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EXPERIMENTAL GALLSTONES: AN ADVENTURE IN BIOLOGICAL GEOLOGY†

> . . . . The Framingham Study was the first attempt to discover just how commonly gall bladder disease occurs . . . in the United States. That such a survey should not have been made before 1966 is sad evidence of an apathetic attitude toward the cause of gallstones. . . . At the age of fifty-five to sixty-four, 10 per cent of men and 20 per cent of women have gallstones, or, in absolute terms, 2.5 to 3.0 million people in a decade of life that qualifies them for peak responsibility but not for Medicare. In the entire population, some 15 million people are probably carrying stones in their little reservoir for bile—or would be carrying them were it not for attrition by surgical removal of about one third of a million gall bladders annually. The hospitalization and medical expenses entailed must come close to half a billion dollars a year. Nor is the threat of gallstones to the national security to be discounted; four of the last six Presidents have had cholecystectomy.[1]

It is entirely appropriate to include the following narrative in a volume dedicated to Professor Hugh Long, for the work to be described had its origins in a study done under Dr. Long's stimulating supervision and reported in this JOURNAL in 1943.[2-5] After Brobeck, Tepperman, and Long[2] had reported a small but statistically significant increase in serum cholesterol concentration in rats with obesity-producing hypothalamic lesions, Drachman and Tepperman[6] re-examined the problem in gold thioglucose obese mice and discovered that obesity was again associated with a modest elevation in serum cholesterol.

Since both of the studies cited were done with animals on "normal" diets it was pertinent to inquire what effect, if any, obesity would have on serum cholesterol in animals fed a diet designed to produce hypercholesterolemia. Accordingly, groups of control mice and gold thioglucose obese animals were put on an "atherogenic" regimen which had been used in many experiments on rats and had been found to produce very high levels of serum cholesterol.[7] The basic features of the regimen were the following (per cent by weight): 30.6% lard content; marginal (15.3%) protein

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† Aided by grant AM 5410, National Institute for Arthritis and Metabolic Diseases, National Institutes of Health, and by a grant from the Hendricks Research Fund of Syracuse University.

content; 51% glucose; 1% cholesterol and 0.5% cholic acid; and 0.04% thiouracil in the drinking water.

At about this time we discovered that the hexose monophosphate shunt dehydrogenase of rat liver showed remarkable fluctuations in activity as a result of starving and re-feeding a fat-free, high carbohydrate diet. We were so entranced by this curious phenomenon that we devoted all of our energies to studying it, and, as a result, 10 surviving forgotten mice continued dutifully to eat their cholesterol and cholic acid for nine months. They were remembered only when a new technician joined our group and began to do cholesterol analyses. We presented the 10 mice to him mainly because we thought a genuine experiment with hypercholesterolemic animals would relieve the tedium of his learning experience.

When we opened the first mouse in order to obtain a blood sample many white concretions were instantly visible through the transparent gall bladder (Fig. 1). A typical harvest of stones from a single animal is shown in Figure 2. Understandably, these objects were of more than passing interest to an investigator with a personal history of biliary colic. Therefore we quickly explored the remaining nine mice and found similar stones in eight of them. Ten laboratory chow-fed controls showed no stones; in retrospect, none of us could recall having seen gallstones in any of the thousands of mice that had been examined in our laboratory for a variety of purposes.

A systematic analysis of the original stone-inducing regimen was made in collaboration with Fred T. Caldwell and Helen Tepperman.[8] By eliminating single parts of the regimen at a time (i.e., cholesterol, cholic acid, high fat, low protein, aurothioglucose, and thiouracil) it was possible to conclude that the combination of cholesterol, cholic acid, weight gain, and good liver function, as indicated by the absence of BSP retention, were necessary and sufficient for stone formation. There was no correlation between extent of elevation of serum cholesterol and stone formation, for many of the groups showed impressive hypercholesterolemia without stones. There was a variable lag period between the time of beginning the stone-forming diet and the appearance of the first stones, which were rarely seen before the 5th or 6th week. On the basis of a rather small sample the suggestion was made that female animals tended to develop stones earlier than did males and at a significantly lower serum cholesterol concentration. After this long study had been completed it was found that the best and easiest method of inducing stone formation was to add 1% cholesterol and 0.5% cholic acid to ordinary ground laboratory chow. The mice accepted and tolerated this diet very well and virtually 100% of them developed stones by the end of the second month.

