Serum Lipoprotein Stability in Atherosclerosis

By Louis Horlick, M.D.

The alpha toxin of Clostridium welchii is a potent lecithinase which disrupts the serum lipoproteins, eventually resulting in complete separation of the lipids from the protein fraction. This reaction is associated with the development of turbidity, the intensity of which is related to the quantitative level of serum lipids. The time of onset of turbidity, however, appears to relate to the stability of the serum lipoprotein bond. Individuals with coronary disease showed early development of turbidity and high final turbidity in 64 per cent as compared with 14 per cent in a control group and 6 per cent in a group of young normal subjects.

There is evidence to suggest that the physicochemical state of the serum lipoproteins may have an important bearing on the genesis and progression of atherosclerosis in man and animals.\(^1\) It has been demonstrated by chemical fractionation techniques that there is a characteristic pattern in the distribution of the lipids among the different protein fractions, and that there are differences in pattern on the basis of sex, age, and the presence of abnormal conditions, such as atherosclerosis, diabetes, nephrosis and hypothyroidism.\(^1\) In atherosclerosis, more of the cholesterol is carried in the beta lipoprotein fraction where the ratio of phospholipid to cholesterol is low.\(^1\) It may be assumed that the beta lipoprotein is more amenable to deposition in the subintimal tissues, or "less stable" than the alpha lipoprotein. Gofman and his associates have demonstrated that several classes of sharply defined lipoproteins (characterized ultracentrifugally) are associated with atherosclerosis in man and animals.\(^2\) Again, these classes of molecules (S\(_{10}\) 10–100) may be considered less stable than those from S\(_{10}\) 0–10.

Another measure of the "stability" of the serum lipoproteins has been the serum cholesterol-phospholipid ratio, that is the ratio of hydrophobic to hydrophilic lipids. Boyd was the first to show that turbidity could be found in sera of normal lipid content where the ratio between the lipids was grossly disturbed in favour of the hydrophobic lipids.\(^3\) The turbidity was due to the formation of aggregates of poorly emulsified fat particles. Ahrens and Kunkel confirmed Boyd's work, and also demonstrated that by the enzymatic destruction of serum lecithin they could render previously clear sera turbid.\(^4\) Numerous observers\(^6\) have since reported that a relatively high cholesterol-phospholipid ratio is found in individuals with atherosclerosis and in experimental animals fed cholesterol.\(^9\) The converse, namely, that a low cholesterol-phospholipid ratio tends to protect against the development of atherosclerosis, has been demonstrated in animal experiments with such diverse substances as Tween,\(^12\) alloxan,\(^13\) cortisone\(^14\) and stilbestrol.\(^15\)

Estimation of the stability or solubility of serum lipoproteins may inferentially provide a method for estimating the susceptibility of individuals to atherosclerosis. Although the serum lipoproteins constitute only one factor in a very complex interaction of serum and tissue factors, yet measurements of lipoprotein stability may serve to detect individuals with disorders of lipid transport and isolate them for further study. The methods employed to date are cumbersome and time consuming and do not lend themselves to mass application. The method which we have developed and

From the Departments of Medicine and Pathology, McGill University, Montreal, Canada.

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which is described below is simple and rapid and may be useful in clinical work.

**Historical Introduction to Method**

In 1939, Nagler observed that when *Clostridium welchii* was grown in human serum, opalescence developed and eventually a layer of fat rose to the surface. This reaction was specific for *Cl. welchii* and was inhibited by *Cl. welchii* antitoxin. Macfarlane and co-workers studied the reaction further and noted that a large number of refractile fat globules could be seen under darkground illumination during the course of development of turbidity. Their chemical studies revealed that the course of the reaction ran parallel with the breakdown of serum lecithin to phosphorylcholine and a diglyceride, and they were able to follow its progress by measuring the amount of water soluble phosphorus set free. They concluded that the alpha toxin of *Cl. welchii* was a highly specific lecithinase, and that the appearance of turbidity must be due to the breakdown of lipid-protein complexes which occurred when the stabilizing effect of lecithin was removed. Further work by Macfarlane demonstrated that sphingomyelin, too, could be hydrolyzed by the *Cl. Welchii* toxin, and that other members of the Clostridia could elaborate toxins which gave the Nagler Reaction.

In 1946, Petermann studied the effect of *Cl. welchii* lecithinase on human serum globulins using electrophoretic and ultracentrifugal technics. She noted complete disappearance of the "X protein" (or massed lipoproteins) from the serum following incubation with lecithinase, and in addition a decrease of 13 per cent in the $\beta_1$ boundary in the electrophoretic diagram. The lipid material separated off carried with it 5 to 10 per cent of the total nitrogen. This confirmed previous studies by Crook who had demonstrated that the material separated consisted of one third protein, and two thirds fat (fat, cholesterol, phosphatides, etc.).

