Observations Concerning the Production and Excretion of Cholesterol in Mammals

XIV. The Relationship of the Hepatic Reticuloendothelial Cell (Kupffer Cell) to Endogenously Produced Cholesterol

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The possible role of the hepatic reticuloendothelial system in the synthesis as well as the disposition of endogenously produced cholesterol was studied in the rat by various means. Our results suggest that this system does not synthesize significant quantities of cholesterol nor participate in the egress of endogenously produced cholesterol from the plasma into the liver. It does however, play an important, perhaps indispensable, role in ridding blood of cholesterol derived from the diet.

Previous morphologic, chemical and physiologic studies done in this laboratory\textsuperscript{1,2} indicate that the hepatic reticuloendothelial system of various mammals plays an essential role in the disposition of cholesterol derived from the intestine. Moreover, if the function of this system is experimentally interfered with by injection of various colloidal "blocking" agents, an accumulation not only of cholesterol but also of lipid and chylomicra occurs in the plasma of the treated animal.

Preliminary evidence obtained in the above studies, however, strongly suggested that the reticuloendothelial cells of the liver did not participate in the disposition of endogenously derived cholesterol. It was thought important then to investigate this point in more complete detail. The results of such a study presented herein confirm the initial observations in that they indicate that the hepatic reticuloendothelial cell does not appear to participate either in the synthesis of cholesterol or in the later disposition of that increment of cholesterol synthesized by the animal itself.

I. The Role of the Hepatic Reticuloendothelial System in the Synthesis of Cholesterol

Previous studies from this laboratory\textsuperscript{3,4,5} have demonstrated that the liver of the animal maintained upon a cholesterol-free diet is constantly discharging into and later removing cholesterol from the plasma and, accordingly, at any given time the cholesterol content of the plasma is dependent chiefly, if not solely, upon the functional integrity of this organ. In other words, for all practical purposes the chief site of cholesterol synthesis from a quantitative standpoint is in the liver. Subsequent studies\textsuperscript{6} demonstrated that an estimate of the rate of this hepatic function could be obtained by a determination of the daily biliary cholesterol excretion. A second indirect estimate of this function also could be obtained by measurement of the plasma cholesterol increase occurring after the intravenous administration of Triton because this latter substance when given in sufficient amount...
causes the accumulation in the plasma of an animal, of all or almost all cholesterol manufactured and previously discharged by the liver. The possible effect then of partial interference with the function of the hepatic reticuloendothelial cells by India ink injection upon the rate of the hepatic synthesis of cholesterol was studied by employing these two different methods of assay.

Methods

The bile ducts of 21 rats were cannulated and the bile subsequently collected for 24 hours was analyzed for its total cholesterol content. Eleven of these animals had received 0.5 ml. of 20 per cent India ink intravenously twice daily for 48 hours immediately prior to the duct cannulation.

A second series of 10 starved rats was given 100 mg. of Triton WR 1339 intravenously daily for 48 hours. Half of these rats also received India ink injections as described above. Plasma samples obtained before and 48 hours after the beginning of injections were analyzed for total cholesterol.

Results

The results obtained in the two different types of experiments indicated that any possible interference effected in hepatic reticuloendothelial cells by the injection of India ink had no effect upon the apparent rate of endogenous cholesterol synthesis as gauged by these two different methods.

The average cholesterol content of the 24-hour bile collection of the 11 rats injected with India ink was 1.7 mg. (range, 0.8–2.5), with a standard error of the mean of ±0.14, and the biliary cholesterol content of the 10 control rats was 1.8 mg. (range, 1.4–2.2) with a standard error of the mean of ±0.09.

The average plasma cholesterol of the five rats given Triton and ink rose from 65 to 637 mg. per 100 ml. (range, 509–704) in 48 hours, and in the control animals it rose from 65 to 620 mg. per 100 ml. (range, 589–653).

