

Experimental Formation of Gallstones in the Squirrel Monkey¹ (35350)

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(Introduced by Arthur Malley)

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Gallstones are rare in animals, although the natural occurrence of biliary calculi in ox, rabbit, elephant, monkey, etc., has been reported (1). Among nonhuman primates, gallstones have been found in the marmoset (2), baboon (3), and orangutan (4). In a group of 300 vervet monkeys (*Cercopithecus aethiops*), however, no calculi were found (5). On the other hand, cholelithiasis is a common disease in man, necessitating numerous cholecystectomies annually. Despite this prevalence in man, research on the pathogenesis of gallstones has long been hampered by the difficulty of producing gallstones in suitable experimental animals. There are several reports of the experimental production of calculi in the biliary systems of various kinds of animals (6-11), but in all cases the composition of the diets or of the stones or both were atypical of human cholelithiasis. However, we have noticed for some years that squirrel monkeys (*Saimiri sciureus*) maintained for a time on diets [compositions, Ref. (12)] that induce a mild hyperlipemia frequently have gallstones. In this study of the incidence and severity of cholelithiasis in monkeys fed different diets, we conclude that the squirrel monkey is a valuable model for the formation of gallstones composed of cholesterol.

Experimental. Animals used. Ninety-four sexually mature male and female squirrel monkeys, weighing from 540 to 885 g, were divided into four diet groups. The first group

of 19 monkeys was fed a natural diet (Purina monkey chow-25) for 4 months and killed at one time. The second group of 33 monkeys was fed a basal semipurified diet (840 c) not associated with atherosclerosis, which supplied 15% of the calories as corn oil (12). The third group of 33 monkeys was maintained on a semipurified diet (high butter +), which was consistently associated with hypercholesterolemia and atherosclerosis and contained 0.1 g of additional cholesterol/100 kcal (12). Monkeys of the second and third groups were killed from 2 weeks to 13 months after the start of the semipurified diets. The fourth group of 9 monkeys was maintained on the atherogenic-hypercholesterolemic diet for 3 months and then changed to the basal semipurified diet (840 c) for a second period (3 and 5 months) before sacrifice. The mean body weights of all groups were similar at the beginning and end of the feeding periods.

Gallbladders were removed immediately after the monkeys had been sacrificed under phencyclidine anesthesia and arterial specimens for study of atherosclerosis had been taken. The contents of the gallbladders were placed in a centrifuge tube. The volume of viscous but not turbid bile was 0.2 to 0.8 ml. The bile minus stones, which were separated upon standing, was transferred to chloroform-methanol for analysis; the gallstones were washed four times with distilled water, dried at 80°, and weighed.

Analysis of gallstones. Weighed amounts of gallstones were dissolved in chloroform. The chloroform solution was applied to silica gel H plates with or without 5% AgNO₃ and subjected to thin-layer chromatography (13,

¹ Publication No. 487 from the Oregon Regional Primate Research Center, supported in part by Grant No. FR 00163 from the National Institutes of Health. This work was supported by a grant-in-aid from the National Institutes of Health (HE 09744).

TABLE I. Incidence of Gallstones in Squirrel Monkeys Fed Different Diets.^a

Duration of feeding	Group 2: Basal diet (840 c)			Group 3: High butter + cholesterol		
	No. of monkeys	No. with stones	Mean wt of stones (mg)	No. of monkeys	No. with stones	Mean wt of stones (mg) ^b
(weeks)						
2	2 F	0		2 F	0	
4	4 M	0		5 M	0	
6	2 M	0		2 M	0	
10	2 M	0		2 M	0	
(months)						
3	7 F	2	5.5	3 F	1	23.0
4	2 M, 2 F	0		2 M, 2 F	1 M, 1 F	16.5
6	4 F	1	5.0	6 F	2	85.0
8	3 F	0		3 F	0	
9	2 F	0		2 F	2	86.5
10	1 F	0		2 F	1	73.0
13	2 F	0		2 F	2	162.0
Totals	33	3		33	10	

^a There were no gallstones in 7 female and 12 male squirrel monkeys fed the natural diet (Group 1). There were also no stones in 5 females fed the high butter + diet for 3 months followed by 840 c for 3 months nor in 4 females fed high butter + for 3 months and 840 c for 5 months (Group 4).

