Biosynthesis of cholesterol in rabbit reticulocytes and its exchange with plasma

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ABSTRACT When rabbit reticulocytes were incubated in normal blood plasma containing mevalonic acid-2-^{14}C, radioactivity was incorporated into cholesterol, cholesteryl esters, and squalene in the cells. The squalene reached a steady level of radioactivity much more rapidly than did cholesterol.

Rabbit reticulocytes which were labeled as a result of previous incubation with mevalonic acid-2-^{14}C were incubated with normal autologous blood plasma. The specific activity of the cholesterol in the plasma rapidly became higher than that of the cells. This suggests that there is compartmentation of cholesterol in the reticulocyte and that a pool involved in exchange with plasma cholesterol has a specific activity which is much higher than the average for the whole cell.

SUPPLEMENTARY KEY WORDS squalene · mevalonic acid · cholesterol exchange · cholesteryl ester

It has been rather convincingly demonstrated that the mature mammalian erythrocyte does not carry out the synthesis *de novo* of lipids (1, 2). The reticulocyte can synthesize fatty acids, cholesterol (1, 3), glycerides, and glycerophosphatides (4). It is uncertain whether the cholesterol of the erythrocyte is derived mainly from the synthetic activity of the cell during its earlier developmental stages, or if it obtains a considerable amount from the cholesterol of the blood plasma (5). Blood plasma contains much cholesteryl ester while the red blood cells contain very little. In this laboratory, a recent study of red blood cell preparations containing reticulocytes from infants revealed that mevalonic acid-^{14}C was incorporated into cholesterol (6). The present investigation with rabbit reticulocytes which are more readily available was performed to study more fully the biosynthesis of cholesterol and related compounds from mevalonic acid, and to obtain information on the relation between the cells and blood plasma with respect to these substances.

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MATERIALS AND METHODS

Blood (ca. 40 ml) was obtained by cardiac puncture from normal, untreated and unanesthetized albino rabbits (weighing about 3 kg); heparin was used as anticoagulant, and the plasma was separated by centrifugation. On the following day, reticulocytosis was induced by the daily subcutaneous injection for 4 days of 1 ml of a 2.5% solution of acetylphenylhydrazine in 50% ethanol. Reticulocyte suspensions were prepared under sterile conditions from the blood of these treated rabbits as previously described (2). Examination of stained smears showed that more than 90% of the cells were reticulocytes and that no leukocytes were detectable.

The dibenzylethylenediamine salt of DL-mevalonic acid-2-^{14}C (3.1 mci/mmole) was obtained from the New England Nuclear Corp., Boston Mass. A weighed portion was dissolved in water in a calibrated tube, and dilute sodium hydroxide solution was added carefully until the mixture was faintly alkaline to phenolphthalein indicator. The solution was then extracted four times with an equal volume of ether to remove the organic base. The aqueous solution was evaporated gently with a stream of nitrogen to remove the ether. To this solution was added one-tenth volume of 9.0% sodium chloride solution in order to make the final solution isotonic with blood plasma.

The rate of incorporation of mevalonic acid-2-^{14}C into cellular lipids was measured by the periodic removal of

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2-ml aliquots from the incubation flask which initially contained 8 ml of reticulocyte suspension (hematocrit of 83%), 10 ml of normal rabbit plasma, 1 ml of 5% glucose in 0.9% sodium chloride, 2 drops of heparin solution (1000 units/ml), and 50 μc of mevalonic acid-2-^{14}C. The incubation was performed under sterile conditions in a Dubnoff shaker at 37° with 5% CO_2–95% O_2 as gas phase. The cells in each aliquot were quickly separated and washed three times with cold, buffered (pH 7.4) isotonic solution, and the lipids were then extracted with mixtures of methanol and chloroform as previously described (4). The last aliquot was removed after 3 hr of incubation. The remaining cells were then separated and washed with cold saline. These cells were used in the study of the exchange of cholesterol and other substances between these labeled reticulocytes and unlabeled plasma as described below.

In the exchange study the incubation flask contained 4 ml of the radioactive reticulocyte suspension (hematocrit of 80%), 8 ml of normal autologous rabbit plasma, 0.5 ml of 5% glucose in 0.9% sodium chloride solution, and one drop of heparin solution. The hematocrit of the final mixture was 26%. This mixture was incubated at 37°C under 5% CO_2–95% O_2 in a Dubnoff shaker, and aliquots were removed at intervals. Plasma was separated from each aliquot, and the cells were washed. Lipids were extracted from measured volumes of the plasma samples and of the washed cell suspensions of known hematocrit.