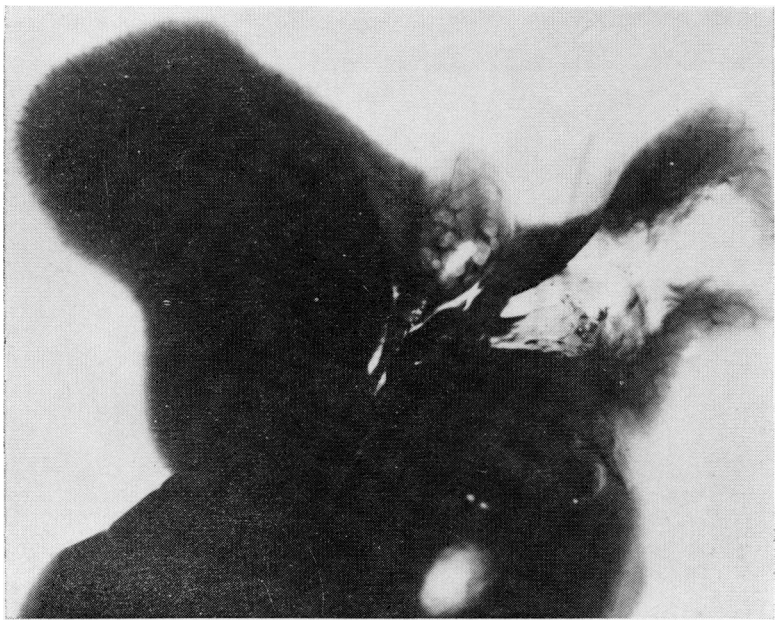

FIG. 1. Gallstones *in situ.*

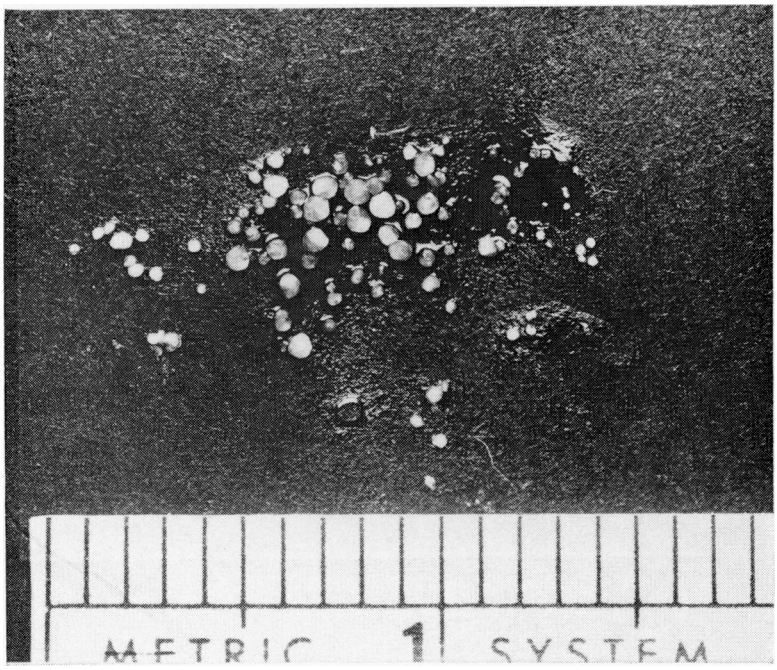

FIG. 2. Typical cholesterol gallstones harvested from a single mouse after nine months on a cholesterol-cholic acid (C-CA) diet.