II. MORPHOLOGIC STUDY OF THE POSSIBLE ROLE OF THE HEPATIC RETICULOENDOTHELIAL SYSTEM IN THE DISPOSITION OF ENDOGENOUSLY DERIVED CHOLESTEROL

Methods

A series of 10 young male rats (average weight, 245 Gm.) of the Long-Evans strain was given intravenously 20 mg. of cholesterol in the form of hypercholesteremic serum. This serum was obtained from rats which had been subjected to biliary obstruction 72 hours before and then given 200 mg. of cholate per day plus a sterol-free diet. Prior to its injection, the serum was centrifuged at 18,000 revolutions per minute to remove any possible chylomicra. Six of the 10 rats were sacrificed at six hours and four at 24 hours, after each had been injected one hour previously with 0.5 ml. of 20 per cent India ink solution. This latter injection was done in order to identify reticuloendothelial cells in the liver. A portion of the liver was fixed in 10 per cent formalin for 24 hours and frozen sections were made. One section of the liver was stained with Sudan III. A second section of each organ was heated for one hour at 55 C. and then examined with the polarizing microscope. For control purposes, six rats (average weight, 252 Gm.) were given orally 100 mg. of cholesterol dissolved in 3 ml. of Tween and three of the rats were sacrificed at six, and the remainder at 24 hours. Portions of their liver were treated and examined in similar fashion. The livers of three rats starved for 24 hours also were obtained and similarly fixed, stained and examined.

Results

The liver of the starved rats showed no sudanophilic granules (see fig. 1) either in the reticuloendothelial or the hepatic parenchymal cells. Neither were any doubly refractile bodies observed.

The liver of the rats given endogenously derived cholesterol by intravenous injection exhibited at the end of six hours a moderate amount of sudanophilic granules which were contained almost exclusively within the parenchymal cells (see fig. 2). Little or none was found between the parenchymal cells or in the reticuloendothelial cells. However the liver of the rats given oral cholesterol six hours previously, exhibited sudanophilic granules which were almost exclusively between the parenchymal cells (see figs. 3a and 3b) and either in or about the reticuloendothelial cells. Examination of liver sections under polarized light revealed the deposit of refractile bodies within the hepatic parenchymal cells of the rats which had received the endogenously derived cholesterol and their aggregation either in or about the hepatic reticuloendothelial cells of the rats which had received oral cholesterol.

A similar selective localization within the
Fig. 1. Liver of starved rat. The reticuloendothelial cells are identified by the ink particles contained in them. Note the absence of stainable lipid. (Sudan III and H. & E. X600.)

Fig. 2. Liver of starved rat, six hours after the intravenous injection of 20 mg. of cholesterol in form of hypercholesteremic serum. Note lipid bodies (red) within the hepatic parenchymal cells with none in or about the reticuloendothelial cells. (Sudan III and H. & E. X600.)

Fig. 3. (a) Liver of rat six hours after oral ingestion of 100 mg. of cholesterol. Note lipid accumulation in and about ink-containing reticuloendothelial cells. Little or none is observed in parenchymal cells at this time. (Sudan III and H. & E. X600.) (b) Isolated, ink-containing reticuloendothelial cells obtained from liver of rat fed 100 mg. of cholesterol 24 hours previously. Note the lipid granules within the cytoplasm of these cells. (Sudan III X800.)

(The use of color in figures 1, 2 and 3 is made possible by a grant from Winthrop-Stearns, Inc., to the publication fund of the American Heart Association.)
Fig. 4. Liver of rat, 24 hours after intravenous injection of 20 mg. of cholesterol in form of hypercholesteremic serum. Observe the refractile bodies (cholesterol) distributed entirely in the parenchymal cells and away from the ink-containing reticuloendothelial cells. The photograph represents a double exposed film. The first exposure was to ordinary light and the second to polarized light. (Unstained ×400.)

parenchymal cell also was found to occur (see fig. 4) in the liver of rats, 24 hours after the intravenous injection of cholesterol. Conversely, in the rats given oral cholesterol 24 hours previously, the greatest deposition of cholesterol still appeared in and about the reticuloendothelial cells (see fig. 5), although at this time, a considerable amount of cholesterol also appeared to be present in the parenchymal cells.

