Isolation of an Agent Causing Bilirubinemia and Jaundice in Raccoons. (20852)

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An agent causing bilirubinemia, jaundice, and frequently death in raccoons (*Procyon lotor*) has been isolated from the liver of a sick raccoon trapped in Maryland. This agent is considered to be a virus as tests for bacteria and for leptospirae have been negative and it is filterable. Thusfar, there has been no indication of relationship between this agent, which will be designated Raccoon Jaundice Virus (RJV) and other viruses, including those of feline distemper, canine distemper and canine hepatitis. RJV may be responsible for epizootics which occasionally affect raccoon populations. The present report describes a few attributes of the agent and of the disease which it produces in raccoons as observed in 5 continuous passages.

Materials and methods. Raccoons used for experiments were live-trapped in Maryland or obtained from a dealer in Florida. They were maintained on a diet of horse meat plus bread, lettuce, apples, and bananas. Preparations of the agent were from livers of diseased raccoons, made into 10% suspensions with meat infusion broth. Such suspensions were stored in a dry ice cabinet in sealed glass ampoules. Unground portions of infected livers were also stored at –40°C in an electric deep freeze. Both methods of storage were effective. In routine passages, raccoons were given 1 ml of a 10% suspension intraperitoneally.

Description of disease. Two isolations of the agent (RJV) were made on first passage from the original raccoon, one from the liver and one from the kidney. This raccoon was weak and had exudate about the eyes when first observed. On necropsy an empty intestinal tract but no jaundice or gross pathology of any kind was encountered. This original animal had convulsive movements of head and paws, signs which have not been observed in inoculated animals. Raccoons inoculated intraperitoneally with suspensions of infected liver usually became ill within 9 to 14 days. In 5 continuous passages, virulence remained high and almost all raccoons not killed when severely ill died from the disease. Illness began with cessation of feeding and was often accompanied by appearance of exudate about the eyes; sick animals remained quiet, eating little or nothing. A few had a bad smelling, scanty anal discharge. Vomiting was not observed. After 5 days, jaundice was sometimes detectable in the gums. Almost all inoculated raccoons, if not sacrificed earlier, were dead or moribund within 4 to 8 days of the time illness was first observed. On necropsy raccoons ill 5 or more days usually had ample stores of fat, although intestinal tracts were empty. Judged by full urinary bladders often encountered and the fact that sick raccoons drank freely, the animals were not dehydrated. Jaundice frequently was of an intense degree in raccoons severely ill or recently dead from the disease. In other animals, jaundice was slight or absent in spite of an elevated serum bilirubin (Table I) and a positive foam test in the urine. Neither petechiae nor hemorrhages were observed. Liver, spleen, kidneys, intestinal tracts and other organs appeared normal, except that occasionally sick animals had small patches of pulmonary consolidation. Histologic studies, to be reported later when more material has been accumulated, have so far given scanty evidence of pathologic changes in liver, kidneys, or other organs. Pathologic changes were found in the liver of one raccoon which had a total serum bilirubin of 11 mg %, the highest level encountered in any animal. These changes consisted of necroses of single liver cells widely scattered throughout the parenchyma. There was no attendant inflammatory reaction. Some few raccoons developed purulent con-

TABLE I. Quantitative Serum Bilirubin Determinations in Relation to Course of Hepatitis* in Raccoons and a Ferret Inoculated Intraperitoneally.

Animal No.	Day of disease	Quantitative serum bilirubin 1 min.	Total	Clinical icterus	Clinical condition	Incubation, days	Inoculum
732	†	.04	.16			21	Kidney unfiltered,
	5	.18	1.06				1st passage
	8	1.59	3.34	Present	Moribund, killed		
842	†	.01	.17			8	Liver unfiltered,
	3	.16	.11				2nd passage
	7	1.44	2.38	None	Died under observation		
846	†	.12	.11			14	Liver filtered,
	6	2.95	4.56	"	*idem*		3rd passage
803	†	Too fatty	.05			12	Liver filtered, pool
	7	2.35	5.38	Present	Moribund, killed		of passages 1, 2, and 3
717‡	†	.12	.13			12	Liver filtered,
	2 (?)	1.48	2.20	None	*idem*		3rd passage

* Tests in laboratory of Dr. Roderick Murray. Biologics Control, Laboratory of Infectious Diseases, National Microbiological Institute.