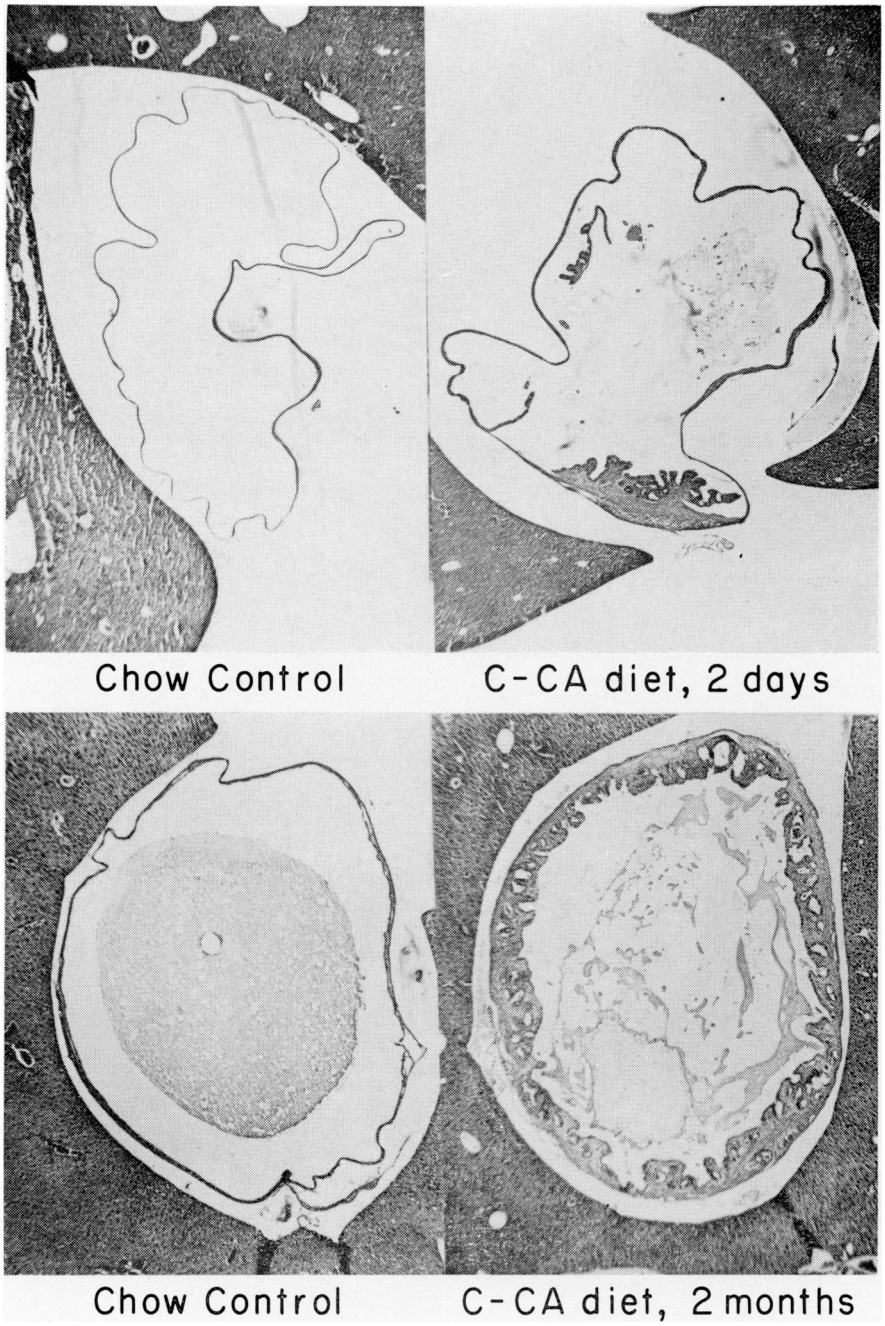

FIG. 3. Histological appearance of mouse gallbladder after two days and after two months on a C-CA diet. (Courtesy of Barbara Rosenberg and Fred T. Caldwell.)