III. EFFECT OF INTERMITTENT INJECTION OF INDIA INK UPON THE HEPATIC DEPOSITION OF ENDOGENOUSLY DERIVED CHOLESTEROL

The preceding observations strongly suggested that the reticuloendothelial cells participated only in the disposition of exogenously derived cholesterol and that these cells were “bypassed” by endogenously derived cholesterol when injected in soluble, chylomicron-free serum. If this were so, then interference with reticuloendothelial cell function by injection of India ink should not impede the rate of hepatic deposition of this type of cholesterol. The following experiment therefore was carried out to determine this point.

Methods

Ten young male rats (average weight, 230 Gm.) were given intravenously, 20 mg. of endogenously derived cholesterol (in the form of hypercholesteremic serum) per day for 48 hours. Half of these rats also were injected intravenously twice daily with 0.5 ml. of 20 per cent India ink suspension. At the end of 48 hours, a portion of the liver was removed, fixed and stained as described above. For control purposes, eight rats (average weight, 246 Gm.) were given 100 mg. of cholesterol dissolved in 3 ml. of olive oil daily for 48 hours. Five of these control rats also received similar injections of India ink. Portions of the liver were treated and examined as described above.

Results

The injection of India ink was not found to influence the apparent rate of cholesterol deposition.
deposit in the rats which had been injected with endogenously derived cholesterol. Thus the liver of the rats injected with India ink exhibited as intense sudanophilic staining as the liver of the rats which had received no ink. The density of the refractile bodies also appeared (see and compare figs. 6 and 7) to be about the same in the two groups of rats.

On the other hand, the liver of rats which were given oral cholesterol and also ink exhibited a profound reduction in sudanophilic staining as compared with the liver of rats given oral cholesterol alone. This same difference also was observed in the comparative density of refractile bodies in the liver of the two groups (see and compare figs. 8 and 9).

These results suggested that "blocking" of hepatic reticuloendothelial cells interfered with the hepatic deposition of dietary but not endogenously derived cholesterol; a finding indirectly confirming the previous morphologic ones concerning the differences in the cellular mode of entrance of the two differently derived cholesterol moieties.

Fig. 6. Liver of rat, 48 hours after the daily intravenous injection of 20 mg. of endogenously derived cholesterol for 48 hours. Note the distribution of refractile bodies is chiefly within parenchymal cells. The photograph is also a doubly exposed one. (X400.)

Fig. 7. Liver of a rat treated exactly as that whose liver is shown in figure 6 except that it was also given 0.5 ml. of 20 per cent India ink twice daily. Note that the quantity and distribution of the refractile bodies is almost identical with that shown in figure 6. Photograph obtained as described in figure 6. (X400.)

IV. EFFECT OF INJECTION OF INDIA INK UPON THE PLASMA CHOLESTEROL OF RATS GIVEN EXCESS CHOLESTEROL OF ENDOGENOUS ORIGIN BY PARENTERAL INJECTION

The above demonstrated inability of injected India ink to prevent or retard the hepatic deposition of endogenously derived cholesterol strongly suggested that the hypercholesteremia observed in animals receiving India ink and exogenously derived cholesterol in our previous studies1,2 would not occur if endogenously derived cholesterol were substituted. Experiments therefore were done to determine this point.

Methods

A series of 21 normal, male, Long-Evans rats (average age, 15 weeks) were given intravenously 25 mg. of cholesterol daily for 48 hours in the form of hypercholesteremic serum obtained and rendered chylomicron free as described in section II. This quantity of cholesterol was given parenterally as this is the approximate amount which was found3 to be
of oral cholesterol did not exhibit hypercholesteremia (see table 1), turbidity or chylomicronemia, the other rats of series II which had received India ink injections with their oral feeding of cholesterol exhibited hypercholesteremia, a moderate turbidity, and intense chylomicronemia.

