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Role of Reticulo-Endothelial System and Disposition of Dietary Cholesterol in Various Mammalian Species.* (20941)

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In a recent report from this laboratory(1), it was shown that the hepatic R-E cell is essential in the disposition of *exogenously* derived cholesterol in the rat. This was demonstrated not only by microscopic detection of cholesterol in the Kupffer cell within 6 hours following administration of cholesterol, but also by the production of hypercholesteremia and persistence of a chylomicronemia in rats given parenteral injection of various colloidal suspensions believed capable of interfering with hepatic reticulo-endothelial cell function.

In view of these results, it was decided to determine whether this hypercholesteremia following interference with the integrity of the R-E system could be duplicated in other species than the rat. Therefore, the present study was undertaken in which rabbits and dogs, as well as rats, received India Ink intravenously together with high cholesterol feedings.

Effect of intravenous administration of India Ink on plasma cholesterol and total lipid of animals on high cholesterol feedings. Plasma samples of all species studied were taken before and 48 hours after the beginning of the experiment. These were analyzed for their total cholesterol and total lipid content by methods previously described(2,3). In addition, most of the samples were examined microscopically under darkfield illumination for the presence of chylomicra.

Rats. Methods. Male rats of the Long-Evans strain, approximately 15 weeks old (avg wt 244 g), were used. Two series of rats were studied. Series A (15 rats) was given 100 mg of cholesterol in 3 cc of olive oil by stomach tube per day for 2 days. Series B (13 rats) was given 100 mg of cholesterol in 3 cc of olive oil by stomach tube intubation per day, *plus* 0.5 cc of 20% India Ink intravenously twice daily for 2 days.

Results. The control rats given cholesterol feedings alone, showed a rise in plasma cholesterol (Table I) but the average plasma cholesterol concentration at 48 hours was still only 64 mg/100 cc and the plasma lipid concentration was not found to change. However, when India Ink was injected into the rats of Series B in addition to the oral administration of cholesterol, a striking increase in both their plasma cholesterol and total lipid occurred. Thus, the average plasma cholesterol rose from 52 mg % before the experiment to 100 mg/100 cc 48 hours after the beginning of the experiment. The average plasma lipid content rose from 242 to 322 mg/100 cc in the same period of time.

As observed earlier(1), chylomicra invariably were absent from the sera of the control rats and always present in great numbers in the sera obtained at 48 hours from ink-injected rats. The latter sera, also, were invariably turbid.

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TABLE I. Effect of Injection of India Ink upon the Plasma Cholesterol and Lipid Content of Rat, Rabbit and Dog after Ingestion of Cholesterol in Olive Oil.

	Series	No. of animals	Plasma cholesterol (mg/100 cc) Before feeding	Plasma cholesterol (mg/100 cc) 48 hr after feeding	Plasma lipid (mg/100 cc) Before feeding	Plasma lipid (mg/100 cc) 48 hr after feeding
			Rats			
Avg	A	15*	49	64	170	173
Range			(33–74)	(40–82)	(133–200)	(148–250)
S.E. mean			± 2.5	± 2.9	± 10.0	± 11.2
Avg	B	13†	52	100	242	322
Range			(32–74)	(79–140)	(157–295)	(227–388)
S.E. mean			± 3.3	± 4.8	± 13.4	± 12.8
			Rabbits			
Avg	A	8*	53	204	107	354
Range			(32–90)	(129–251)	(73–135)	(218–545)
S.E. mean			± 5.6	± 4.5	± 6.4	± 12.4
Avg	B	10†	73	316	137	1323
Range			(46–152)	(158–558)	(91–204)	(435–4100)
S.E. mean			± 9.0	± 33.6	± 10.4	± 14.6
			Dogs			
Avg	A	7‡	161	217	360	455
Range			(102–196)	(167–262)	(208–477)	(317–568)
S.E. mean			± 11.4	± 11.2	± 36.3	± 28.4
Avg	B	8‡	164	258	460	738
Range			(115–196)	(210–304)	(310–640)	(640–820)
S.E. mean			± 8.6	± 12.9	± 34.9	± 19.0

* Animals given 400 mg cholesterol/kg/day dissolved in olive oil.
† *idem*, but oil and India Ink.
‡ Dogs given 600 mg cholesterol/kg/day dissolved in olive oil.