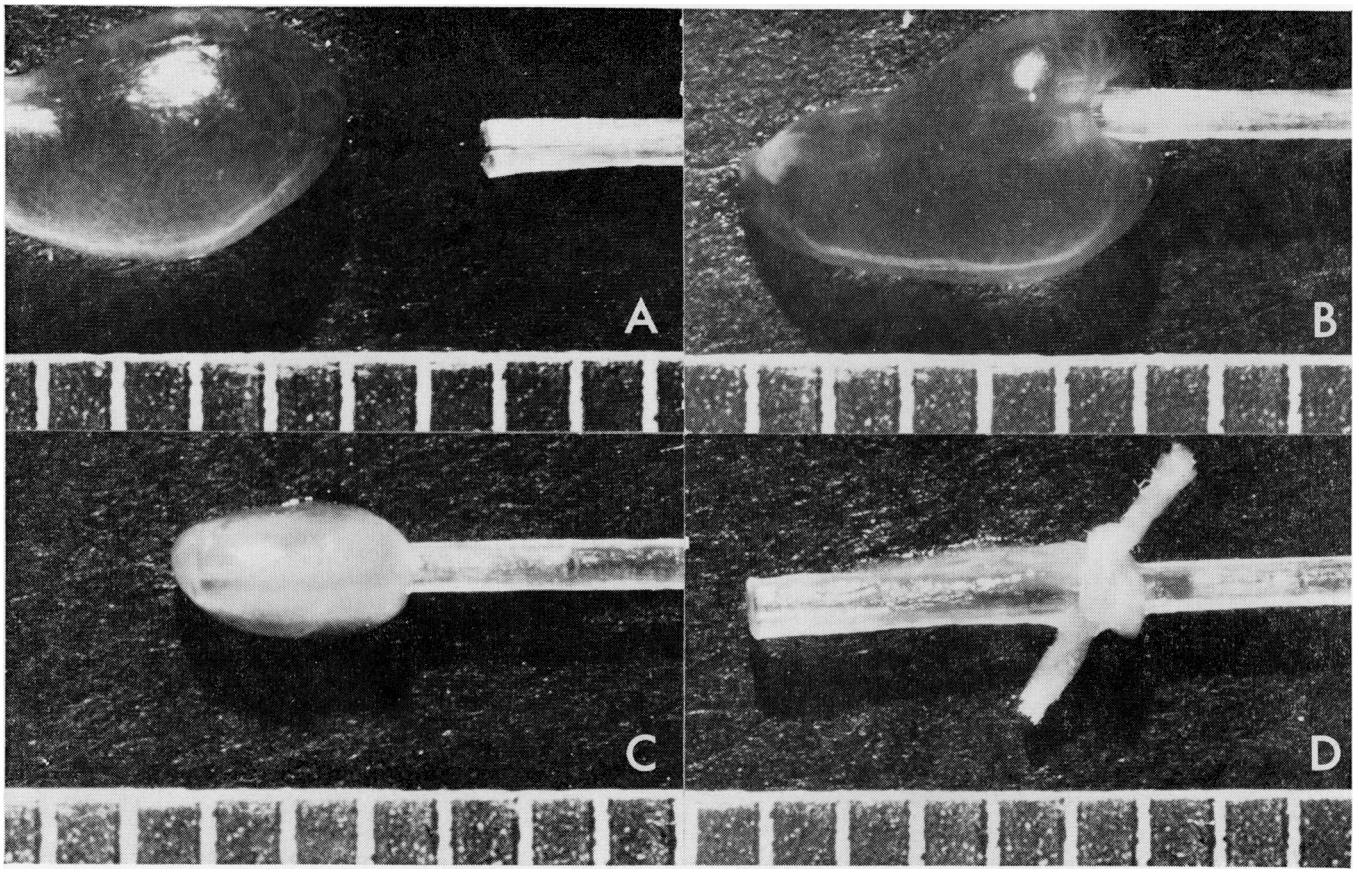

FIG. 4. Serial views of preparation of everted gallbladder under a dissecting microscope. A millimeter scale is shown in each frame. Cuts in the end of the tubing are clearly visible in frame A.

An interesting variety of cholesterol concretions was observed and photographed during the course of this study.[8] Some of the more solid stones are shown in Figure 2. Analysis of these and other stones revealed about 95% of cholesterol. Many other kinds of concretions were seen from single cholesterol crystals through loosely aggregated crystals to spheres that still contained visible edges of individual crystals. The variety of crystal aggregates suggests that the primary event in stone formation is crystal precipitation. This is followed by progressively more tightly bound aggregations of crystals until the "mature" stones (some of which achieve a diameter of more than 0.5 mm.) appear. The nature of the forces that bind one crystal to another are presently unknown. Historically there have been many "nidus" hypotheses which implicate dead tissue or bacteria as "seeds" of crystal growth.[9] An examination of the loosely aggregated crystals in the model under discussion suggests that "nidus" theories may not be pertinent here. Alternatively it has been suggested that each cholesterol crystal may be coated with a thin layer of proteins (or as Womack, *et al.*[10] have proposed, mucopolysaccharide) and that crystal aggregation, growth, and stone "maturation" may then proceed on the basis of protein-protein interactions. An experimental test of this hypothesis is now in progress.

In one series of mice a comparison was made between the cholesterol concentration of *gall-bladder* bile in chow fed animals and in animals adapted to the cholesterol-cholic acid diet for several weeks. An attempt was made to exclude animals with visible stones from the latter group. The control value was 91 mg./100 ml. while that in the lithogenic diet-adapted group was 550 mg./100 ml. This experiment stimulated us to devise a convenient method for collecting *hepatic* bile in order to compare the cholesterol concentration of newly secreted bile in normal and stone-inducing diet adapted mice. Inserting a length of No. 50 polyethylene tubing (internal diameter 0.023 inch) in the gall bladder of a common duct-ligated mouse and tying it in place with a pre-placed ligature proved to be a task that almost brought the study to a halt. When it suddenly occurred to us that we could anchor both our mosquito hemostats and the plastic tubing in proper position by means of bars of modelling clay ("internes"), the operation became ridiculously easy. Moreover, the first successful bile collection was the occasion of an accidental observation that changed the course of the study and supplied a substantial dividend of information.

When the polyethylene tubing had been successfully tied in the gall bladder and after the abdominal wound had been closed with wound clips, we noticed that bile appeared to be flowing into the tubing at a remarkably constant rate. To test our impression we marked the advancing fluid edge

at 10 minute intervals and plotted cumulative centimeters of tubing filled against elapsed time. The rate of flow was indeed linear and remained so for over an hour! The polyethylene tubing was carefully tested and found to have a remarkably constant internal diameter. Thus, this simple piece of apparatus proved to be a precision flow meter, a volume meter (since internal diameter was known, volume could be obtained simply by measuring the length of the fluid column), and a convenient storage tube in which samples awaiting analysis could be frozen. One does not have to be a Luddite to be comforted by simplicity in an age when the ultracentrifuge room in a well-known institute is affectionately known as "The Laundromat" for obvious reasons.

Pedreira and Tepperman[11] were able to make highly reproducible observations on the effect of cholesterol-cholic acid diet adaptation on bile flow rate in the mouse. They found that there was a progressive increase in the rate of hepatic bile production over a two-month period in both male and female mice after the regimen of cholesterol-cholic acid was begun. Table 1

TABLE 1. BILE FLOW RATES IN MICE ON CHOW AND CHOLESTEROL-CHOLIC ACID (C-CA) DIETS

Diet	*Chow*	*C-CA 5 days*	*C-CA 2 months*
Males	6.89 ± 0.14 (12)	15.46 ± 1.48 (8)	34.76 ± 3.80 (10)
Females	9.51 ± 0.82 (10)	16.40 ± 1.49 (10)	60.50 ± 7.10 (7)

Values are expressed as cm. of tubing ± SE/10 minutes. Figures in parentheses represent numbers of experiments. Volume conversion factor for the tubing: 0.1 ml. = 38 cm. (Abbreviated from Ref. 8.)

contains an abbreviated summary of previously published data.[8] The progressive increases in flow rate are highly significant ($P < 0.001$) in both sexes. The mean differences in flow rate between male and female on a chow diet (column 1) and on the C-CA diet for two months (column 3) are also highly significant ($P < 0.01$ and $P < 0.001$ respectively). Parenthetically, the sex difference in bile flow rate was found to be readily reversible if female mice were given androgen or male mice were treated with estrogen.

