their variations in shape and high refractility should be noted. × 730.

FIG. 10. Fracture forms of the same nuclei = rosettes. × 730.

FIGS. 11 and 12. Deposits of almost colorless carbonate crystals upon pigmented calcium nuclei like those just figured. There are many free crystals as well. The preparation shown in Fig. 11, which is magnified 600 diameters, came from the bile of the same animal furnishing those of Figs. 9 and 10. It was obtained on the 2nd day after operation. Fig. 12 is from the bile of another dog, collected 4 days after operation. × 730.

FIG. 13. Demonstration of calcium nuclei lying within the small gall stones found at post mortem on the tube wall in a dog sacrificed 14 days after intubation. The animal had previously furnished the material of Fig. 12. The stones came from just below the cannula. They are treated with weak hydrochloric acid which dissolved the layers of calcium carbonate surrounding the nuclei and rendered the shadows of the latter more distinct. × 730.

FIG. 14. Sediment of coarse carbonate crystals from the later bile of the dog furnishing the material of Figs. 9, 10, and 11. The specimen pictured was procured on the 4th day. × 600.

PLATE 18.

FIG. 15. Carbonate sediment in the later bile of the dog furnishing the material of Figs. 12 and 13; specimen of the 6th day. About one-half natural size.

FIG. 16. A layer of crystalline carbonate enclosing a group of calcium nuclei. There was in this instance an especially brilliant color contrast, scarcely visible in the reproduction. × 730.

FIG. 17. Cannula with interpolated bulb, as removed at autopsy on the 14th day after intubation from the dog furnishing the material of Figs. 12, 13, and 14. The punctate distribution, dark color, and angular shape of the calculi should be noted. Nearly all of them were on the side of the cannula that was lowest during life. There are many more in the bulb than elsewhere but they are smaller there, doubtless because of the fact that they soon became overlaid with mucus. The duct end of the cannula is free from stones. × 5.

FIG. 18. A heavily pigmented carbonate bilirubinate calculus of the sort pictured in Fig. 17, after treatment with hydrochloric acid. The included nuclei are well shown. The liver had been severely damaged with chloroform 2 days prior to death, which may account for the presence of peripheral nuclei. × 450.

FIG. 19. Minute concretions of calcium carbonate from a sterile specimen of bile obtained on the 19th day of intubation. The concentric deposition is evident. × 730.

PLATE 19.

FIGS. 20 and 21. Minute concretions consisting almost wholly of calcium carbonate, as observed in the gall bladder bile of a patient with mild cholecystitis and many large gall stones. The bile was sterile. × 150.

FIG. 20 shows a "morning star" form, as also some cholesterol plates and organic débris. Fig. 21 shows only calcium spheroliths, but these with concentric zone varied by color differences. All are much larger than the nuclei found in dog bile (Figs. 9 and 10). × 150.