† Pre-inoculation.

‡ Ferret.

junctivitis with gluing of the eyelids. This complication was presumably due to secondary invasion by pyogenic organisms, for it even appeared in one raccoon fully protected from bilirubinemia by immune serum.

Laboratory findings. As shown in Table I in the case of a few animals used as examples, elevation of serum bilirubin has been marked in diseased raccoons and in a single ferret. Hematocrits run on diseased or dying raccoons have averaged between 42 to 45% and, in absence of signs of dehydration, support the conclusion that anemia, hemolytic or otherwise, plays no role in the disease picture, especially as blood smears have appeared normal. Leucocyte counts made for 5 sick raccoons were not remarkable. Raccoon 846 (Table I), for example, had a total white cell count of 16,400 cu mm, of which 72% were neutrophiles, 13% lymphocytes, 10% monocytes, 1% eosinophiles, and 4% young forms, when moribund on its 6th day of disease.

Transmission. The raccoon disease caused by (RJV) has been passed parenterally by suspensions of infected liver, kidney, spleen and on one occasion by blood obtained on the first day of illness and passed directly to a recipient raccoon by intraperitoneal inoculation. This last animal became ill in 13 days. Although most passages were made by the intraperitoneal route, one raccoon became ill 11 days after subcutaneous inoculation. In other experiments, confined to single animals, oral transmissions were not successful with eye exudate and saliva, stool specimen from a raccoon on the 2nd day of disease or from a dilute suspension of infected liver given by stomach tube. At the outset of present investigations one raccoon appeared to have developed bilirubinemia from spontaneous transmission. As no other cases of this sort have arisen among inoculated raccoons or among uninoculated animals kept for many weeks in the same room as controls, it is felt that precautions taken have been effective. These precautions have included placing actually diseased raccoons on bottom shelves in one room and spacing cages of other experimental animals well apart on upper shelves. In special experiments raccoons have been maintained in separate laboratory rooms.

Neutralization tests. In these tests 4 sera were heated at 56°C for ½ hour, then mixed with an equal volume of 10% liver suspension which had been centrifuged at 4000 rpm for 1 hour. These mixtures were inoculated separately into peritoneal cavities of 7 young raccoons in amounts of 1.5 ml. Two of these raccoons which had received pre-inoculation phase serum from raccoon No. 842, became severely ill and had elevations of serum bili-

TABLE II. Results of Neutralization Tests Performed with Raccoon Sera in Raccoons Inoculated Intraperitoneally with Serum-Virus Mixtures.

Raccoon	Serum phase	No. of raccoons tested	Fate of test raccoons
842	Pre-inoculation	2	Severe illness + bilirubinemia
842	7 days after onset of illness	2	Remained well
846	*idem*	1	*idem*
829	Convalescent	2	"

rubin when killed (Table II). The other raccoons never became ill and were apparently fully protected by sera from 2 raccoons (No. 846 and No. 842) which had had illnesses of 7 days duration, as evidenced by jaundice at necropsy. Convalescent serum from a third raccoon (No. 829) was also protective on 2 occasions. In additional tests with commercial sera one raccoon was not protected by a combined anticanine distemper, anti-canine hepatitis serum, and 2 raccoons were not protected from death by anti-feline distemper serum.

Properties of agent. Preparations of the agent have survived storage in a dry ice cabinet for 6 months and liver suspensions frozen and thawed twice, and in one instance, 4 times, have remained infective. Three successive passages were carried out with liver suspensions passed through Selas .03 filters shown to be bacteria tight. As leptospirae can cause hepatitis and also pass through such filters, special efforts were made to determine whether they might be involved. Convalescent serum from raccoons No. 829, which had neutralizing antibodies as shown in Table II, were negative in complement-fixation and in agglutination tests for leptospirae.* Leptospirae could not be cultured from infected raccoon livers in Shüffner's modification of Vervoort's medium. Furthermore, leptospirae could not be found when histologic sections from diseased raccoon livers were stained with a spirochaetal strain shown to be effective in control tissues known to contain leptospirae. It is concluded, therefore, that this agent is a virus.

