(16) or leukemia (16). In animals, ASA (12, 17), phenylbutazone (5, 17), and sulfinpyrazone (5) prolonged the bleeding time of transected mesenteric blood vessels, and a very severe hemostatic defect was evident when these drugs were given to rabbits receiving oral anticoagulants (17) or dogs with Factor IX deficiency (18).

Summary. Secondary aggregation of platelets was produced in 8 of 10 normal subjects by a critical concentration of adenosine diphosphate (ADP) ranging from 1.0–2.0 μM. Shortening of the Russell Viper Venom clotting time and release of serotonin-^{14}C were also observed with the critical concentration of ADP or with higher levels, but did not occur in the two subjects who showed only primary aggregation in response to ADP. Ingestion of 1.3 gm of acetylsalicylic acid in a divided dose 1 and 2 hours prior to testing abolished secondary aggregation, serotonin release, and platelet factor-3 activation induced by ADP, as well as aggregation and serotonin release induced by connective tissue particles, but did not affect primary ADP-induced aggregation.

Addendum. O'Brien (Lancet **1**, 204, 1968) has recently shown that ingestion of as little as 0.15 gm of aspirin can abolish secondary aggregation induced by epinephrine for as long as 6 days.

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Gallbladder Damage and Prolonged Excretion of Cholera Vibrios in *Erythrocebus patas* (32738)

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According to Pollitzer (1), Pirogoff, Kulescha, and Gregg were the first students of the relationship of cholera and the gallbladder, but it remained for Snijders to assert that this organ is invaded by vibrios from the intestinal tract rather than the lymphatic channels or the bloodstream. Valk (2) was perhaps the first to relate prolonged excretion of cholera vibrios and their survival in the gallbladder. Gohar (3), Barua (4), Gangarosa *et al.* (5) and Wallace *et al.* (6) used duodenal intubation or oral administration of magnesium sulfate to detect gallbladder carriers of cholera vibrios. Hasan *et al.* (7) found the biliary system also involved in experimentally-infected rhesus monkeys.

These observations stimulated the extension of our studies of cholera in nonhuman primates in the direction of the relationship of prolonged vibrio excretion (carrier status), immunization, gallbladder pathology and circulating vibriocidal and agglutinating antibodies in patas monkeys *(Erythrocebus patas)*. The immunological responses of this species to *V. cholerae* biotype El Tor have been studied previously in this laboratory (Greer and Felsenfeld, 8). These animals excrete cholera vibrios after oral administration, usually for 0–6 days and about 5–30% of them become ill with diarrhea according to the vibrio strain.

In view of the spread of the El Tor pandemic into the Near East, it seemed advisabe to concentrate on the El Tor biotype of *V. cholerae* which, moreover, is known to cause cholecystitis in artificially-infected rodents (Sayamov, 9). To the best of our knowledge, data have not yet been published concerning the frequency of gallbladder damage in primates, including man, in disease caused by El Tor vibrios. An attempt to investigate this problem was the principal aim of the present study.

Materials and Methods. Experimental animals. Adult patas monkeys of both sexes, weighing 8–14 lbs, were used. There was no history of disease in their records within the year preceding the experiments. The animals were considered free from illness and infection by clinical observation, by bacteriological and parasitological examination of blood, stools and swabs from the upper respiratory tract, and by tuberculin tests. The body temperature, RBC and WBC counts, Hb determination, the cephalin floculation and SGOT were normal for the species.

Preparatory surgery. The monkeys were divided into four groups of four animals. One group served as control of the challenge. The second group underwent sham surgery. The abdominal cavity was opened under general anaesthesia, and the gallbladder gently handled with sterile gloved hands; then the peritoneum and the abdominal wall were closed. Sterile pumice stones were implanted in the gallbladders of the third group. The mucosa of the gallbladder was squeezed with flat pincers over an area approximately 1 × 1 cm in the fourth group. Strictly humane treatment was employed in all operations, and the rules of the Animal Resources Board of the National Institutes of Health were obeyed. All animals recovered within 1 week without ill effect.

Immunization. The antigen was prepared from an agar-grown, 18 to 20-hour-old culture of *V. cholerae,* biotype El Tor, strain No. 201. This strain was known to cause diarrhea in about 20% of patas monkeys when approximately 10^9 live cells were administered through a duodenal sonde. The growth was suspended in 0.85% saline to contain 1.2 to 1.5 × 10^{11} organisms per ml and exposed to ultrasonic disruption. The cellular debris was centrifuged off at 15,000*g* at 0°C. One ml of the supernate was used for intramuscular immunization of one of the four patas in each group. The same amount was given by duodenal sonde to another monkey in each group.