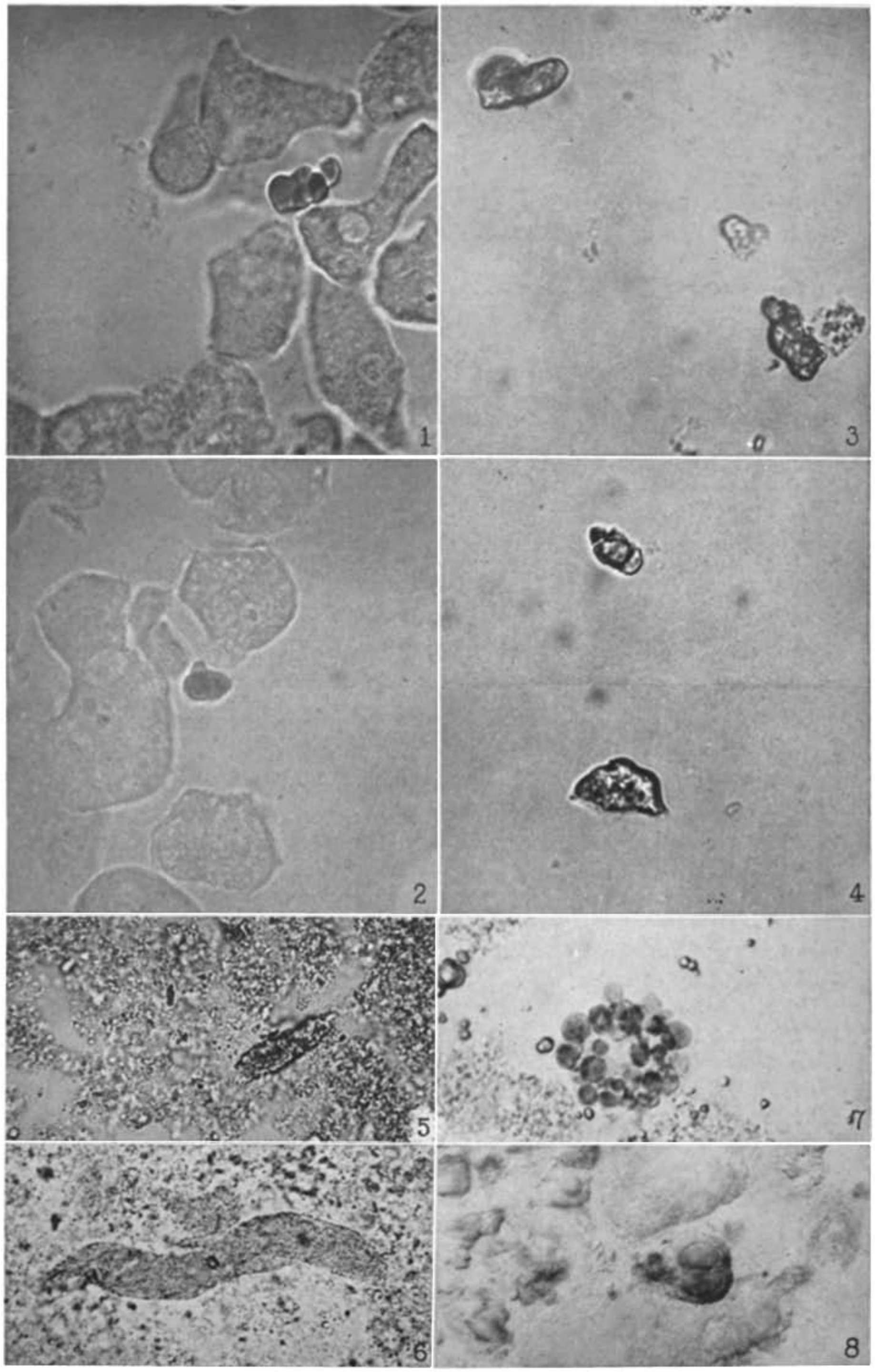
FIG. 22. Carbonate concretions from the bladder bile of another patient with cholelithiasis and mild chronic cholecystitis. In addition to spheroliths, boat forms and forms of lemon shape were present. The bile was infected. × 150.

FIG. 23. A carbonate spherolith from the bladder bile of yet a third patient with cholelithiasis. The bile was uninfected. A rind of stained organic material covers the little concretion. Owing to the use of a color filter to bring out the shape of the nucleus, this latter appears far darker than it really was, and the covering layer much lighter. × 290.