Host range. Ferrets are susceptible to infection with (RJV) as shown in Table I for Ferret No. 717. This animal, when found moribund 12 days after inoculation, had experienced a rise of serum bilirubin in the course of its disease. When passed to raccoons, suspensions of this ferret's liver resulted in bilirubinemia and jaundice. Animals inoculated without evidence of a resultant disease included suckling and weanling mice and hamsters, rats, cotton rats, rice rats, deer mice (Peromyscus) domestic rabbits, cottontail rabbits, guinea pigs, 4 puppy dogs, 5 Rhesus monkeys, 2 suckling pigs and embryonated eggs.

Discussion. The present report demonstrates that raccoons are susceptible to a disease which, under laboratory conditions, is highly fatal and is accompanied by bilirubinemia, jaundice, and conjunctivitis. As the etiologic agent is filterable and resistant to repeated freezing and thawing, it appears to us to be a virus, especially as tests for leptospirae have been negative. Whether it is a hitherto undescribed virus or is related to one already known in some other host cannot be stated at the present time.

In the disease of raccoons described above, jaundice has appeared to be due primarily to liver dysfunction rather than to any hemolytic type of anemia. A puzzling feature of the disease, however, has been the meagerness of pathologic findings considering the severity of the disease. It is hoped that continued study of the disease under varied conditions including intracerebral inoculations will make possible a more complete description of histologic changes which must take place in such a severe and fatal disease. Of other mammals so far studied, only the ferret has developed bilirubinemia and jaundice similar to that found in raccoons. Efforts are also being made to find out what relationship, if any,

* Tests made at Army Medical Service Graduate School, Veterinary Division, Washington, D. C.

raccoon hepatitis, if such it is, may bear to infectious hepatitis of man.

Summary. An infectious agent, which appears to be a virus (RJV) has been isolated from the liver of a wild raccoon which has led to a highly fatal type of disease characterized by conjunctivitis and an elevated serum bilirubin frequently accompanied by jaundice on inoculation of raccoons. Ferrets also appear to be susceptible to infections with this agent.

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Calcification. XII. Cation-Linked Inhibition by Fluoride and Cyanide Ions in β-Glycerophosphate Medium.* (20853)

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In a previous report it was shown that in the presence of magnesium ions in solutions employed for calcification *in vitro,* both cyanide and fluoride ions exert a marked inhibitory effect. In the absence of magnesium ions, however, fluoride actually enhances the degree of mineralization, while cyanide appears to have neither a blocking nor an enhancing effect(1). Magnesium, in the amounts used, either exerted no measurable influence on the degree of mineralization or only a mild inhibition when cyanide or fluoride ions were not present. Recently Marks and his associates commented on the cyanide inhibition, stating that magnesium inhibition in itself accounts for the findings of cyanide inhibition(2). For this reason our experiments were carefully repeated and the data presented in Table IV of reference 1 were confirmed. It was shown at that time that only in the presence of magnesium ions added to the calcifying fluid will cyanide block the mineralizing process *in vitro.* The present work is concerned with an extension of the studies of cyanide and fluoride inhibition to calcifying media containing β-glycerophosphate as the source of phosphorus. In the course of these experiments it was found that cations other than magnesium, such as strontium, barium, and manganese, also cause cyanide to act as an inhibitor.

Robison and his co-workers(3,4) were first to observe inhibition of *in vitro* calcification by fluoride and cyanide ions in inorganic phosphate solution.‡ No cyanide effect was observed with β-glycerophosphate, although a block by fluoride was still evident. A reinvestigation of the problem by Gutman, *et al.* (5-7), served to confirm the earlier work of Robison, with one exception: neither cyanide nor fluoride interfered with calcification in mineral solutions containing phosphorus as β-glycerophosphate.‡ This point of discrepancy seemed to warrant further inquiry, particularly in view of the significance attached to the apparently dissimilar behavior of the ions in inorganic and organic phosphate medium(5-7).

Methods. A modified USP rachitogenic diet replaced the diet previously used(8). The rations, on which the Wistar strain albino rats were placed at the age of 22-24 days, consisted of 76% degermed corn meal, 20% wheat gluten, 3% $CaCO_3$, and 1% NaCl. The rachitic animals were sacrificed 17 to 23 days later. Slices of bone cartilage were removed from their tibiae and suspended in the designated solutions in 25 ml Erlenmeyer

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‡ In addition to calcium, phosphate, sodium, potassium, and bicarbonate, magnesium was included in the solutions of both Robison and Gutman to simulate its presence in serum. The critical role of magnesium in the inhibitory process has only recently been recognized(1).