Rabbits. Methods. Male rabbits, approximately 16 weeks old, were used. Two series of rabbits were studied. Series A (8 rabbits) was given 400 mg of cholesterol (33.5 mg cholesterol/cc of olive oil) per kg body weight by stomach tube daily for 48 hours. Series B (10 rabbits) received the same amount of cholesterol, *plus* 5 cc of 20% India Ink suspension intravenously daily for 48 hours.

Results. As was anticipated, the increment of increase in the plasma cholesterol of both Series A and Series B (Table I) was greater than that in the rat experiments. However, once again there was a greater increase (333%) of plasma cholesterol in those rabbits receiving the R-E "blocking agent," India Ink, than that (285%) observed in the rabbits which received only the cholesterol in olive oil feedings. The total plasma lipid content also showed a more marked increase in those animals receiving the intravenous India Ink.

Unlike the control rats, the control rabbits fed only cholesterol, exhibited many chylomicra in their plasma at the 48-hour period. However, even more chylomicra were observed in the plasma of the rabbits fed cholesterol and injected with India Ink.

Dogs. Methods. Two series of mature mongrel dogs were used. Series A (7 dogs), the control series, received cholesterol (0.6 g in 10 cc olive oil/kg body weight) by gastric intubation daily for 2 days. Series B (8 dogs) received an intravenous injection of one cc of 20% India Ink solution/kg body weight per day, in addition to the above-described gastric feedings of cholesterol in olive oil. The India Ink was given within 2 hours following the administration of the cholesterol feeding. Blood samples, taken before and 48 hours after the beginning of the experiment, were analyzed and examined as described above.

Results. The results of these experiments indicated that the dog exhibited a moderate increase in both its plasma cholesterol and total lipid content following the administration of cholesterol in oil alone. Thus (Table I), the average plasma cholesterol and lipid

content of the 7 control dogs was 161 and 360 mg/100 cc respectively before, and 217 and 455 mg/100 cc 48 hours after feeding of cholesterol alone. This represents an increase of 35 and 26% respectively in the cholesterol and lipid plasma content. The experimental series of 8 dogs, however, given the same amount of cholesterol in oil, plus intravenous injection of India Ink, exhibited an even greater rise in both the cholesterol and total lipid content of their plasma. Thus the average plasma cholesterol increased (Table I) from 160 to 258 mg/100 cc (an increase of 57%) and the average plasma total lipid increased from 460 to 738 mg/100 cc (an increase of 60%).

As in the rat, the plasma samples obtained at the 48-hour period from the ink-injected dogs, showed turbidity as well as myriads of chylomicra, whereas the plasma samples of the control dogs were clear and showed few or no chylomicra.

Discussion. The present observations indicate that the intravenous injection of India Ink, when *combined* with the gastric intubation of cholesterol dissolved in olive oil, leads to frank hypercholesteremia in the rat, intensifies the expected hypercholesteremia of the rabbit and elevates the plasma cholesterol of the dog. The plasma lipid of these 3 species is similarly affected by the combination of ink[1] injection and cholesterol feeding. These results suggest that our earlier finding of the essentiality of the hepatic reticulo-endothelial system for proper metabolism of *dietary* cholesterol in the rat(1) is a phenomenon not limited to this species, because when this system is interfered with in other mammalian species, the latter show an interference in their usual handling of cholesterol.