^b Values are the mean weights of stones per animal for those animals which had stones.

14). The extracts were chromatographed alone or with various combinations of cholesterol purified by crystallization through the dibromide and cholestan-3 β -01 (from Applied Science Laboratories, State College, Pa.). Each spot was detected and the cholesterol was quantified by charring as previously described (15, 16), except that a Farrand VIS-UV thin-layer analyzer was used. The concentrations of cholesterol were also determined with the Lieberman-Burchard reagent (17).

Analysis of plasma and biliary lipids. Plasma and biliary cholesterol concentrations were determined by the method of Abell *et al.* (17), phospholipid concentrations by the method of Bartlett (18).

Results. Incidence of gallstones. No stones were found in the 19 monkeys of the first group fed the Purina monkey chow diet. In the monkeys of the second group which were fed the basal semipurified diet for 3 and 6 months, three monkeys had stones, the size and number of which were much smaller than those in Group 3 on the atherogenic diet. The relatively small mass of stones from

these three monkeys of Group 2 is shown in the mean weight column of Table I. There were no stones in monkeys fed the basal semipurified diet for longer than 6 months. In the third group on the atherogenic diet gallstones appeared within 3 months and increased with the duration of the feeding (Table I). Of the monkeys maintained on the hypercholesterolemic diet for 9 months or more, 83% (five out of six) had gallstones, compared with 0% in the first group with a natural diet and the second group with the basal semipurified diet. The gallstones were greenish-white or white and round, ovoid, or polyhedral. The size and number of gallstones increased with time (Fig. 1 and Table I), and the biggest stones at 3, 6, 9, and 13 months were 0.9, 1.5, 2.3, and 3.3 mm in diameter, respectively. The difference in the size of the stones from animals sacrificed at different times indicates that the increase in size paralleled the increase in age. In the fourth group, whose original atherogenic diet had been changed to the basal diet, no calculi were found. Fine black pigment stones, weighing little, were found in a few monkeys