These results indicated that rats injected with India ink failed to become hypercholesteremic when given endogenously derived cholesterol despite the fact that they were given parenterally the same amount of cholesterol known to be absorbed in rats fed oral cholesterol and which became hypercholesteremic.

V. Effect of Injection of India Ink upon the Plasma Cholesterol of Rats Given Excess Cholesterol of Mixed Origin by Parenteral Injection

The preceding observations strongly suggested that although two series of animals

Results

As table 1 demonstrates, the rats of series I which only received 25 mg. of endogenously derived cholesterol daily by intravenous injection failed to exhibit hypercholesteremia. Similarly, those rats receiving India ink together with their injections of endogenous cholesterol also failed to exhibit hypercholesteremia, turbidity or chylomicronemia.

On the other hand, whereas the control rats of series II which received only 100 mg.
Table 1.—The Effect of Intravenous Injection of India Ink upon the Plasma Cholesterol of Rats also Given (I) Intravenous Cholesterol of Endogenous Origin and (II) Oral Cholesterol

Average Plasma Cholesterol (mg./100 ml.)

<table>
<thead>
<tr>
<th>No. of Rats</th>
<th>Av. Wt. (Gm.)</th>
<th>Before Injection</th>
<th>48 Hours after Injection</th>
<th>Plasma Turbidity (48 hours)</th>
<th>Chylomicronemia (48 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Rats Given Intravenous Cholesterol of Endogenous Origin (25 mg./Day)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>A. With Injections of India Ink</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>295 (252-414)</td>
<td>48</td>
<td>55</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Range</td>
<td>±5.2</td>
<td>(35-76)</td>
<td>±4.1</td>
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</tr>
<tr>
<td>S.E.-Mean</td>
<td></td>
<td></td>
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<tr>
<td>B. Without India Ink Controls</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>281 (250-340)</td>
<td>49</td>
<td>68</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Range</td>
<td>±4.8</td>
<td>(50-78)</td>
<td>±3.6</td>
<td></td>
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<tr>
<td>S.E.-Mean</td>
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<tr>
<td>II. Rats Given Oral Cholesterol (100 mg./Day)</td>
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<td></td>
<td></td>
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<tr>
<td>A. With Injections of India Ink</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>268 (244-340)</td>
<td>49</td>
<td>100</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Range</td>
<td>±4.2</td>
<td>(83-114)</td>
<td>±3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.E.-Mean</td>
<td></td>
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<tr>
<td>B. Without India Ink Controls</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>256 (212-306)</td>
<td>55</td>
<td>68</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Range</td>
<td>±4.8</td>
<td>(50-88)</td>
<td>±3.3</td>
<td></td>
<td></td>
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<tr>
<td>S.E.-Mean</td>
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</tbody>
</table>

received eventually the same quantity of cholesterol in their blood per day, “blocking” of the reticuloendothelial cells with India ink led to hypercholesteremia only in those rats which were fed cholesterol. One possible explanation for the observed hypercholesteremia in the latter rats might be that orally derived cholesterol is first discharged into the blood enmeshed as an insoluble increment of a chylomicron, and that India ink competes with this latter particle for reticuloendothelial cell ingestion and in so doing leads to the observed chylomicronemia and a consequent hypercholesterolemia. On the other hand, those rats given endogenously derived cholesterol receive it directly into their blood stream as soluble cholesterol and not entangled in lipid rich chylomircia. Accordingly, the latter type of cholesterol may not need to be ingested by a process of phagocytosis, hence may not be capable of being interfered with in its transit from plasma to hepatic parenchymal cell by India ink injection. In order to investigate this possibility, the following experiments were done.

Methods

Pooled hypercholesteremic serum was obtained from hypercholesteremic rabbits which had been placed upon a high cholesterol diet two months previously. A portion of the pooled serum was centrifuged at 18,000 revolutions per minute for 30 minutes. At this speed, the chylomircia were forced into an upper, very creamy layer occupying no more than 10 per cent of the total volume. This upper layer was removed and discarded, and the lower layer was checked routinely before its use for the absence of chylomircia by microscopic examination under darkfield illumination. This type of centrifuged separation was carried out four times. The average cholesterol content of the four different unchanged sera was 1525 mg. per 100 ml. (range, 1120–1940), and that of the lower layer component of these same sera after centrifugation was 1195 mg. per 100 ml. (range, 885–1570).

