In Vitro Cholesterol Esterification in Human Serum

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We report a simple, convenient, and reproducible method, involving the use of radiolabeled cholesterol dispersed in Tween 20 as a tracer and endogenous lipoproteins as a substrate, for measuring the rate of serum cholesterol esterification in vitro. The reaction of lecithin acyltransferase (EC 2.3.1.43) was enhanced by the presence of Tween 20, which probably accelerates the exchange between radiolabeled cholesterol and endogenous lipoprotein cholesterol. In sera from 65 normal subjects, the in vitro cholesterol esterification rate was significantly correlated (r = 0.47, P = 0.001) with age. The mean rate of esterification of 31 subjects 30 years old or younger was significantly lower than that of 34 subjects 31 to 64 years of age. We found no significant difference in the rate of esterification between men and women. The rate of cholesterol esterification (nmol/ml per h) was significantly correlated with the concentration of endogenous free cholesterol in serum, but the fractional rate (the percentage of radiolabeled cholesterol esterified per hour) was inversely proportional to the endogenous free cholesterol. The fatty acid composition of the cholesteryl esters formed by the acyltransferase reaction may provide an index in recognizing some specific disorder.

Additional Keyphrases: lecithin acyltransferase activity normal values age- and sex-related effects lipoproteins

The ability of serum (or plasma) of man to esterify cholesterol was first reported by Sperry (1, 2). Subsequent studies (3, 4) suggested that this reaction was catalyzed by a plasma fatty acid transferase (lecithin acyltransferase, EC 2.3.1.43; LCAT) and that most of the transesterified fatty acids were from the C-2 position of the plasma lecithin. A study of the distribution of transference-like activity of various tissues has shown that the acyltransferase is mainly present in the plasma (5).

When plasma is incubated with radiolabeled cholesterol, the cholesteryl esters of all major lipoprotein classes become labeled (6). The lecithin-rich high-density lipoprotein, however, appears to be the preferred substrate (6, 7). Glomset et al. (8) suggested that the acyltransferase reaction plays an important role in the production of cholesteryl esters in high-density lipoproteins.

In recent studies of the serum (or plasma) acyltransferase reaction the rate of cholesterol esterification has been measured by one of the following three techniques.

In the first method, a small amount of fresh plasma is incubated for 1–6 h at 37 °C with a substrate composed of heat-inactivated plasma and of labeled, unesterified cholesterol added as an albumin-stabilized emulsion (9) or coated onto Celite particles (10).

In the second method, trace amounts of labeled cholesterol are pre-incubated with serum in the presence of disulfide inhibitor (11). The exogenous labeled substrate approaches equilibrium with endogenous lipoprotein cholesterol during the preincubation period while the enzyme activity is inhibited by disulfide. Finally, the enzyme is reactivated by excess of thiol reagent.

In the third method, the concentrations of unesterified cholesterol are measured by gas-liquid chromatography (11, 13, 14).

None of these three methods is ideal for reliable measurement of the acyltransferase activity.

In the first method, the heat-inactivated serum substrate carriers are much less efficient than their native substrates (15). The use of common heated substrates, however, may possibly lead to spurious estimates of enzyme activity, because the rate of serum cholesterol esterification in vivo depends not only on the enzyme activity but also on the amount of circulating lipoproteins (11).

In the second method, the preparation of labeled cholesterol/albumin emulsion is a crucial step (12, 14, 16), requiring specialized techniques not generally used in clinical laboratories.

In the third method, the procedure is laborious and cannot be used when the formation of cholesteryl esters is low (16).