The extracted lipids were fractionated on columns containing 3 g of silicic acid (Bio-Sil BH, 100–200 mesh; Bio-Rad Laboratories, Richmond, Calif.) which were eluted successively with hexane, hexane–ether mixtures, and chloroform–methanol mixtures as previously described (4). Those fractions from the column which contained cholesterol were identified on thin-layer chromatograms that were sprayed with an anisaldehyde reagent (7) which distinguishes sterols from other lipids. Radioautograms of the chromatograms were made on Kodak no-screen medical X-ray film using an exposure time of about 4 wk.

Cholesterol was analyzed by the method of Zlatkis and Boyle (8). Alkaline hydrolysis of lipid fractions was done in a manner similar to that described by Hanahan, Watts, and Pappajohn (9).

RESULTS

In Table 1 are shown results obtained from the fractionation on a silicic acid column of the lipids extracted from reticulocytes which had been incubated with mevalonic acid-2-^{14}C. The fraction eluted with hexane was identified as squalene by thin-layer chromatography on Silica Gel H using two different solvent systems which gave good separations of cholesterol, a cholesteryl ester, and squalene. The first system consisted of toluene–hexane 60:20 for which R_f values are 0.92 for squalene, 0.50 for cholesteryl palmitate, and 0.0 for cholesterol; and the second solvent system was hexane–diethyl ether 99:1 for which the R_f values are 0.63, 0.26, and 0.0, respectively. Radioautographs of the chromatograms showed a single spot with R_f values identical with that of an authentic sample of squalene (Sigma Chemical Co., St. Louis, Mo.). The chromatographic behavior of this fraction was not changed after alkaline hydrolysis. Further confirmation of its identity as squalene was obtained by bromination (10) of the fraction after the addition of carrier squalene. The product had the correct melting point (178°C) for squalene dodecabromide, and its specific radioactivity remained constant after recrystallization from ethyl acetate.

Radioautographs of the thin-layer chromatograms of the fraction eluted with 2% diethyl ether in hexane showed a somewhat streaked spot with a R_f value similar to that of cholesteryl palmitate. After alkaline hydrolysis, the unsaponifiable material yielded a single, well-defined radioactive spot identical in position with that of cholesterol.

The fraction eluted with 8% ether in hexane contained most of the radioactivity and was identified as cholesterol by thin-layer chromatography using three different solvent systems. It produced a single radioactive spot which gave the characteristic color with anisaldehyde reagent. Its chromatographic behavior was unchanged by alkaline hydrolysis, and it was precipitable by digitonin. An ether solution of the material in this fraction and carrier cholesterol was treated with bromine in acetic acid, and the precipitated cholesterol dibromide was recrystallized from methanol. The cholesterol dibromide from the reticulocyte lipids and from the plasma had specific activities which were 81 and 85%, respectively, of those calculated on the basis of all the radioactivity in cholesterol.

The fraction containing cholesteryl esters also contained some triglyceride, and the cholesterol fraction contained diglyceride. Thin-layer chromatograms were developed with hexane–ether–acetic acid 80:20:1, which separates cholesteryl ester (R_f 0.75) from triglyceride (R_f 0.50), and with chloroform–methanol 95:5, which separates cholesterol (R_f 0.55) from diglyceride (R_f 0.65). Radioautograms of the chromatograms showed that no measurable amount of radioactivity was present in triglyceride or diglyceride. About 10% of the total radioactivity was eluted from the silicic acid column with 100% ether. This radioactivity was not present in the monoglyceride which is present in this eluate (4). The remaining radioactivity was eluted from the column with chloroform–methanol mixtures which remove the

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TABLE 1 Incorporation of Mevalonic Acid-2-^{14}C into Lipids of Rabbit Recticulocytes In Vitro after 3 Hr at 37°C*

Eluting Solvent	% of Total Radio-activity	Radioactive Component	Cells	Cells	
			cpm/ml	*mg/ml*	*cpm/mg*
Hexane	3	squalene	16,300	†	—
2% ether in hexane	1	cholesteryl ester	9,200	†	—
8% ether in hexane	70	cholesterol	319,000	1.52	210,000

* Incubation vessel contained 8.0 ml reticulocyte suspension (hematocrit of 83%), 10.0 ml of normal plasma, 1.0 ml of 5% glucose in saline solution, and 50 μc of mevalonic acid-2-^{14}C. The lipids extracted from 1.65 ml of cells were fractionated on a column (12 mm diameter) containing 3 g of silicic acid.

† Estimated to be of the order of magnitude of 0.01 from the size of the spot on thin-layer chromatograms.

phospholipids. However, no appreciable amount of this radioactivity was present in the major phospholipids of the reticulocytes (4).