Studies with purified serum protein fractions demonstrated that the enzyme attacked $\beta_1$ globulin lipoproteins found in fraction III-1 of the serum proteins with the production of intense turbidity. Very little turbidity was produced when the enzyme was incubated with fraction IV-1 (albumin, $\alpha_1$, $\alpha_2$ and some $\beta$). This would suggest that the beta globulin lipoproteins are perhaps more susceptible to breakdown by the enzyme and that they contribute the lion's share of the turbidity produced by the enzyme.

Both Nagler and Crook noted that sera varied in their sensitivity to lecithinase (that is, some developed turbidity sooner than others when incubated with lecithinase). Crook also noted that this variation in end points was quite wide.

More recently the Nagler reaction has been restudied by Ahrens and Kunkel, and by Zameunik and associates. The former investigators noted that when serum was incubated with lecithinase, considerable enzymatic destruction of lecithin could be demonstrated prior to the development of measurable increases in optical density of the serum. They also noted that the final turbidity of the lecithinase-treated serum correlated reasonably well with the total lipid content of the serum as determined chemically. Zameunik and colleagues have carefully investigated and described the enzyme kinetics of the interaction between lecithinase and lecithin.

Our interest was aroused by the finding of Ahrens and Kunkel of the correlation between turbidity and total lipid content of the serum. This study was repeated using both normal individuals and individuals with coronary atherosclerosis and their findings were confirmed. During the course of this work it became apparent that turbidity appeared to develop earlier in the sera of individuals with coronary disease than in normal controls. It was decided to investigate this further, and to this end an attempt was made to standardize a lecithinase preparation so that a comparable amount of enzyme would be added to each serum tested. With this standardized toxin, groups of young normal persons, middle-aged and elderly normal persons, and individuals convalescent from coronary disease have been investigated. The results suggest that there is a distinct difference between normals and individuals with atherosclerosis in the response of their serum to incubation with lecithinase, and
we believe that this is a reflection of the degree of stability of their serum lipoproteins.

**Materials and Methods**

**Materials**

1. *Cl. welchii* lecithinase* in fluid and dried state was used. Zamecnik and co-workers* had assayed similar material from the same source and had found it to contain in addition a small amount of α toxin and approximately 2000 mucin clot prevention units of hyaluronidase per milliliter. The dry toxin was prepared by saturation of the toxic filtrate with ammonium sulfate. The toxin was assayed by Led-erle Laboratories using the mouse subcutaneous half lethal dose test, at from 350 to 600 minimal lethal doses (mld.) per milliliter.

Liquid toxin was diluted with borate buffer diluent immediately prior to use. The usual dilution was 100 times. Powdered lecithinase was dissolved in borate buffer diluent to make a 0.025 per cent solution. Fresh solutions were made up at least once a month and were stored in the refrigerator.

2. Lecithin was prepared from egg yolks by ether extraction and acetone precipitation following the technic of Macfarlane and Knight.* A 2.5 per cent solution in distilled water was used. It soon became apparent that there might be differences in the lecithin obtained from different batches of eggs. To obviate this difficulty a large amount of lecithin was prepared by Pangborn’s method,* which yields a purer product, and stored in vacuum-sealed ampules in the refrigerator.

3. Borate buffer was prepared as described by Adams.* It contained: boric acid 12.5 Gm., sodium chloride 8.12 Gm., calcium chloride 1.12 Gm., gelatin 5 Gm. and Merthiolate 0.1 Gm. per liter. Its pH was adjusted to 7.2 which is the optimum for the enzyme. The calcium content serves to accelerate the enzyme reaction, and the gelatin serves to prevent inactivation of the enzyme by shaking and dilution.

**Methods**

(a) Serum Lipid Determinations. All sera studied were subjected to lipid analysis. In all, except the young control group, venous blood was drawn before breakfast, and complete lipid analysis was done. In the young control subjects blood was drawn one to two hours after breakfast, and only serum cholesterol was determined. Cholesterol was determined by Sperry and Webb’s modification of the Schoenheiner-Sperry method; lipids phosphorus by Youngburg’s method as modified by Hawk, Oser and Summers.* and neutral fat by the method of Man and Gildea.*

(b) Enzyme Standardization. Standardization of the enzyme preparations was carried out as described by Macfarlane and Knight.* One ml. of a 2.5 per cent lecithin solution was added to 3 ml. of buffer in thin-walled glass tubes in a water bath at 37 C. To this was added 1 ml. of enzyme solution, and the mixture incubated for 15 minutes. The reaction was stopped by adding 1 ml. of 20 per cent trichloracetic acid. The solution was filtered from the flocculated lecithin within 15 minutes through no. 43 Whatman paper and refiltered until clear. In the blank tubes, the trichloracetic acid was added before placing them in the water bath. Total phosphate was determined on 1 ml. aliquots of filtrate by Youngburg’s method.