A rapid and simple method for preparing serum lipoproteins labeled with [4-14C]cholesterol, in Tween 20 dispersion, was described by Whereat and Staple (17).
Table 1. Relation between Age and Sex and Concentration of Serum Cholesterol and Rate of Serum Cholesterol Esterification

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Group</th>
<th>Sex</th>
<th>Serum cholesterol concentration (mean ± SD)</th>
<th>Rate of esterification (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total mg/dl</td>
<td>Free mg/dl</td>
</tr>
<tr>
<td>7-20</td>
<td>M + F</td>
<td>11</td>
<td>162 ± 17</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>21-30</td>
<td>M + F</td>
<td>20</td>
<td>189 ± 30</td>
<td>50 ± 8</td>
</tr>
<tr>
<td>31-40</td>
<td>M + F</td>
<td>13</td>
<td>193 ± 21</td>
<td>56 ± 6</td>
</tr>
<tr>
<td>41-50</td>
<td>M + F</td>
<td>11</td>
<td>221 ± 33</td>
<td>57 ± 10</td>
</tr>
<tr>
<td>51-64</td>
<td>M + F</td>
<td>10</td>
<td>226 ± 24</td>
<td>59 ± 8</td>
</tr>
<tr>
<td>7-30</td>
<td>M + F</td>
<td>31</td>
<td>179 ± 29*</td>
<td>47 ± 8*</td>
</tr>
<tr>
<td>31-64</td>
<td>M + F</td>
<td>34</td>
<td>212 ± 31</td>
<td>56 ± 8</td>
</tr>
<tr>
<td>7-30</td>
<td>M</td>
<td>12</td>
<td>172 ± 32</td>
<td>45 ± 9</td>
</tr>
<tr>
<td>31-64</td>
<td>F</td>
<td>19</td>
<td>185 ± 25</td>
<td>49 ± 7</td>
</tr>
<tr>
<td>10-64</td>
<td>M</td>
<td>18</td>
<td>210 ± 27</td>
<td>54 ± 7</td>
</tr>
<tr>
<td>7-62</td>
<td>F</td>
<td>37</td>
<td>199 ± 33</td>
<td>53 ± 9</td>
</tr>
<tr>
<td>7-64</td>
<td>M + F</td>
<td>65</td>
<td>196 ± 34</td>
<td>52 ± 9</td>
</tr>
</tbody>
</table>

*P < 0.001.

Contrary to what we report here, no cholesterol esterification was found to have occurred during the first four hours. Later, Shah et al. (18) and Stefanovich (19) applied this same technique to a study of plasma cholesterol esterification over prolonged periods of time in rats and rabbits, respectively.

The aim of the present study was to find simple and reliable assay procedures to determine the initial rate of serum cholesterol esterification in the presence of Tween 20 dispersion, and to obtain reference values for normal human serum.

Materials and Methods

For comparison with normal, we obtained data on the serum of 65 healthy subjects without manifestations of atherosclerosis, lipidosis, diabetes mellitus, nephrosis, or other disease known to be associated with abnormal serum lipid profiles (Table 1). All the samples were obtained after an overnight fast. The dietary habits of the control subjects were not evaluated or controlled.

In a typical experiment to assess the appropriate conditions for cholesterol esterification, 1 ml of fresh serum was added to a 15-ml centrifuge tube containing about 0.1 µCi of [4-14C]cholesterol (specific activity, 56 kCi/mol; Amersham/Searle Co.; radiochemical purity, as tested by thin-layer chromatography, >98%) previously dissolved in 0.1 ml of ethanol containing Tween 20 (5 ml/liter) (18, 19). For the determination of normative acyltransferase activity of human sera, 0.25 ml of a serum sample was incubated with 25 nCi of [4-14C]cholesterol dispersed in 30 µl of ethanol containing Tween 20, 5 ml/liter. The ethanol was evaporated under nitrogen before the serum was added. Heat-inactivated serum (30 min at 60 °C) or pH 7-8 buffer solution was used as controls. The incubation was done in a Dubnoff Metabolic Shaking Incubator (Precision Scientific Corp., Chicago, Ill. 60647) at 37 °C for 5 h (about 45 strokes/min) or as indicated. The enzyme activity was stopped by adding 10 ml of a chloroform/methanol (2/1 by vol) mixture. The contents were vortex-mixed and allowed to stand at room temperature for 30 min. Then 2 ml of 0.1 mol/liter KCl solution was added to the tube. The two liquid phases were completely mixed before centrifugation. The precipitated protein formed a thin layer at the interface of the two liquid phases. The chloroform (lower) phase was transferred to a 50-ml round-bottom flask by means of a capillary siphon. The upper phase was washed once more with another portion of chloroform solution, and the combined chloroform solutions were dehydrated over Na2SO4. The lipid extracts were then filtered and evaporated in a rotary evaporator. The residue was redissolved in 0.4 ml of chloroform and 0.04 ml of this solution was separated into free cholesterol and esterified cholesterol fractions by thin-layer chromatography using a developing system consisting of hexane/diethyl ether/acetic acid (112/35/2.5 by vol).