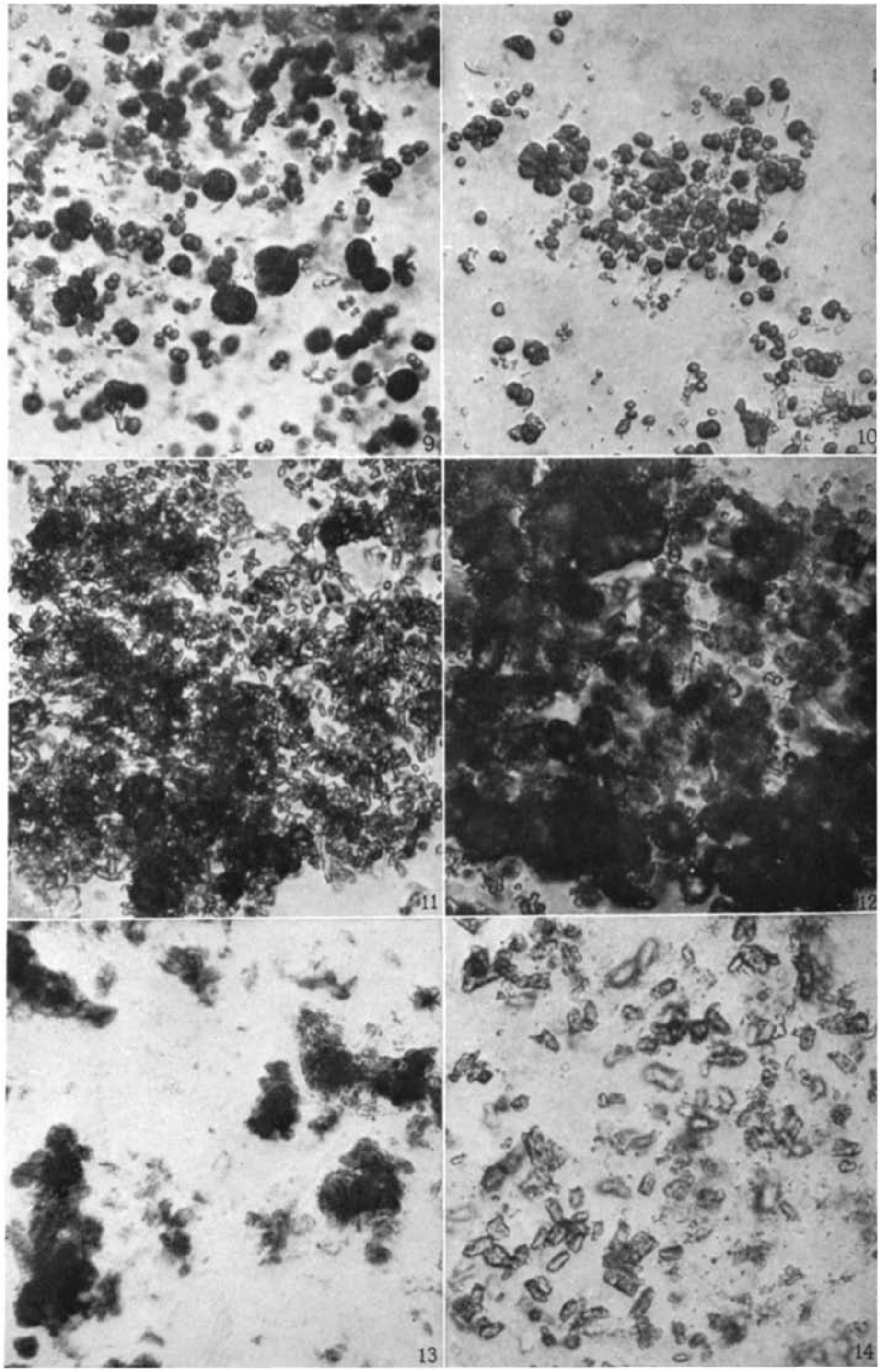
FIG. 24. Another spherolith from the same bile specimen, upon which cholesterol has been thickly deposited. Plates of the substance can be seen here and there, and those between the observer and the spherolith make it seem irregularly striated; but the bulk of the cholesterol deposit is only evident in the photograph as a highly refractile zone. There is much free organic débris round about, and at one side a smaller spherolith. × 290.

Fig. 25. Small calculus from the bladder specimen furnishing the material of Figs. 20 and 21, to show the pigmented center and an outer layer of cholesterol. × 70.

Fig. 26. Carbonate spherolith from the interior of such a stone as is shown in Fig. 25. The calculus had been submitted to weak acid which dissolved out a carbonate matrix, causing it to fall apart into cholesterol plates and organic débris. Such treatment left only the stained shadow of the spherolith. Its concentric striation, plainly visible in the original, cannot be seen in the photograph. × 150.