When the collected samples were analyzed for cholesterol we were surprised to discover that there were no consistent increases in the cholesterol concentration of hepatic bile in animals on the stone-forming regimen for two days, five days, or two months in spite of the fact that, by two months, the animals uniformly showed an impressive hypercholesterolemia as well as the aforementioned increase in *gall bladder* bile cholestrol concentration.

In fact, the female mice at two months exhibited a statistically significantly lower cholesterol concentration than did similarly treated males. It will be recalled that such animals had no fewer stones than males and, indeed, tended to develop them at a somewhat earlier time. Thus, on the basis of cholesterol concentration of hepatic bile alone it was impossible to differentiate a stone-forming situation (two months on the C-CA diet) from one in which stones were never found (chow controls, C-CA diet for two and five days).

When the differences in flow rate were taken into consideration (total cholesterol secreted into the biliary tract per unit of time; i.e., concentration x flow rate) both male and female mice after two months of C-CA feeding secreted about three times as much cholesterol per hour as did either control mice of C-CA mice on the lithogenic diet for only 2 or 5 days.[11] (Table 2.) Thus, the stone forming circumstance could be differentiated from the others on the basis of the aggregate amount of cholesterol secreted into the biliary system.

TABLE 2. EFFECT OF FEEDING C-CA DIET FOR 2 MONTHS ON GALLBLADDER WEIGHT

	Chow (15)	*C-CA diet (15)*	*P*
Wet wt., mg ± SE	1.26 ± .75	2.96 ± .276	$<.001$
Dry wt., mg ± SE	.27 ± .015	.54 ± .052	$<.001$
% water ± SE	81.28 ± 1.33	78.3 ± 1.25	$>.2$

( ) signifies n.

The observations in C-CA diet adapted mice of 1) high rates of hepatic bile production, 2) no difference in cholesterol concentration of hepatic bile, and 3) a striking increase in gall bladder bile cholesterol concentration suggested the strong possibility that one of the events that had to occur during the latent period between initiation of C-CA diet administration and cholesterol precipitation was a change in the fluid transport capacity of the gall bladder. Barbara Rosenberg and Fred Caldwell had already demonstrated a striking hypertrophy of both muscularis and mucosal epithelium in C-CA diet adapted mice (Fig. 3). It remained to be demonstrated whether or not the ability of the gall bladders of the C-CA diet adapted mice to transport fluid increased *pari passu* with the increase in bile volume characteristically seen in this circumstance.

The nature of the technical challenge involved in this demonstration is well illustrated by the effect of the C-CA diet on gall bladder wet and dry weight. As one might expect from examination of Rosenberg's and Caldwell's prior histologic examination, two months of C-CA feeding resulted

in highly significant differences in both wet and dry weight of the organ, although there was no significant difference in the mean per cent water content of the tissues. It should be noted that the control gall bladders weighed 1.26 mg. (wet) while the hypertrophic ones weighed 2.96.

Diamond[12] and others had studied transport of fluid through the wall of gall bladders filled with various salt solutions and incubated *in vitro* under favorable conditions of salt composition, buffering, and substrate and oxygen availability. All of these experiments had been done with the gall bladders of species larger than the mouse (mainly fish and rabbit). We devised a three-way needle technique of emptying bile from the distended gall bladder of mice fasted overnight and refilling the bladder with a glucose-containing Krebs-Henseleit buffered salt solution. We attempted to incubate these preparations for two hours, periodically removing, blotting, and weighing them much as had been done with those of larger species. However, the small size of the organ and the small volumes of fluid transported made it difficult to perform reproducible experiments, and this approach was abandoned.