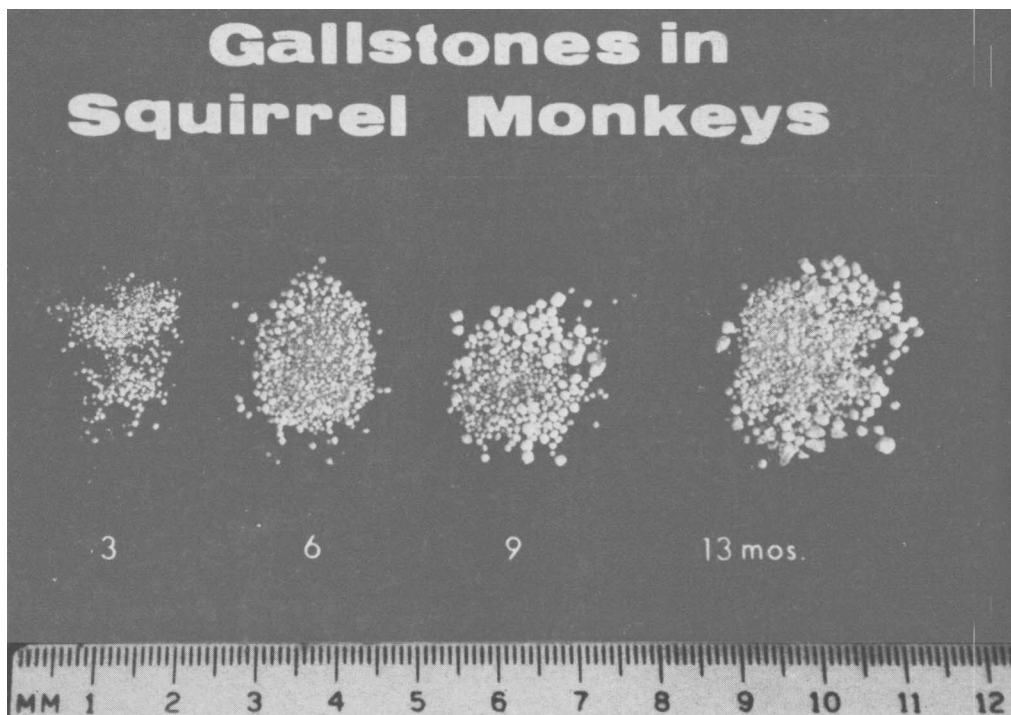


FIG. 1. The gallstones found in single gallbladders from squirrel monkeys in Group 3 (high butter + cholesterol) which were fed experimental diets for 3, 6, 9, and 13 months. The quantity of stones, the heterogeneity of sizes, and the maximum size of stones all increased with duration of the experiment.

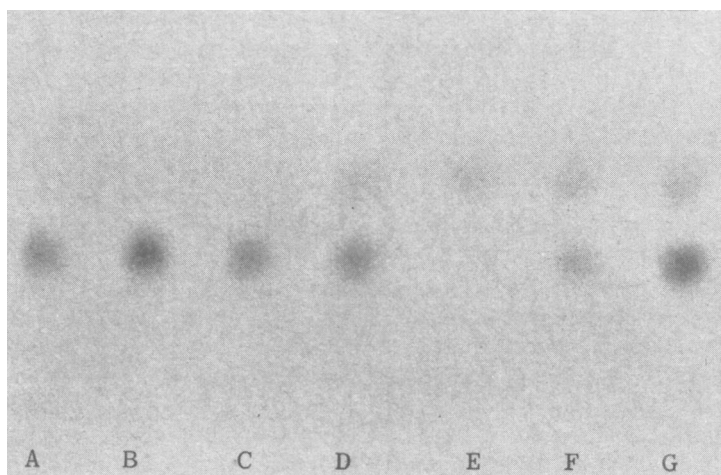


FIG. 2. Thin-layer chromatography of chloroform:methanol (2:1) solutions of gallstones together with various mixtures of cholesterol and cholestan- 3β - α 1. (A) 5 μ g of cholesterol; (B) 5 μ g of cholesterol + 5 μ g of gallstone; (C) 5 μ g of gallstone; (D) 5 μ g of gallstone + 5 μ g of cholestanol; (E) 5 μ g of cholestanol; (F) 5 μ g of cholesterol + 5 μ g of cholestanol; (G) 5 μ g of cholesterol + 5 μ g of gallstone + 5 μ g of cholestanol.

from all four groups with and without cholesterol gallstones. The white stones appeared to be completely separate from the pigment stones.

Determination of the composition of gallstones. The gallstones, which were completely soluble in chloroform, showed only one spot with AgNO₃ thin-layer chromatography (Fig. 2). Cochromatographed with authentic cholesterol, the material in the gallstones had the same R_f of cholesterol and was easily separated from cholestan-3β-o1. The latter compound was not considered to be a likely constituent of the squirrel monkey gallstones but was used in the study to indicate the resolution of the analytical system. Quantitative thin-layer chromatography (99%) and direct application of the Lieberman-Burchard reagent (111%) showed the entire mass of the gallstones to be cholesterol.

Plasma and biliary cholesterol and phospholipid concentrations. Cholesterol stones in the bile of these monkeys may be related primarily to the composition of the secreted bile and secondarily to the composition of the plasma. The mean plasma cholesterol, phospholipid, and cholesterol:phospholipid ratios (Table II) were all highest in Group 3 (high butter + cholesterol).