Serological tests. Blood specimens were collected weekly, before and during the experiment, and 1 week after its termination. The sera were tested for vibriocidal antibodies in twofold dilutions beginning with 1:50 according to the method of Sack *et al.* (10).

Agglutination tests were carried out with sera in twofold dilutions beginning with 1:40. The serum–antigen mixtures were incubated at 50°C for 1.5 hours, then refrigerated overnight. Only 3+ and 4+ reactions were considered positive.

Challenge. Four weeks after immunization, all patas, including the control group, were given, through a duodenal sonde, 2 ml of a saline suspension of *V. cholerae,* biotype El Tor, strain No. 201, containing 1.3 to 1.8 × 10^9 live organisms.

Gallbladder extirpation. Four weeks after the challenge, the gallbladders of all patas were removed and examined histologically after staining with hematoxylin–eosin, and the Masson trichrome technique. Microbiological examination of the bladder bile was carried out by culturing it first in 2% peptone water with 1% sodium chloride, pH 8.6, for 6–8 hours, then streaking on meat extract agar (Baltimore Biological Laboratory) pH

TABLE I. Relationship of Gallbladder Damage to Duration of Vibrio Excretion.[a]

Duration of excretion (days)	Patas monkeys with															
	Mechanical damage				Implanted stones				Sham operation				No surgery			
	im	po	N1	N2	im	po	N1	N2	im	po	N1	N2	im	po	N1	N2
3						+			0	0				+	0	
3–6	+				+						+	++				+
7–14								++								
15–18		++	++	++			++									
27													+			
Diarrhea after challenge	Ø	D	D	Ø	Ø	Ø	D	Ø	Ø	D	Ø	Ø	Ø	Ø	Ø	Ø

[a] Abbr.: im = antigen given intramuscularly; po = antigen given by duodenal tube; N1, N2 = not immunized; 0, +, ++ = degree of histological damage; Ø = no diarrhea; and D = diarrhea.

8.6. Sections of the gallbladder were stained with the method of Gram.

After cholecystectomy, the abdominal cavity was closed. All monkeys recovered without ill effect.

Stool examinations. These were carried out in 1–2 day intervals for 5 weeks beginning with the day after the challenge. Rectal swabs were collected, immersed into alkaline peptone water, incubated for 6–8 hours, and streaked on one alkaline meat extract agar and one cholera medium plate (Oxoid Laboratories).

Results. Table I shows the duration of the excretion of vibrios in relation to the histological findings.

The histological changes in the gallbladder were graded ++, +, and 0, according to the extent of the edema of the mucosa, the presence and intensity of foci of infiltration with lymphocytes, plasma cells, histocytes, and polymorphonuclear cells, and the extension of these infiltrates into the muscularis. Necrosis was seen only once, in an unimmunized patas (N2) with implanted pumice stones. The cellular infiltrates contained relatively small amounts of polymorphonuclear cells. In severe (++) damage, the infiltrates reached the subserous connective tissue.

Table I shows that more nonimmunized animals developed severe (++) gallbladder pathology than immunized (5:1). Relatively more patas that had surgery excreted vibrios for a period longer than 2 weeks (4:1).

Diarrhea developed in 4 of the 16 animals after challenge. It lasted less than 36 hours and required neither rehydration nor treatment with antibiotics. It did not appear in parenterally immunized patas, but orally vaccinated monkeys were, evidently, less protected at the time of the challenge (4 weeks after vaccine administration).

Vibrios were not cultured from the bile specimens, nor seen adhering to the wall of the gallbladder in the gram-stained sections.

Table II shows serum vibriocidal activities and agglutinin titers. Measurable vibriocidal titers were present in 2 patas before vaccination or challenge. These titers reached their peak during the second or third week after the intramuscular administration of the antigen. Peroral immunization caused the appearance of antibodies at low levels.

After challenge, the antibody titers, principally the vibriocidal capability levels, soon increased to relatively high titers in intramuscularly immunized animals. The vibriocidal antibody levels remained elevated for a longer time in most (3 out of 4) parenterally vaccinated monkeys and in nonimmunized animals which excreted vibrios for more than 2 weeks (N1 and N2 in the groups "mechanical damage" and "implanted stones"). The agglutination reactions followed the pattern of the results of the vibriocidal antibody determination to a limited extent.