Unknown to us at the time these experiments were done, Dietschy[13] was using everted rabbit gall bladder sacs in elegant studies of water and salt movement across the wall of the gall bladder. We were stimulated to attempt an everted gall bladder preparation by our prior experience with the Wilson-Wiseman everted intestinal sac.[14] Accordingly, we prepared coils of #10 polyethylene tubing (O.D. 0609 cm.; capacity, 0.6047 microliters per cm.) so that they could be placed in the bottoms of standard incubation vessels fitted with soft rubber baby bottle caps through which they could be conveniently oxygenated. One end of the tube was scored at right angles with a sharp razor blade under a dissecting microscope as shown in Figure 4A. Then the gall bladder was everted and tied over the scored end as shown in the sequence of photographs in Figure 4B, C, and D. The end of the tube opposite the scored end was tightly ligated to prevent the entry of fluid into the tube during preparation. If a small amount was trapped at the beginning of the experiment, the fluid level was noted. These preparations were then incubated for two hours in a Krebs-Henseleit buffer containing glucose at 37°C. At the end of the incubation time the length of the fluid column was measured with a millimeter rule and an appropriate correction for initial fluid was made. Since it had previously been established that the internal diameter of the tubing was uniform, linear measure constituted an accurate measure of volume. Fourteen mice fed the C-CA diet for 24 days and 15 chow-fed controls were studied in this way. The results are presented in Table 3. The 47 per cent increase in fluid transport by the gall bladders of C-CA diet adapted rats was statisti-

TABLE 3. EFFECT OF FEEDING A C-CA DIET FOR TWO MONTHS ON TRANSPORT OF FLUID BY EVERTED GALLBLADDERS *in vitro*

	No.	*Fluid transported/ 2 hrs. (cm ± SE)*
Control	15	2.52 ± .181
C-CA	14	3.71 ± .393
P		< 0.01

Preparations as described in text were incubated in Krebs-Henseleit bicarbonate buffer (containing 200 mg. p.c. glucose) in an atmosphere of 95% oxygen and 5% CO_2.

cally significant at a P of < 0.01. We do not mean to suggest that these figures represent the comparative fluid transport performance of these gall bladders *in vivo*, for we observed that many of the gall bladders of the C-CA diet group showed very prominent vascular markings. This suggests that there may be important differences in rate of blood perfusion of the gall bladders of the two diet groups. If this were the case, the *in vitro* performance data would represent a serious understatement of the difference in "concentrating ability" between the bladders of control mice and of those on the lithogenic diet. The present findings are consistent with the conclusion that the gall bladders of the C-CA diet animals have a demonstrably greater fluid transporting capacity than do those of chow-fed controls.

We cannot now identify the signal or signals that initiate the adaptive changes in the structure and function of the gall bladder described above. Since frequent distention of other hollow viscera—especially the stomach and intestine[15]—has been seen to result in similar hypertrophy of both muscularis and mucosa and since distention of the gall bladder was often observed in C-CA diet mice, one of us (M.W.) suggested that hypertrophy of the gall bladder might occur in some circumstance other than C-CA feeding in which periodic distention of the organ might be expected to take place—namely, periodic starvation and overfeeding. Accordingly, one group of 13 mice was fed a laboratory chow diet and fasted on alternate days for 28 days. A control group was never without chow pellets in the cage. In neither case did the diet contain cholesterol or cholic acid. At the end of the experimental period wet and dry weights of gall bladders were obtained with results as shown in Table 4. It will be seen that although the mice on the "stuff and starve" regimen weighed significantly less than did the controls, their gall bladders were clearly hypertrophic. Regrettably, no functional or histologic studies were made on hypertrophic gall bladders of this type.

TABLE 4. EFFECT OF FEEDING AND STARVING ON ALTERNATE DAYS FOR 28 DAYS ON BODY WEIGHT AND GALLBLADDER WEIGHT

	Control (13)	*Intermittently starved (13)*	*P*
Mouse body wt, g. ± SE	30.8 ± .93	25.4 ± .516	< .001
Gallbladder, wet wt, mg ± SE	1.49 ± .064	2.31 ± .172	< .001
Gallbladder, dry wt, mg ± SE	0.284 ± .014	0.464 ± 0.59	< .01

SOME SPECULATIONS ON THE ETIOLOGY OF GALLSTONES

There have been many epidemiological studies of the incidence of gallstones of which the most recent is a comparison of two population groups, Pima Indians and the residents of Framingham, Massachusetts.[16] All of these studies have supported the view that gallstones, like atherosclerosis, coronary thrombosis, hypertension and many other diseases, represent a complex mosaic (in Irvine Page's phrase) of many contributory factors, both genetic and environmental. Sex, parity, obesity, and diabetes are among possible contributory factors suggested, but since the last three of these are themselves mosaics, many other forces could contribute, directly or indirectly, to the formation of gallstones. Among these are: exercise habits, composition of the diet, pattern of food intake (as well as total calories). It is virtually impossible to study isolated factors in population groups; even in the well-controlled study of Comess, *et al.* cited above[16] one cannot easily differentiate between genetic and environmental factors responsible for a sixfold greater incidence of gallstones among Pima Indians as compared with Framinghamers. The appeal of animal models, such as those of Christensen and Dam,[17] of Hikasa and his colleagues,[18] and of ours[8,11] lies in the fact that the experimenter can study comparatively small numbers of variables and thus conceptualize mechanisms that are at least useful in thinking about the human disease.