The absolute amounts of cholesterol and phospholipid in the gallbladder bile varied considerably, probably because of different amounts of water removed by the gallbladder wall. The ratio of cholesterol to phospholipid varied less (see Table II). Group 3, with the highest mean ratio of biliary cholesterol to phospholipid, had the highest incidence of gallstones. Furthermore, within individual groups, the animals with gallstones had the highest ratios. For example, from Group 3, 9 of the 12 animals on the experimental diet for 3 months or more and with a ratio greater than 0.166 (0.33 on a molar basis) had stones, whereas only one of 10 with a ratio less than 0.166 had stones. The most remarkable finding was the very low cholesterol:phospholipid ratio in the biles from the group fed a natural diet. The concentration of biliary cholesterol was lower and that of phospholipid was higher in Group 1 than in any other group.

Discussion. Calcium phosphate stones have

TABLE II. The Composition of Plasma and Gallbladder Bile of Squirrel Monkeys from Four Different Diet Groups.*

Diet group	Plasma (mg/100 ml)		Bile (mg/100 ml)		C:P
	Cholesterol	Phospholipid	Cholesterol	Phospholipid	
1. Purina chow	121.2 ± 18.0	254.2 ± 27.1	207.1 ± 28.9	4467.5 ± 320.7	0.047 ± 0.006
2. 840 c	143.4 ± 5.8	161.1 ± 7.0	363.9 ± 44.4	2136.7 ± 162.3	0.157 ± 0.013
3. High butter +	315.3 ± 29.5	282.6 ± 30.0	591.2 ± 71.4	3291.1 ± 271.6	0.185 ± 0.021
4. High butter + then 840 c		not available	314.8 ± 30.4	2601.9 ± 387.2	0.123 ± 0.015

* All values are means ± standard errors of the mean.

been induced in guinea pigs fed a high cholesterol diet (6), cholesterol stones in young Syrian hamsters fed a fat-free, high sucrose diet (7), glyco-allodeoxycholic acid stones in rabbits (8) and guinea pigs (9) fed 5 α -cholestan-3 β -ol, cholesterol stones in mice fed cholesterol plus cholic acid (10), and lithocholic acid stones in rats fed a low protein diet with lithocholic acid (11). Like man, squirrel monkeys with dietary regimens and plasma cholesterol levels which are similar to those of some human populations develop gallstones composed of cholesterol and thus are a good model for the study of gallstones in man. The size and number of gallstones increased with the duration of the atherogenic diet, *i.e.*, the larger stones were present in the longer term monkeys. The fact that the most heterogeneous distribution of sizes was also present in that group (Fig. 1) indicates that gallstones grow gradually, with new small stones continuously forming in lithogenic bile. Thus the stones begin very small and enlarge and solidify only gradually.

Several major factors in the pathogenesis of gallstones in squirrel monkeys remain to be investigated. Knowledge of the effects of bile salts and phospholipid on the stabilization of cholesterol in bile (19) has led to a study of the relationship of hepatic and gallbladder bile composition to plasma and liver composition and stone formation. The fact that gallstones are more prevalent in women than in men and that most of the monkeys in our study of the lithogenic diet were females suggests the need to investigate the sex distribution of gallstones in squirrel monkeys. We must also define the nutritional factors responsible for gallstone formation in these monkeys. Although the level of cholesterol and the saturation of the fat are important factors, sucrose or some other component of the semipurified diets must also be investigated; the significant difference between the cholesterol:phospholipid ratios in the bile of monkeys on the chow and those on the semipurified diets cannot be a coincidence. We previously observed sizable differences between the bile salt turnover (20) in rats or monkeys on Purina chow and those on a low residue semipurified diet containing simple

sugars.

Summary. Squirrel monkeys fed a semipurified diet to induce atherosclerosis showed a high incidence of gallstones, which began to appear 3 months after the start of the atherogenic diet. The incidence in animals maintained on this diet for over 9 months was 83%. The gallstones consisted essentially of pure cholesterol and increased in size, number, and total mass of stones during the period of feeding. The cholesterol:phospholipid ratio in bile was highest in the group and in the individuals with stones. Monkeys fed a natural diet had very low ratios and were free of gallstones.

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Received Oct. 15, 1970. P.S.E.B.M., 1971, Vol. 136.