Discussion. The results presented in this paper are based on experiments carried out on a limited number of animals. Possible

TABLE II. Reciprocal Vibriocidal and Agglutinin Titers Before and After Challenge.[a]

Week	Patas monkeys having: Mechanical damage im	po	N1	N2	Implanted stones im	po	N1	N2	Sham operation im	po	N1	N2	No surgery im	po	N1	N2
Before vaccination	0[b] / 0	0 / 0	50 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	50 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
After vaccination 1	100 / 0	50 / 40	50 / 0	0 / 0	200 / 80	50 / 0	0 / 0	0 / 0	50 / 40	50 / 40	0 / 0	0 / 0	400 / 40	0 / 40	0 / 0	0 / 0
2	400 / 160	50 / 80	0 / 0	0 / 0	400 / 160	50 / 40	0 / 0	0 / 0	800 / 80	50 / 80	0 / 0	0 / 0	800 / 320	100 / 40	0 / 0	0 / 0
3	200 / 80	0 / 40	50 / 0	0 / 0	400 / 40	0 / 0	0 / 0	0 / 0	200 / 160	50 / 40	0 / 0	0 / 0	1600 / 160	50 / 0	0 / 0	0 / 0
4 (Before challenge)	100 / 80	0 / 40	100 / 0	0 / 0	200 / 0	0 / 0	0 / 0	0 / 0	50 / 0	50 / 0	0 / 0	0 / 0	200 / 80	0 / 0	0 / 0	0 / 0
After challenge 1	800 / 160	200 / 80	200 / 0	100 / 40	1600 / 320	400 / 80	100 / 0	400 / 40	800 / 80	200 / 40	100 / 40	200 / 40	1600 / 80	400 / 40	200 / 40	200 / 0
2	3200 / 320	800 / 80	400 / 160	200 / 80	6400 / 160	800 / 160	800 / 80	400 / 80	1600 / 160	800 / 80	400 / 80	400 / 160	1600 / 160	800 / 40	200 / 80	400 / 80
3	800 / 80	800 / 80	1600 / 80	800 / 160	1600 / 40	800 / 40	400 / 80	800 / 80	400 / 40	200 / 40	100 / 0	200 / 40	1600 / 80	200 / 0	200 / 0	200 / 0
4	200 / 0	400 / 40	800 / 40	400 / 80	400 / 40	100 / 0	200 / 40	400 / 0	100 / 0	0 / 0	0 / 0	50 / 0	800 / 80	0 / 0	0 / 0	0 / 0
5	100 / 0	200 / 0	400 / 0	200 / 40	100 / 0	0 / 0	200 / 0	100 / 0	0 / 0	50 / 0	0 / 0	0 / 0	400 / 80	0 / 0	0 / 0	0 / 0

[a] Abbr.: im = antigen given intramuscularly; po = antigen given by duodenal tube; and N1, N2 = not vaccinated.

[b] Vibriocidal titer/agglutinin titer; 0 = negative or lower than 1:50 vibriocidal or 1:40 agglutination titer.

variations in the challenging organisms, and the need to keep all experimental animals under strictly identical environmental conditions, prevented our using a greater number of monkeys. The statistical evaluation of the results is, therefore, not easy, as in the unusual case of prolonged (27 days) excretion of vibrios by one of the control animals. It seems, however, that the ratio of immunized and nonimmunized animals with extensive gallbladder pathology, as well as the ratio of monkeys, with and without surgery, excreting vibrios for more than 14 days, represents true differences.

The development of cholecystitis in animals not exposed to mechanical damage or implantation of stones indicates that at least this strain of *V. cholerae* biotype El Tor is able to produce gallbladder pathology in patas monkeys. This observation demonstrates that not only rodents, but also monkeys, which are phylogenetically nearer to man, may suffer gallbladder damage when infected with El Tor vibrios. This damage, evidently, aggravated the results of the surgery.

It was interesting to note that none of the 4 parenterally immunized animals developed diarrhea, while 2 out of 4 orally vaccinated and 2 out of 8 nonimmunized monkeys had this symptom. The role of vaccination with this antigen in the prevention of diarrhea in nonhuman primates is the subject of further studies.

Serological studies revealed generally lower antibody titers than reported in man by Sack *et al.* (10), but such a difference is not exceptional in nonhuman primates under these experimental conditions (Felsenfeld *et al.* 11). The maintenance of significant serum antibody levels, principally of vibriocidal activity titers, in animals excreting vibrios for a long time, is of interest and indicates the possible usefulness of that test in a search for vibrios carriers.

Summary. Mechanical damage and implantation of pumice stones into the gallbladder of patas monkeys seemed to extend the period of excretion of vibrios, principally in animals not vaccinated parenterally with the homologous strain. The administration of live El Tor vibrios, without surgery, also appeared to induce pathological changes in the gallbladders of some unvaccinated animals. The vibriocidal activity titers remained elevated for a longer time in animals that excreted vibrios for an extended period.

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