The central event in cholesterol gallstone formation is cholesterol precipitation. It is less remarkable that cholesterol precipitates out of bile than that it remains in solution, for it is a remarkably insoluble material. When it fails to precipitate in bile it is solubilized by the combined detergent action of bile salts and lecithin (i.e. "the cholesterol holding capacity of bile"). (The soapy nature of bile has been recognized for over 200 years, for Coe,[19] in his 1757 treatise, mentions the fact that bile solutions had been used for many years to clean grease stains from various kinds of cloth.) The physical consequences of changing the relationships among the bile salts, lecithin, and cholesterol have been admirably described in a recent review article by

Hofman and Small.[20] Many investigators have described decreases in both the bile salt:cholesterol ratio and the lecithin:cholesterol ratio that occurs under cholesterol stone forming conditions. Caldwell, Levitsky, and Rosenberg[21] established the occurrence of such changes in the gall bladders of mice fed a C-CA diet. They were impressed by the concurrence of a decrease in these ratios and the appearance of thickening and inflammation of the gall bladder wall.

In the last analysis, it is the distinctive chemical composition of gall bladder bile that determines whether or not precipitation of cholesterol will occur. Gall bladder bile composition represents a vector of many potential forces. In the *liver*, water and solutes are transported into the bile and in the *gall bladder* an isosmolar sodium chloride solution is returned to the blood so that nonabsorbed solutes are concentrated. As we have shown, the rate of hepatic bile secretion may be an important determinant of the aggregate amount of cholesterol secreted into the biliary tract. In our model we propose that the cholic acid in the diet has a powerfully choleretic effect, but that there is an adaptive increase in the capacity of the system to respond to the choleretic stimulus. As more and more bile is produced, more and more intense dilation of the gall bladder occurs. Since the inflammatory reaction in the wall of the gall bladder noted by Caldwell, Levitsky, and Rosenberg[21] was seen before detectable cholesterol precipitation occurs, it seems unlikely that it could be due to irritant properties of cholesterol. We suggest that the stretching of the organ secondary to high rates of bile flow may cause cell damage, release of lysosomal enzymes, and the inflammatory sequelae of this event. At the same time, persistent dilation results in hypertrophy of the gall bladder and an increase in the size of the mucosal absorbing surface of the organ. Thus the basically adaptive changes in the gall bladder might set the stage for subsequent precipitation of cholesterol crystals by 1) concentrating large volumes of bile and 2) selectively reabsorbing bile acids and rejecting cholesterol so that the unfavorable bile acid-cholesterol ratios in gall bladder bile observed by Caldwell, *et al.*[21] would result. It is, of course, possible that the ratio of bile acid-cholesterol in hepatic bile may be altered in an unfavorable direction under circumstances in which choleresis is occurring, but there is no information on this point. There is a precedent for suggesting that the gall bladder may contribute to an unfavorable bile acid:cholesterol ratio for Riegel, Ravdin, and Johnston[22] demonstrated an increase in bile acid reabsorption by inflammed gall bladders in dogs when the mucosa remained impermeable to cholesterol.

In addition to the main contributory factors to stone formation mentioned, there is another that deserves attention; i.e., biliary stasis (see

Rains[9]). On the face of it, one would assume that turbulence of the gall bladder contents and/or frequent emptying of the viscus would inhibit crystal aggregation and stone growth, whereas biliary stasis would predispose to stone formation both by permitting more time for "concentrating" gall bladder bile and by allowing maximal opportunity for crystal-crystal interaction. Investigators who have looked at the distended gall bladders of pregnant rabbits have been impressed by their atony and have, in fact, claimed that such gall bladders are less responsive to emptying stimuli than are gall bladders of non-pregnant animals. (See Imamoglu[23] for discussion of this point.) Atony of the gall bladder wall cum biliary stasis may be yet another tile in the mosaic of gallstone formation.

There are many other potential contributory factors to stone formation which have been discussed by other investigators. For example, falling bile lecithin:cholesterol ratios may predispose to cholesterol precipitation since lecithin (which is readily soluble in bile salt micelles) has the effect of "swelling" the micelles and thus increasing the volume of their cores which function as isolated little pools of nonpolar solvent. (The physical chemistry of this phenomenon has been beautifully described in a review by Alan Hofmann.[24]) Hisaka's school suggests that disturbances in phospholipid metabolism may, in fact, be central in the changes in bile chemistry that precede cholesterol precipitation.

Similarly, the protein or mucopolysaccharide "cement" problem has been stated but not explored extensively. Whether there is one or many "cements" that bind crystals to one another or whether the variable activity of mucous secreting cells of the gall bladder epithelium can either predispose to or protect from stone formation is not now known. In addition, Christensen, *et al.*[25] have demonstrated in their hamster model that the incidence of gallstones is affected by the composition of the dietary salt mixture.