The rates of incorporation of mevalonic acid-2-^{14}C into cholesterol and squalene are shown in Fig. 1. The results are plotted as cpm/ml of cells. Since we found that the concentration of cholesterol in the cells did not change during the course of the incubation, the curve also shows a plot of the specific activity of the cholesterol. We have no precise measurement of the very low concentration of squalene in the cells, but it appeared from the stained spots of thin-layer chromatograms that there was no marked change during the incubation. Therefore, the curve shown for squalene is also probably a fair estimate of the specific activity of the squalene. As might be expected, the curve indicates that squalene reached a steady level of specific activity long before that of the large pool of cholesterol in the cells.

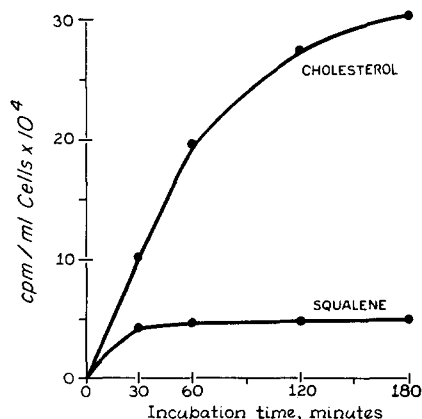

Fig. 1. Rate of incorporation of mevalonic acid-2-^{14}C into cholesterol and squalene of rabbit reticulocytes incubated in vitro at 37°C in blood plasma. Extracted lipids were fractionated by chromatography on silicic acid columns, and the identities of cholesterol and squalene fractions were verified by radioautograms of thin-layer chromatograms.

The results obtained from the experiments in which labeled reticulocytes were incubated with normal autologous blood plasma are shown in Table 2. Only a very slight amount of hemolysis occurred during the course of the incubation, so it is likely that no appreciable transfer of radioactive material from the cells to the plasma was due to a process other than exchange. This is supported by the data of Table 2 which shows that there was no significant difference in the concentrations of cholesterol and cholesteryl esters in cells and plasma at different incubation times. It is noteworthy that the specific activity of the cholesterol of the plasma became rapidly higher than that of the cells. This unexpected result has been confirmed by subsequent independent experiments.

Samples of the cholesterol and cholesteryl ester fractions from both the incorporation and exchange experiments were subjected to alkaline hydrolysis. In all experiments more than 90% of the radioactivity was present in nonsaponifiable materials, and only traces were found in water-soluble materials. This indicates that only small amounts of radioactivity could be present in the fatty acid moieties of cholesteryl esters.

DISCUSSION

London and Schwarz (5) have shown that mature human erythrocytes do not synthesize cholesterol as indicated by the fact that they did not incorporate radioactive acetate into cholesterol. O'Donnell et al. (1) also found that mature rabbit erythrocytes did not incorporate radioactive acetate into cholesterol but that rabbit reticulocytes did. However, they did not attempt to determine whether there was any radioactivity in cholesteryl esters. Since they measured the radioactivity of cholesterol after saponification of the extracted lipids, no distinction could be made between the activity of cholesterol and cholesteryl esters in their experiments.

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TABLE 2 Exchange In Vitro at 37°C of Cholesterol and Cholesteryl Esters between Labeled Rabbit Reticulocytes and Normal Autologous Blood Plasma*

	Incubation Time		
	30 min	60 min	120 min
Cells:			
Cholesterol, mg/ml	1.43	1.46	1.50
Cholesterol, cpm/mg	179,000	177,000	165,000
Cholesteryl esters, cpm/ml	2,000	1,900	1,900
Plasma:			
Cholesterol, mg/ml	0.08	0.10	0.08
Cholesterol, cpm/mg	194,000	355,000	420,000
Cholesteryl esters, mg/ml	0.20	0.22	0.19
Cholesteryl esters, cpm/ml	1,300	1,500	1,600
Cholesteryl esters, cpm/mg	6,500	7,300	8,400

* The incubation vessel contained 4.0 ml of labeled reticulocyte suspension (hematocrit of 80%), 8.0 ml of normal autologous plasma, and 0.5 ml of 5% glucose in saline solution. The lipids extracted from 1.0 ml of cells and from 4.0 ml of plasma were fractionated on columns (12 mm diameter) containing 3 g of silicic acid.