(Rous, Drury, and McMaster: Causes of gall stone formation. II.)



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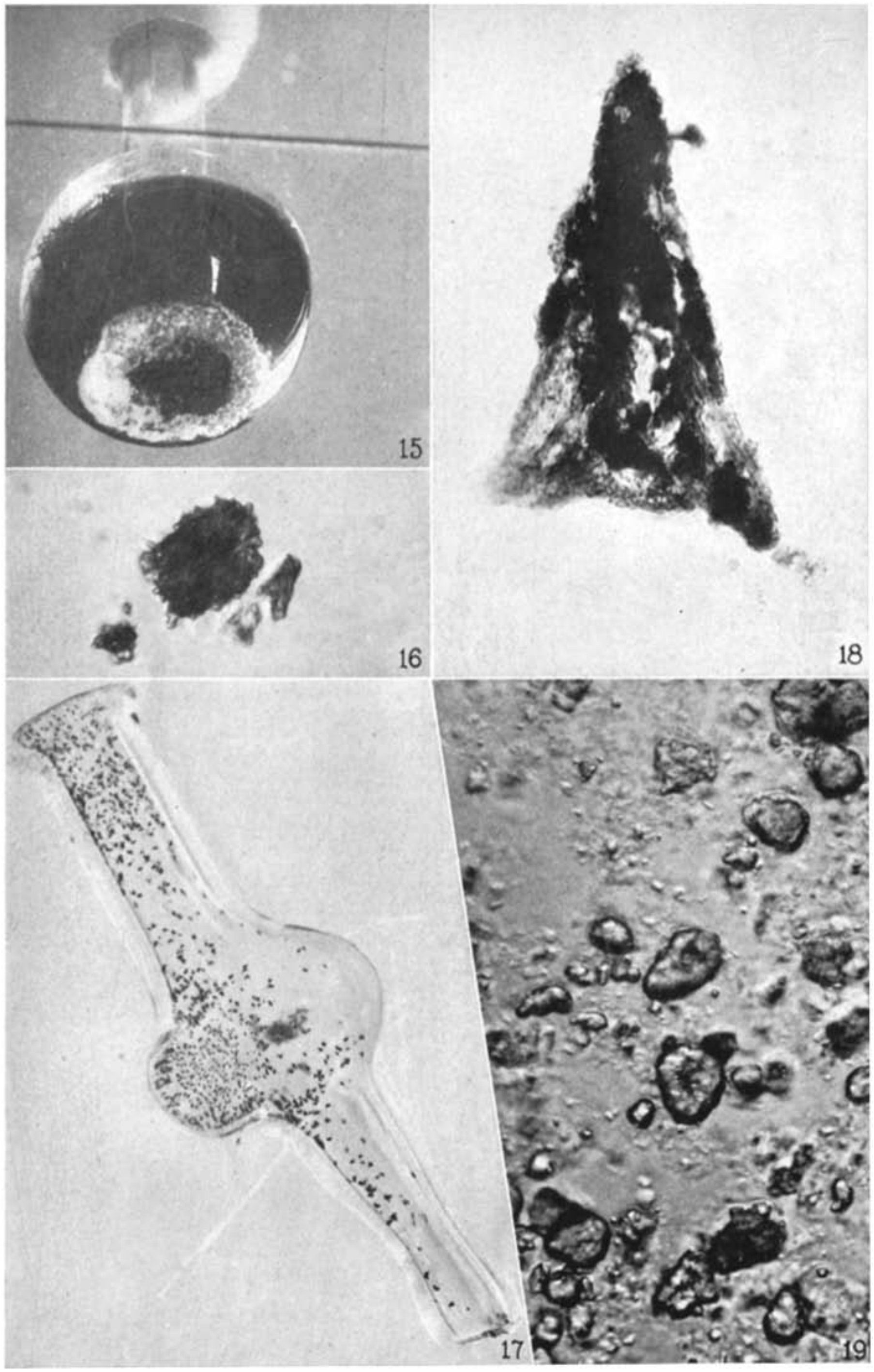