Macfarlane has defined an enzyme or lecithinase unit as the amount releasing 0.1 mg. phosphorus from excess lecithin at pH 7.6 and 37 C.

(c) Lecithinase Turbidity Test. In our early work we followed the technique described by Ahrens and Kunkel.* Two-tenths ml. of serum was added to 1 ml. of borate buffer containing 10 mld. of toxin and readings were made at 0, 1, and 2 hours, using the Evelyn microcolorimeter attachment and filter 620. Later on, when we became interested in observing the “lag period” and time at which turbidity first appeared, we employed 0.5 ml. of serum, 0.16 ml. of lecithinase solution (0.5 lecithinase unit) and 5.34 ml. of buffer (total volume 6 ml.). Readings were made at half hour intervals using the Evelyn colorimeter and filter 620. Galvanometer readings were converted to density readings \((L = 2 \log G)\). These were multiplied by 100 to give “turbidity units.” An increase of three units from the first reading was taken to indicate the point of first development of turbidity. In most cases this occurred at the same time that turbidity could first be made out by the eye. Turbidity readings were recorded at 24, 48 and 72 hours. The 72 hour reading was adopted as final turbidity point.

**Clinical Material**

Two groups of normal individuals, one young (mean age 22.1 years) and one older (mean age 50.2 years.), and a group of individuals with coronary disease (mean age 57.7 years) were investigated. The composition of the groups is shown in table 1. Normal individuals were selected on the basis of lack of complaints referable to the cardiovascular system, and a normal physical examination. In some instances they were in hospital for surgical procedures, but most of them were seen in the course of routine annual health examinations in an industrial medical service. Individuals with coronary disease were selected from patients hospitalized with bona fide myocardial infarction as judged by clinical and
electrocardiographic evidence.* Blood was drawn at least three weeks after infarction. Individuals with diabetes or hypertension or with a history of liver disease were excluded. Turbidity tests and serum lipid determinations were carried out on all sera.

**RESULTS**

1. **Enzyme Assays**

Standardization of the lecithinase proved to be a difficult problem because of the impossibility of securing a standard lecithin substrate. Using lecithin prepared from egg yolks by the method of Macfarlane and Knight, it soon became obvious that the results of enzyme assays were variable within fairly wide limits. Pure lecithin is commercially unobtainable. It has been prepared synthetically by Baer in forms in which the constituent fatty acids are known and constant, but we were unable to obtain enough of this product to carry out standardization procedures. Fairly pure lecithin was prepared by Pangborn's method, and was stored in evacuated ampules in the refrigerator. Table 2 demonstrates the results obtained with seven solutions of enzyme, made up between April 2, 1953 and Oct. 14, 1953. The standardization values obtained on Aug. 9, 1953 show a modest variation between samples. The retest on Oct. 26, 1953 (two and one half months later) shows lower values for the solutions dating from April to July, and only slight change in the solution of Aug. 8, 1953. The solutions of September 18 and October 14 are well within the range obtained with the other solutions on August 9th.

2. **Characteristics of the Lecithinase Reaction.**

(A) In figure 1 the time course of the development of turbidity against the amount of water-soluble phosphorus released in the course of the reaction has been charted. It will be noted that there is a lag period varying between two and four hours during which the major amount of hydrolysis of lecithin occurs, but which is characterized by no essential change in turbidity. Immediately following this period there is a very marked increase in turbidity with only slight changes in the amount of water-soluble phosphorus released.

In serum G which is from an individual with coronary atherosclerosis there is an earlier onset of turbidity (that is, a shorter lag period) than in serum F which is from a control, two and one half hours against four and one half hours. In addition, the final turbidity attained is in excess of 80 units for G as compared with about 65 in F.

The higher final turbidity readings in G are taken to indicate the greater amount of unstable lipoprotein present in this serum as compared to F. These values correlate fairly well with the actual serum lipid values, as will be shown below.