There is disagreement as to the existence of cholesteryl esters in mammalian erythrocytes. Since blood plasma contains much cholesteryl ester, retention of small amounts of the plasma constituents by washed erythrocytes could account for the relatively low concentration of cholesteryl ester previously reported (9). Nelson (11) has carefully examined the neutral lipids of erythrocytes of several mammalian species and found trace amounts of triglyceride and cholesteryl esters. He concluded that it is uncertain that these lipids are true constituents of the erythrocytes. It is possible that the differences in results are related not to contamination of the erythrocytes by plasma but to different contents of reticulocytes and young erythrocytes in the erythrocyte samples which were investigated. Our results show that a small amount of radioactive cholesteryl ester was present in the rabbit reticulocyte after incubation with radioactive mevalonic acid. This suggests that cholesteryl ester is synthesized by the reticulocyte. However, it is possible that radioactive cholesterol was esterified in the plasma (12) and that the esterified cholesterol found its way back into the cell. It is of some interest that there is also disagreement as to the presence of cholesteryl ester in brain, a tissue which also contains much unesterified cholesterol. Wells and Dittmer (13) have reported that a significant amount of cholesteryl ester was present in the brain of the newborn rat but that this disappeared within 6 days after birth.

The existence of squalene in red blood cells has not been previously reported. Our results (Fig. 1) agree with the known precursor relationship of squalene to cholesterol. Since the concentration of squalene in the reticulocytes is much lower than that of cholesterol, it is highly probable that the specific activity of squalene was considerably higher than that of cholesterol at all incubation times.

The formation of squalene in the reticulocyte may be of particular interest because of the low rate of oxidative metabolism in this cell. Popják, Gosselin, Gore, and Gould (14) have reported that liver preparations readily converted mevalonic acid to cholesterol aerobically, but that under anaerobic conditions only squalene was produced from mevalonic acid. The reticulocyte and the mature erythrocyte under aerobic conditions are actively glycolytic and consume very little oxygen. Perhaps in reticulocytes the rate of conversion of squalene to cholesterol is limited by the level of oxidative metabolism. Thus they may be interesting cells in which to study the synthesis of cholesterol from squalene.

Our values for cholesterol content of rabbit reticulocytes (Table 1) calculated as mg/ml of packed cells are somewhat higher than those reported for mature rabbit erythrocytes (11). Since the reticulocyte is a considerably larger cell than the erythrocyte (15), this means that the reticulocyte contains more cholesterol per cell than does the erythrocyte. It seems likely that the organelles in the interior of the reticulocyte contain cholesterol which represents one or more metabolic compartments separate from that of the cell envelope. It is of interest that Kabara and Werthessen (16) found that cholesteryl esters had a much higher specific activity than cholesterol in the brain of the baboon after injection of radioactive acetate. They suggested that this indicates the existence of at least two free sterol pools.

The moderately rapid exchange in vitro of cholesterol between erythrocytes and blood plasma was demonstrated by Hagerman and Gould (17) in their experiments with dog cells and plasma which had been labeled

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in vivo by administration of acetate-^{14}C. A significant result of our exchange study is the finding that the specific activity of the unesterified cholesterol of the plasma rapidly became higher than that of the cells. After 2 hr of incubation, the plasma cholesterol specific activity was 2.5 times that of the cell (Table 2). A possible explanation for this finding is the existence of two or more populations of reticulocytes in the labeled cells. If this were so, one population having a high specific activity in its cholesterol might be exchanging its cholesterol at a higher rate than the other populations. Thus, the specific activity of the cholesterol in these cells might be at all times higher than that of the plasma cholesterol. This might be a likely possibility if the reticulocyte preparations included cells of various stages of maturation and mature erythrocytes. However, our reticulocyte preparations, which had been produced as a result of an acute hemolytic process, contained few mature erythrocytes (less than 10% of the total), and microscopic examination of the cells stained with new methylene blue showed that nearly all the reticulocytes were young and contained large amounts of stained reticulum. Of course, this relative morphological uniformity does not exclude the possibility of the existence of metabolic heterogeneity in these cells. A more likely explanation of our results is the existence of metabolic compartmentation of cholesterol within the reticulocyte. In this case the plasma cholesterol might exchange more rapidly or to a greater extent with a compartment in the cell in which the specific activity of the cholesterol is considerably higher than the average for the whole cell. It is also possible that the reticulocyte releases newly synthesized cholesterol into the plasma by a process other than exchange.

The interpretation of these exchange phenomena is further complicated by the probable existence of metabolic compartments for cholesterol in the plasma. Ashworth and Green (18) found that the exchange of cholesterol between erythrocytes and α-lipoproteins of plasma was different from that with β-lipoproteins. Another indication of the heterogeneity of plasma cholesterol is the marked difference in turnover rate of cholesteryl esters between high and low density lipoproteins (19). It is evident that further investigation is needed to elucidate the behavior of this complex system.

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