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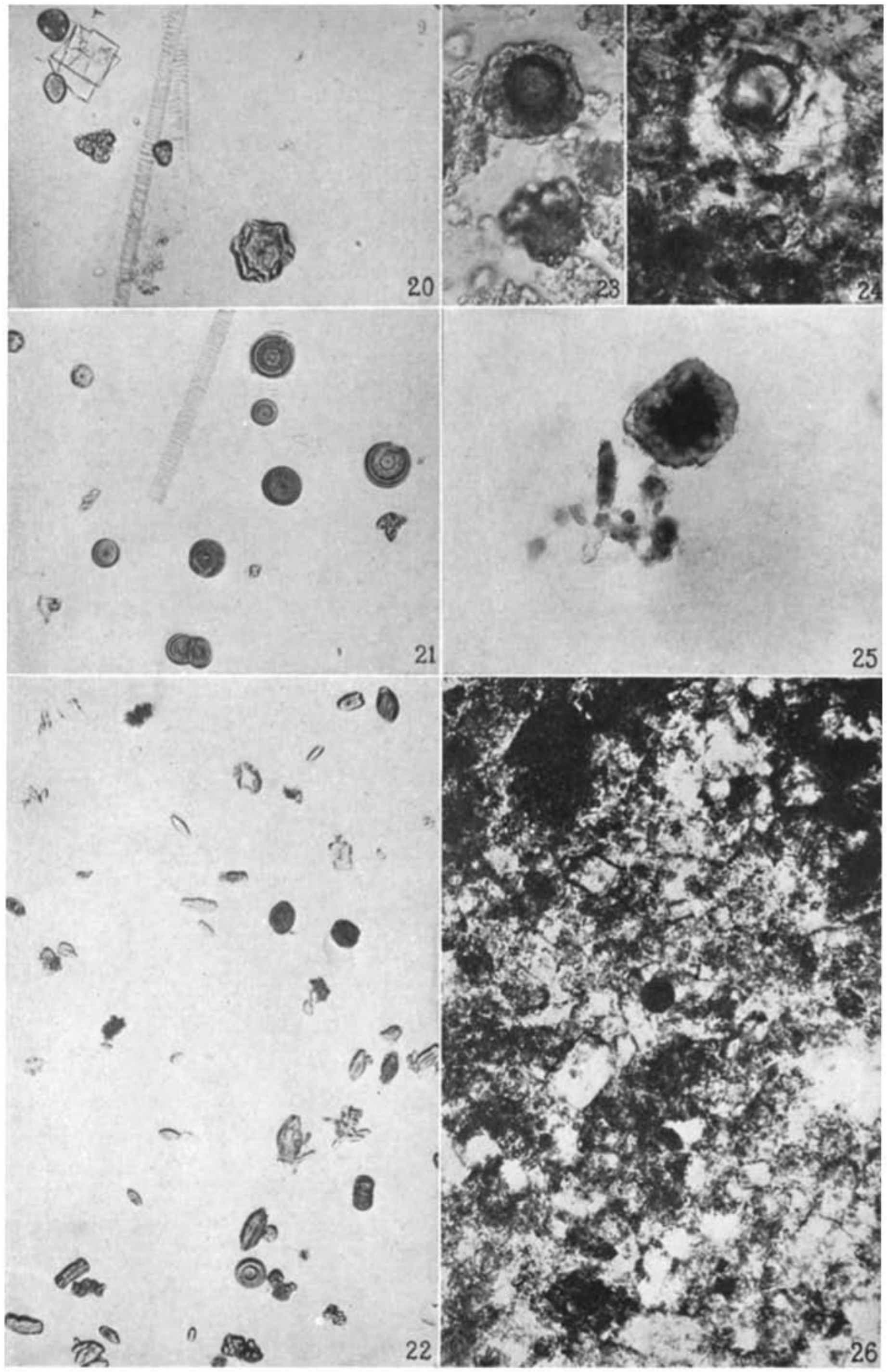

(Rous, Drury, and McMaster: Causes of gall stone formation. II.)