(B) Attempts have been made to arrest the reaction at various time intervals so that the reaction products could be examined more closely. *Cl. welchii* antitoxin or potassium

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* From the wards of the Royal Victoria Hospital, and Queen Mary Veterans Hospital, Montreal, Canada.
Discussion

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added to serum before development of turbidity in serum incubated with lecithinase at 37°C. Breakdown of lecithin is followed by determination of water soluble phosphorus liberated. Serum G is from a patient with myocardial infarction, serum F is from a normal individual. Discussion in text.

oxalate will inhibit the Nagler reaction when added to serum before exposure to lecithinase. If serum is exposed to lecithinase for a period which varies from 5 to 20 minutes the Nagler reaction can no longer be inhibited by antitoxin or potassium oxalate. By the addition of potassium oxalate at intervals of 1, 2, 5, 10, 20 minutes of the mixing serum and enzyme, the reaction was arrested after varying degrees of turbidity had developed. The sera were subjected to paper electrophoresis and stains for protein (0.1 per cent bromphenol blue) and lipoprotein (Sudan black) were carried out. Figure 2 shows a representative sample. In the normal control serum sample the lipoproteins are concentrated in the α1 and β bands. Exposure of the lipoproteins to enzyme for one minute followed by addition of oxalate results in striking changes in the protein and lipoprotein patterns. The α1 globulin band disappears from its expected position, and the α2 and β globulin bands show considerable retardation in their migration rate. The albumin and gamma globulin show no change from the control. Immediately ahead of the α2 is an indistinct band which might be α1. There is complete disappearance of the α1 lipoprotein band and a single band is now seen parallel with the α2 globulin band. It trails a diffuse Sudan staining deposit all the way to the line of origin. Exposure of the lipoproteins to enzyme for 24 hours, without addition of oxalate, results in no further changes in the protein pattern, but markedly

FIG. 1. Relationship of breakdown of lecithin to development of turbidity in serum incubated with lecithinase at 37°C. Breakdown of lecithin is followed by determination of water soluble phosphorus liberated. Serum G is from a patient with myocardial infarction, serum F is from a normal individual. Discussion in text.

* I am indebted to Dr. J. H. Quastel of the Montreal General Hospital Research Institute for the idea of using oxalate to retard the lecithinase reaction.

Fig. 2. Paper electrophoresis of serum from a normal young female age 23. Upper paper stained for protein (0.1 per cent bromphenol blue), lower paper stained for lipid (Sudan black). The top pair of papers are the untreated controls. The middle pair represent the serum after exposure to enzyme for 1 minute followed by addition of potassium oxalate and further incubation for 24 hours, and the bottom pair represent serum in which no oxalate was added (i.e. no attempt was made to slow down the reaction). For discussion see text.
modifies the lipoprotein pattern. All the lipid staining material remains at the line of origin (fig. 2).

It is of interest that despite the marked change in the distribution of the lipoproteins in the sample exposed to enzyme for one minute, the serum was quite translucent, whereas longer exposure resulted in grossly turbid sera. It appears, therefore, that the lecithinase enzyme alters the α-β lipoproteins, probably producing new classes of lipoproteins of altered mobility, and releasing a considerable amount of lipid from the protein binding. If the reaction is permitted to go to completion, there is a complete release of all the lipid material from protein linkage, and it remains as a single band at the point of origin, together with a variable quantity of protein.

### 3. Variability of Lecithinase Test Data

(a) **Reproducibility of Results Using Identical Serum and Lecithinase Solutions, and Repeating Tests on Different Days.** Results obtained with samples of serum from three patients are shown in table 3. In all instances there was good agreement between the data obtained when tests were repeated at intervals of from two to eight days.

(b) **Reproducibility of Results Using Identical Serum Samples but Different Lecithinase Preparations.** Results of five sera tested with lecithinase solutions, one of which was three weeks old, and the other two days old, are given in table 4.

There is a moderately good agreement between the two sets of data. There is a variation of a maximum of one hour in onset of turbidity in one serum, one-half hour in three, and no variation in one. Final turbidity readings show only a very narrow range of variation (range 1 to 3.2 units, average 2.3 units).

(c) **Reproducibility of Results on Retest of Individuals at Varying Time Intervals, Using Different Lecithinase Solutions.** Data are available on eight patients, and are shown in table 5. The maximum variation in time of onset

### Table 4.—Variability of Lecithinase Test Results.

#### B. Identical Serum with Different Enzyme Preparations

<table>
<thead>
<tr>
<th>Enzyme Solution 1 (July 16/53)</th>
<th>Enzyme Solution 2 (Aug. 8/53)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of onset of turbidity in hours</td>
<td>Final turbidity units</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Hoo 5</td>
<td>55.7</td>
</tr>
<tr>
<td>Hor 5</td>
<td>60.2</td>
</tr>
<tr>
<td>Co 5</td>
<td>48.8</td>
</tr>
<tr>
<td>St 5</td>
<td>60.4</td>
</tr>
<tr>
<td>La 4</td>
<td>63.8</td>
</tr>
</tbody>
</table>