The radiolabeled cholesteryl esters formed during the incubation period were isolated by thin-layer chromatography according to the procedure already described. Cholesteryl esters were eluted from silica gel with two 5-ml aliquots of CHCl3 followed by another two aliquots of hot benzene/diethyl ether (1/1 by vol) (20). The procedure used to measure the distribution of radioactivity among the different cholesteryl esters in each sample was essentially the same as described by Morris (21) and as modified by Goodman and Shiratori (20). The saturated, monounsaturated (Δ1), diunsaturated (Δ2) and tetraunsaturated (Δ4) cholesteryl esters were separated by thin-layer chromatography on silica gel G impregnated with AgNO3, with benzene/hexane (1/1 by vol) as the developing system.

The radioactivity of total and separated cholesteryl esters was determined after thin-layer chromatography
by scraping the appropriate portion of the developed thin-layer chromatography plate into counting vials containing 10 ml of scintillation mixture [7 g of 2,5-diphenyloxazole (PPO) and 0.6 g of 1,4-bis[2-(4-methyl-5-phenyl oxazolyl)] benzene (dimethyl-POPOP) in 1 liter of scintillated grade toluene]. To eliminate the quenching, probably caused by floating gel and spray reagent, we stabilized all the vials in the counter at 10 °C overnight before counting. The radioactivity was measured in a liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif. 92634) to an error of 1.5%. Quenching in each sample was calculated by an external standard. The counting efficiency for $^{14}$C is about 92%.

The serum total and free cholesterol concentration was measured by gas–liquid chromatography according to the method of MacGee et al. (22). Cholesterol esterifying activity was expressed as either the percentage of labeled cholesterol esterified per unit time ($R_t$) or the number of nanomoles of cholesterol esterified per milliliter of serum per hour of incubation ($R_i$), with the assumption that there was isotopic equilibrium between added labeled cholesterol and endogenous lipoprotein cholesterol.

The method used to measure fatty acid composition of serum cholesteryl esters by gas–liquid chromatography has previously been reported (23).

Results

To obtain an idea of the optimal conditions for assay of lecithin acyltransferase activity, we evaluated the effect of Tween 20, of pH, of serum sample volume, of incubation time, of specific activity of the isotope, and of storing the serum on chipped ice or frozen, using multiple samples of a single serum.

Figure 1 shows the effect of Tween 20 on incorporation of radiolabeled substrate into cholesteryl esters by human serum. Less than 1% of added [4-14C]cholesterol was esterified by 1 ml of serum sample during a 3-h incubation if [4-14C]cholesterol was not dispersed into Tween 20 before incubation. The percentage esterification of labeled cholesterol in the incubation medium dramatically changed in the presence of as little as 0.1 ml of Tween 20. However, the enzymic activity was inhibited by an excess of Tween 20 (≥ 1 ml) in the incubation medium. The maximum rate of esterification was obtained when [4-14C]cholesterol was dispersed into 0.1–0.15 ml of Tween 20 in ethanol (5 ml/liter) corresponding to a final concentration of 0.5–0.75 µl of Tween 20 per milliliter of serum. We detected no difference in the initial rate of cholesterol esterification on using the various amounts of radiolabeled cholesterol. Therefore, the amount of Tween 20 in the incubation medium was related only to the substrate concentration in the serum sample. The optimal ratio of Tween 20 to serum volume is about 1 to 1500–2000.