Two series of normal male rats (average age, 14 weeks) of Long-Evans strain were anesthetized with
ether and operated upon, and an external lumbar vein was cannulated with polyethylene tubing connected to an apparatus delivering approximately 0.3 ml. of fluid per hour. This procedure has been described (in detail) in a previous study.\(^\text{1}\)

The 21 rats of the first series received, by continuous intravenous injection, cholesterol in the form of the *unchanged* rabbit dietary hypercholesteremic serum sufficiently diluted with normal saline solution so that each rat received approximately 35 mg. of cholesterol a day for 48 hours. In addition, 12 of the rats received intravenously 0.5 ml. of 20 per cent India ink suspension twice daily for 48 hours. Plasma samples obtained before and 48 hours after the beginning of the injection were analyzed for total cholesterol content.

The 22 rats of the second series also received 35 mg. of cholesterol daily for 48 hours except that it was given in the form of the lower layer of *centrifuged* rabbit dietary hypercholesteremic serum. Twelve of the rats also were injected with India ink as described above.

**Results**

As table 2 indicates, rats given 35 mg. of cholesterol per day in the form of *unchanged* rabbit dietary hypercholesteremic serum and also injected with ink exhibited a greater degree of hypercholesteremia at 48 hours than the control rats which received the same amount of cholesterol but no ink. Thus the average plasma cholesterol of the 16 ink-injected rats rose from 55 to 158 mg. per 100 ml. (see table 2), whereas that of the un.injected rats rose from 51 to 103 mg. per 100 ml. The difference moreover between the means of both series at 48 hours was found to be significant since on calculation it was found to be 3.2 times the standard error of the difference between the two means.

On the other hand, rats of the second series given the same quantity of cholesterol and in the same type of serum, except that the latter was cleared of chylomicra, exhibited no greater hypercholesteremia when injected with India ink than the controls. Thus the average plasma cholesterol of the eight ink-injected rats rose from 59 to 121 mg. per 100 ml. in 48 hours (see table 2) whereas that of the controls rose from 62 to 107 mg. per 100 ml. Calculation of the standard error of the difference between the two means at 48 hours indicated that it was approximately identical with the actual difference of the means con-

<table>
<thead>
<tr>
<th>Table 2.—The Effect of Intravenous Injection of India Ink upon the Plasma Cholesterol of Rats Given (A) Unchanged and (B) Centrifuged Rabbit Dietary Hypercholesteremic Serum.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma Cholesterol (mg./100 ml.)</strong></td>
</tr>
<tr>
<td><strong>No. of Rats</strong></td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td><strong>A. Injection of Unchanged Rabbit Dietary Hypercholesteremic Serum</strong></td>
</tr>
<tr>
<td>(1) Rats Given India Ink Injection</td>
</tr>
<tr>
<td>16</td>
</tr>
<tr>
<td>Range</td>
</tr>
<tr>
<td>S. E. Mean</td>
</tr>
<tr>
<td>(2) Control Rats</td>
</tr>
<tr>
<td>14</td>
</tr>
<tr>
<td>Range</td>
</tr>
<tr>
<td>S. E. Mean</td>
</tr>
<tr>
<td><strong>B. Injection of Centrifuged Rabbit Dietary Hypercholesteremic Serum (Lower Layer)</strong></td>
</tr>
<tr>
<td>(1) Rats Given India Ink Injection</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>Range</td>
</tr>
<tr>
<td>S. E. Mean</td>
</tr>
<tr>
<td>(2) Control Rats</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>Range</td>
</tr>
<tr>
<td>S. E. Mean</td>
</tr>
</tbody>
</table>

noting that there was actually a good agreement between the two means.