SUMMARY

Cholesterol gallstones can be produced regularly in mice by feeding a diet containing 1.0% cholesterol and 0.5% cholic acid. The disease is characterized by a 4-6 week latent period after the beginning of the stone-forming regimen. During this time there is a progressive increase in the rate of hepatic bile production in common duct-ligated animals and a readily demonstrable decrease in the bile acid:cholesterol ratio of gall bladder bile. At one point in time during the latent period it is possible to demonstrate an increased capacity for fluid transport by gall bladders of cholesterol-cholic acid fed mice. It is suggested that this change in the gall bladder

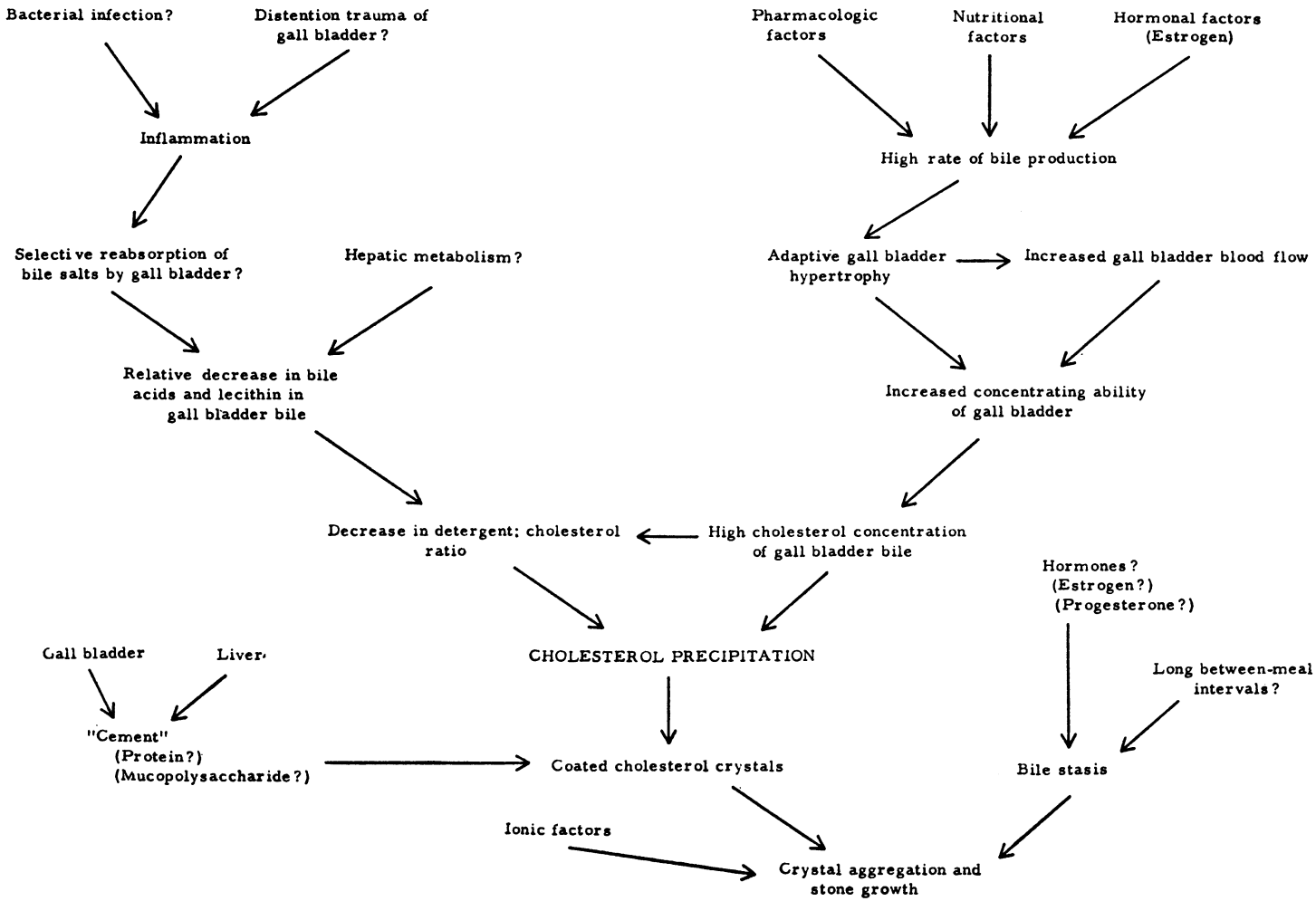

FIG. 5. Diagrammatic summary of possible mechanistic inter-relations in cholesterol gallstone formation.

may be secondary to prior stimulation of choleresis by the bile salt in the diet. The relevance of these observations to the problem of cholesterol gallstone formation in man is, as yet, unknown.

A graphic attempt to suggest possible mechanisms in the etiology of cholesterol gallstones is shown in Figure 5. This flow sheet of events is intended to suggest some of the inter-relations among many of the factors mentioned in the discussion. We realize that it contains a measure of fantasy, but we prefer to regard the guesses it contains as a strategic plan for future investigation. It should be emphasized that this scheme is restricted to cholesterol stones only; factors involved in the formation of pigment and calcium stones are, no doubt, equally complex.

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