The in vitro rate ($R_i$) of esterification of human serum was not linear during the first hour of incubation (Figure 2). However, there was a linear relationship between rate of esterification and incubation time between 1 and 4 h.

Based on our present studies, the best method (method 1, Table 2) for measuring the acyltransferase activity is as follows: First, the serum samples (0.25 ml of serum will suffice) are pre-incubated for 1 h with labeled cholesterol (25 nCi of [4-14C]cholesterol and 30 µl of the ethanolic Tween 20) to establish equilibrium between the labeled and endogenous substrate. The radioactivity of cholesteryl ester is measured and then the increased radioactivity of the cholesteryl esters is measured in the following 1–3 h. Therefore, two incubations and two extractions and separation of lipids or one incubation and two extractions and separations are required for the enzyme assay. To dispense with one extraction and separation and thus to decrease the sample volume required for the assay, we selected a single 5-h incubation period (method 2, Table 2). The rate of serum cholesterol esterification measured by 5-h incubation was very similar to that obtained on using the two-sample incubation method described above (Table 2).

There was no appreciable change in the rate of esterification when the pH of the incubation medium was 6.8 to 8.2. With proper addition of Tween 20 to the in-
Table 2. Rate of Serum Cholesterol Esterification as Measured by Two Different Procedures

<table>
<thead>
<tr>
<th>Sample</th>
<th>Method</th>
<th>Duration of Incubation (h)</th>
<th>Rate of esterification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>I</td>
<td>1</td>
<td>9.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>19.07</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>5</td>
<td>26.15</td>
</tr>
<tr>
<td>2</td>
<td>I</td>
<td>1</td>
<td>9.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>20.09</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>5</td>
<td>27.41</td>
</tr>
<tr>
<td>3</td>
<td>I</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>II</td>
<td>5</td>
<td>37.56</td>
</tr>
</tbody>
</table>

*Method I: Two sample incubation. First the sample was preincubated for 1 h with [4-14C]cholesterol in Tween 20. The radioactivity of cholesteryl esters was measured and then the increased radioactivity of the cholesteryl esters was measured in the following 2 h. Method II: A single 5-h incubation.*

Incubation medium (using the optimal ratio described above), the enzyme activity appears to increase linearly with enzyme concentration contributed by 0.1–2.5 ml of serum (Figure 3).

The effect of varying the specific activity in the incubation medium was assessed with use of a single serum sample (0.25 ml) in the presence of various amounts of radiolabeled substrate. The rate of serum esterification was not affected by a wide range of specific activities (60 to 1200 cpm/µg) in the incubation medium. This result indicates that the sensitivity of the present assay procedure will not be influenced by different concentrations of endogenous free cholesterol in serum samples. The mean value and SD of Rg and Rh in these 20 assays (duplicate with 10 different specific activities) were 6.56 ± 0.14% per hour and 86.43 ± 1.42 nmol/ml per hour, demonstrating good reproducibility of the method.

To test whether the enzyme activity was affected by cooling the serum before incubation, the enzyme activities of serum samples kept at 0–4 °C for as long as 2 h were compared to those of duplicate samples of serum freshly obtained and kept at room temperature. The curves relating per cent cholesterol esterification to incubation time were not affected by these temperature changes.