These results suggested that when cholesterol was given in a serum containing a portion of its cholesterol as an insoluble increment of chylomicra,\(^\text{9}\) "blocking" of hepatic reticuloendothelial cells interfered with the egress of such cholesterol from the plasma.

**VI. The Possible Effect of Injection of India Ink upon the Function of the Mast Cells**

Although the preceding experiments strongly suggested that injection of India ink affected hypercholesteremia only in animals supplied with exogenously derived cholesterol by partially preventing the ingestion of the latter by the hepatic reticuloendothelial cells, a possibility existed that perhaps the heparin discharge of the mast cells\(^\text{12}\) was interfered
with. Such interference conceivably could reduce the circulating heparin increment necessary for the “clearing process” by which a turbid, chylomicron-rich plasma is rendered translucent and in the process has its cholesterol solubilized.

In order to determine this possibility, three of six rats were injected twice daily with 0.5 ml. of 20 per cent India ink suspension for 48 hours, at which time all of the rats were sacrificed and sections of various tissues (heart, liver, spleen, kidney, jejunum and skin) were fixed and stained for their mast cell contents both with toluidine blue and thionine blue. The number and condition of the mast cells of the various tissues of the ink-injected rats appeared to be exactly similar to those of the uninjected rats. No carbon particles moreover were discerned either in or about these same cells.

**Discussion**

The above studies when considered with our previous finding that all dietary-derived cholesterol enters the blood as an insoluble increment of a chylomicron appears to lead to one conclusion, namely that the hepatic reticuloendothelial cell plays an important role in ridding the blood of chylomicra and the cholesterol and lipid contained therein. When this apparent mode of chylomicron ingestion is interfered with by the simultaneous ingestion of various other particulate substances by the Kupffer cells, a retention in plasma of chylomicra with a consequent turbidity, hyperlipemia and hypercholesteremia results. However, the latter phenomena will not occur unless precautions are taken to insure that the “blocking” agent, whether colloidal ink, iron or chromium phosphate, is being injected at the very same time that cholesterol is being absorbed.

Cholesterol that is manufactured exclusively by the body itself, being in a soluble form in plasma, apparently “bypasses” the hepatic reticuloendothelial cells to enter directly, perhaps by diffusion, the hepatic parenchymal cells. Accordingly, experimental interference with the Kupffer cell does not retard the egress of such cholesterol from the plasma.

Heretofore some investigators have not been impressed with the various phenomena dealing with dietary-derived cholesterol because, considering the latter identical with the steroid synthesized in the body, they believed it was but a small increment of the total cholesterol available to the animal. However, with the availability of the present data, it is clear that dietary-derived cholesterol not only exists in the blood (for a time at least) in a different physicochemical state than endogenously derived cholesterol, but also to a certain extent is handled physiologically differently from endogenously derived cholesterol. The arterial wall of the animal, moreover, is exposed in one case to a particle, in the other, to a molecule or ion. Its reaction to either, of course, may be totally different. This difference might have pathogenetic significance in respect to atherosclerotic mechanisms.

**Summary**

The hepatic reticuloendothelial system of the rat appears to play an important role in the disposition of dietary-derived cholesterol but apparently is not involved in either the synthesis or disposition of endogenously derived cholesterol. The studies suggest that the difference in the physicochemical state of dietary as compared with endogenously derived cholesterol may account for the participation of the hepatic reticuloendothelial system in the disposition of the former only.

**Sumario Español**

El posible papel del sistema reticulo-endothelial hepático en el síntesis y disposición del colesterol endógeno fué estudiado en la rata por varios medios. Nuestros resultados sugieren que este sistema no sintetiza cantidades significativas de colesterol ni participa en el traspaso del colesterol endógeno del plasma al hígado. Sin embargo, juega un papel posiblemente indispensable en purificar la sangre de colesterol derivado de la dieta.

**References**

1. Friedman, M., Byers, S. O., and Gunning, B.: Observations concerning the production and excretion of cholesterol in mammals. XII.


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