Earlier studies of this laboratory demonstrated that the liver functions to remove and store the excess cholesterol in the plasma derived from the diet(4) until it is later either discharged again into the plasma or converted and excreted into the bile as bile salt(5,6). However, almost all dietary cholesterol after absorption from the intestine travels in intestinal lymph enmeshed in chylomicra(7) and the relatively huge size of these particles makes it almost impossible to conceive of them as being able to make their way through the intact capillary walls of the liver to the hepatic parenchymal cells. Obviously then, they must either be broken down in plasma (*e.g.* solubilized by "clearing factor") or ingested by a phagocytic cell and solubilized intracellularly. It therefore was no surprise to us to find(1) that the hepatic Kupffer cell and only this type of reticulo-endothelial cell of the rat contained cholesterol demonstrable by Sudan staining and refraction of polarized light soon after the oral ingestion of this steroid. Apparently then, this cell ingests the chylomicron and in so doing solubilizes the cholesterol component contained therein and then transfers it to the hepatic cell. It was the discovery of this cell's similar handling of the lipid content of the chylomicron which caused Gilbert and Jomier(8) to call the Kupffer cell, "l'auxiliare précieux et dévoué de cellule hépatique"—a function later confirmed by Jaffé and Levinson(9). The injection of a "blocking agent" simultaneously with the absorption of dietary cholesterol interferes with this ingestion process and in so doing leads to a hypercholesteremia ostensibly due to the retention in plasma of chylomicron cholesterol. This interference process also explains the frequent associated finding of plasma turbidity and may be responsible for the results recently obtained by LeQuire, Gray, and Cobb(10) who observed that following injection of thorotrast in the dog a marked reduction in the clearing of lipemic plasma occurred following heparin injection.

Summary. Interference with the reticulo-endothelial system by injection of India Ink was found to interfere with the ability of the rat, rabbit and dog to clear their plasma after the ingestion of cholesterol dissolved in olive oil. This interference extended not only to cholesterol, but also to total lipid and chylomicra. These findings suggest that the reticulo-endothelial system plays an essential role in the disposition of exogenously derived cholesterol. The possible mechanism involved is discussed.

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Inhibition of Hyaluronidase Activity by Factors Associated with Blood Clotting. (20942)

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The interaction of hyaluronidase with the clotting mechanism has been suspected for some time. Attempts have also been made to identify complement (C′) with the nonspecific serum hyaluronidase inhibitor and also with prothrombin(1-5). This suggests a possible relationship between the prothrombin complex and inhibitor. Baserga *et al.*(6) noted that hyaluronidase interferes with the prothrombin consumption in sera. The inhibition of the agglutination of platelets by this enzyme was observed by Quattrin(7). Fiala *et al.*(8,9) have shown that dialyzed bovine hyaluronidase acts as an anticoagulant *in vitro*, by virtue of its capacity to counteract the thromboplastic effect of platelets. This interaction between platelets and hyaluronidase was postulated as representing a competition between the two, for a specific plasma protein(9). The latter was found to be stable on storage, and to have the same adsorption affinities as prothrombin, but was nevertheless present in a prothrombin free serum. Fiala has further stated(10) that tissue thromboplastin inhibits the enzymatic degradation of hyaluronic acid. Studies made in this laboratory on the interaction between hyaluronidase and the clotting mechanism have revealed that this phenomenon is more complex than can be explained merely as a competitive inhibition between platelets and enzyme. The data to be presented below suggest that the nonspecific serum (plasma) inhibitor of hyaluronidase may play a specific role in the mechanism of blood coagulation.

Materials and methods. Platelets were isolated by differential centrifugation from plasma containing Sequesterine® (ethylenediamine-tetraacetic acid), and collected in siliconized vessels. The final centrifugate was washed 4-fold with saline, resuspended in distilled water and then shaken for 5 minutes with aid of a mechanical shaker. The shattered and agglutinated platelets were separated by centrifugation at low speed, and the supernatant "platelet extract" poured off. The sediment was washed once by shaking with one-third volume distilled water and the wash water combined with the supernate. The final sediment was discarded. This platelet extract was found to correct the clotting

TABLE I. The Effect of Proconvertin Concentration on "Anticoagulant" Activity of Hyaluronidase.

Proconvertin eluate added (ml)	0	.1	.2	.3	.4	.5	Control
Clotting time on recalcification (sec.)	∞	1800	1450	1100	1000	1040	1060

Each tube contained 0.5 ml plasma with 3×10^8 platelets, 0.1 ml hyaluronidase and saline plus proconvertin eluate, total 1.1 ml. In the control saline replaced enzyme. 0.5 ml (0.025M) $CaCl_2$ added after 10 min. incubation at 37°C. Clotting times are the avg of 3-4 determinations.