To test lecithin acyltransferase activity of serum kept at 0–4 °C for various periods of time, four serum samples with different rates of esterification were serially evaluated for acyltransferase activity for as long as eight days. The percentage esterification of [4-14C]cholesterol in these sera remained fairly constant (5.21 ± 0.08, 6.60 ± 0.08, 7.27 ± 0.15, 7.48 ± 0.09% per h, respectively). Similar studies were performed on sera frozen to −70 °C and stored at this temperature for as long as three months. We saw no appreciable change in enzyme activity during this time.

The activity of the serum of 65 control subjects was measured for comparison purposes and to evaluate the effect of age and sex. The in vitro rate (Rg) of cholesterol esterification in normal subjects correlated significantly (r = 0.47, P = <0.001) with their age (Figure 4). Especially, the mean value (and SD) of Rg in 31 subjects age 30 or under were significantly lower (P < 0.001) than that in 34 subjects 31 to 64 years old. However, no significant differences in both Rg and Rh rate of serum cholesterol esterification were found between men and women.

Fig. 3. The relationship of rate of cholesterol esterification to volume of serum

Each sample containing 0.1 µCi of [4-14C]cholesterol and Tween 20 (0.5 µl/ml serum) was incubated at 37 °C for 5 h.

Fig. 4. Relation of age to rate of serum cholesterol esterification in normal subjects
women, although the \( R_s \) for the 37 women (6.08 ± 0.79%) is lower than that of the 28 men (6.31 ± 0.75%).

Figure 5 (A and B) shows the relationship between the rate of esterification and endogenous concentration of free cholesterol in serum. A positive correlation \((r = 0.65, P < 0.001)\) was observed when rate was expressed as \( R_s \). On the contrary, \( R_a \) was inversely proportional to the concentration of free cholesterol in serum \((r = 0.55, P < 0.001)\).

The radiolabeled cholesteryl esters formed on 1.5 min to 6 h of incubation were isolated and the distribution of radioactivity among the different fractions of cholesteryl esters was measured (Table 3). The percentage distribution of radioactivity, as determined by thin-layer chromatography, among the saturated, \( \Delta 1 \), \( \Delta 2 \), and \( \Delta 4 \)-cholesteryl ester fractions remained unchanged throughout the whole range of incubation times tested.

There was also a fairly constant pattern of the percentage distribution of radioactivity among the saturated, \( \Delta 1 \), \( \Delta 2 \), and \( \Delta 4 \)-cholesteryl esters regardless of age of subjects (Table 4). The distribution of radioactivity in sera of persons of different age was almost identical to that found by gas–liquid chromatography.

**Discussion**

The present studies were designed to test whether with the use of labeled cholesterol and dispersion with Tween 20, a sensitive and reliable method could be developed to estimate the rate of serum cholesteryl esterification. The increase in measured cholesterol esterification in the presence and absence of Tween 20 are explainable on the basis of enhanced exchange of \( [4-^{14}C] \)cholesterol with endogenous lipoprotein resulting from use of Tween 20 (17).

The rate of cholesterol esterification as measured by the presently described method was not altered by changing the pH value of the incubation medium from 8.2 to 6.8, a result in keeping with that others (10, 24) found for human serum but by use of different methods. In the rabbit, on the other hand, Stefanovich (19)
showed that plasma cholesterol esterification was optimal at pH 6.8.

As compared to other, more complex methods in which pre-incubation is used, the present one does not provide linear rates of cholesterol esterification for the first hour (Figure 2). During the first hour, in our method, the endogenous lipoprotein cholesterol has not equilibrated completely with added radiolabeled cholesterol. Therefore the calculated specific activity is lower than the true specific activity of the incubation medium and results in a higher rate of esterification than expected. The equilibrium between exogenous [4-14C]cholesterol and endogenous substrates gradually approaches completion with increased time (Figure 2). Whereat and Staple (17), using [4-14C]cholesterol incubated with serum, also showed the exchange of isotope with low-density lipoprotein cholesterol to be very rapid during the first 30 min and complete within 2 h. We found the reaction of cholesterol esterification to be linear for at least 3 h after the first hour of incubation (Figure 2). A similar period of time of linearity was also found on using heat-inactivated plasma as a substrate (10, 25). On the other hand, serum cholesterol esterification was linear for only 1 h or less if methods involving an inhibitor (11, 12) or gas-liquid chromatography (11, 13) were used. The reason(s) for this difference in times of linearity is unclear. However, in the present method the incubation time is adjusted (Table 2) and the data are reproducible; the lack of linearity of cholesterol esterification during the first hour is not critical.

To prevent in vitro cholesterol esterification before assay, we prepared all the serum samples collected for the study of normal values at about 4 °C. The sudden change (1.5 to 2 min) in temperature from 4 to 37 °C did not affect the acyltransferase activity.

The activity measured by the present method (5-h incubation) was not decreased when the serum samples were stored at 0–4 °C for as long as eight days or at −70 °C for as long as three months. Our results agree well with those of Blomhoff (14), but not those of Christian and Cheung (26), who found that plasma had to be quick frozen and stored in liquid nitrogen to keep the activity of this enzyme constant.

In studying the effect of age on the extent of cholesterol esterification in human serum, Gherondache (27) reported that both the absolute and relative amounts of cholesterol esterified during two-day incubation were greater in the 20–30 year age group than in the 40–49 year age group. These results are similar to those reported by Wagner and his associates (28, 29). However, their results are difficult to interpret because the lecithin acyltransferase was measured by a method that depends both on the enzyme activity and the type and amount of lipoprotein substrate present (30). The present values, which depend on the amount of endogenous lipoprotein substrate, show that this transferase activity in human serum, measured by in vitro rate of cholesterol esterification, is influenced significantly by age (Figure 4). The greater cholesterol esterification in older age is probably related to the higher concentration of free cholesterol in serum of older persons.

The mean value and SD of rate (both Ra and Rb) of serum cholesterol esterification for the male group was not significantly different from the female group. This is in accordance with the data of others (27, 31, 32). By a method based on the measurement of plasma unesterified cholesterol by gas-liquid chromatography (13), and enzymic determination (33), the mean rate of cholesterol esterification in their male group was significantly higher than that for the female group. However, they did not differentiate by age in their studies.

By the method in which heat-inactivated plasma, labeled cholesterol, and fresh active plasma are used as an assay system, a direct relationship between the level of cholesterol esterifying activity and the concentration of plasma cholesterol was reported by Monger and Nestel (25). Using different assay methods, Marcel et al. (34), Lack et al. (12), Blomhoff (14), and we (Figure 5A) also found a direct relationship, but others did not (35–38).

The ratio of radioactivity among the saturated, Δ1-, Δ2-, and Δ4-cholesterol esters follows a steady pattern in normal plasma and remains constant on incubation for 1.5 min to 6 h (Table 3). This finding implies that the transferase is highly specific for each individual fatty acid even during the period of inequilibrium between radiolabeled cholesterol and endogenous lipoprotein cholesterol. Portman and Sugano (10) also indicate that the pattern of cholesteryl esters formed during the incubation was related to the specificity of the enzyme rather than the composition of the fatty acid donor.
The fatty acid composition of the cholesteryl esters formed by the lecithin acyltransferase-catalyzed reaction was very similar to that of the cholesteryl esters of fresh serum (Table 4). In case of familial deficiency of this enzyme (9, 39, 40), the distribution of dienoic acid (i.e., 18:2) in serum cholesteryl esters was severely decreased and that of monoenothenoic acids (i.e., 16:1 and 18:1) markedly increased. However, the percentage distribution of fatty acid composition in serum cholesteryl ester of Tangier disease patients who also had reduced activity of this enzyme appeared to be normal (41). Thus, further separation of radiolabeled cholesteryl esters formed by lecithin acyltransferase reaction into different degrees of unsaturated fatty acids may be helpful in recognizing various specific diseases.

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References