### Table 5.—Variability of Lecithinase Test Results.

#### C. Same Individuals Retested at Intervals of from 1 to 10 Months

<table>
<thead>
<tr>
<th>Serum</th>
<th>Date</th>
<th>Time of onset of turbidity in hours</th>
<th>Final turbidity units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ho</td>
<td>Jan. 31/53</td>
<td>5.5+</td>
<td>63.8</td>
</tr>
<tr>
<td></td>
<td>Mar. 20/53</td>
<td>6.0</td>
<td>58.5</td>
</tr>
<tr>
<td></td>
<td>Aug. 8/53</td>
<td>5.0</td>
<td>60.2</td>
</tr>
<tr>
<td>La</td>
<td>Jan. 31/53</td>
<td>4.0</td>
<td>68.8</td>
</tr>
<tr>
<td></td>
<td>Aug. 8/53</td>
<td>4.0</td>
<td>63.8</td>
</tr>
<tr>
<td>Gr</td>
<td>Jan. 31/53</td>
<td>3.5</td>
<td>84.6</td>
</tr>
<tr>
<td></td>
<td>June 14/53</td>
<td>3.0</td>
<td>87.0</td>
</tr>
<tr>
<td></td>
<td>July 16/53</td>
<td>4.5</td>
<td>92.1</td>
</tr>
<tr>
<td>Hoo</td>
<td>Mar. 20/53</td>
<td>4.5</td>
<td>56.1</td>
</tr>
<tr>
<td></td>
<td>Aug. 8/53</td>
<td>5.0</td>
<td>55.7</td>
</tr>
<tr>
<td>Well</td>
<td>June 14/53</td>
<td>3.5</td>
<td>67.8</td>
</tr>
<tr>
<td></td>
<td>July 16/53</td>
<td>4.5</td>
<td>71.0</td>
</tr>
<tr>
<td>Lep</td>
<td>May 22/53</td>
<td>8.0+</td>
<td>48.5</td>
</tr>
<tr>
<td></td>
<td>June 13/53</td>
<td>9.5+</td>
<td>48.5</td>
</tr>
<tr>
<td>Dio</td>
<td>Mar. 3/53</td>
<td>4.5</td>
<td>44.4</td>
</tr>
<tr>
<td></td>
<td>Mar. 20/53</td>
<td>4.5</td>
<td>62.9</td>
</tr>
<tr>
<td>Ad</td>
<td>Dec. 20/52</td>
<td>4.0</td>
<td>62.9</td>
</tr>
<tr>
<td></td>
<td>Jan. 31/53</td>
<td>3.5</td>
<td>65.8</td>
</tr>
</tbody>
</table>
of turbidity ranges from 0 to one and one-half hours, with most cases showing a variation of one half hour. It is unfortunate that more data were not available on this crucial point. Two of the cases (Gr and Well) tested "normal" on one occasion and "coronary" on another on the "onset" criterion. There was good agreement in final turbidity readings in all except one case (Di).

Summary. There is little variation in results when the same enzyme solution and serum sample are retested at intervals up to eight days. Using different enzyme preparations the results are a little more variable and this may be due to the fact that standardization of the enzyme is not yet sufficiently accurate. Retests at intervals of months on the same individuals show relatively little change in the final turbidity readings, but a somewhat wider variation in time of onset of turbidity, which may be due to spontaneous changes in the physico-chemical pattern of the serum lipoproteins.

4. Results of Lecithinase Test

(A) Time of onset of turbidity in all sera tested is shown in figure 3. The earliest appearance of turbidity was at less than two hours after the beginning of the reaction, and the latest at 48+ hours. Examination of the data for the coronary and older control groups reveals a distinct difference in the time of onset of turbidity in these groups. If a line is drawn between the 4 and 4.5 hour readings, 36 (72 per cent) of the 50 coronary patients fall on one side of it, and 39 (78 per cent) of the 50 controls on the other. In the younger control group 34 out of 123 cases (21 per cent) demonstrated turbidity before the 4.5 hour mark.

In some instances readings were not made until four hours after the beginning of the reaction. Sera from eight patients with coronary disease showed considerable turbidity at the time of this first reading, and it may be assumed that the onset of turbidity was prior to the four hour reading in all of them. In other instances readings were taken for 4.5, 5, 5.5 and 6 hours only, and in these runs, many sera had not yet become turbid when the final readings were made. It may be assumed that the true onset of turbidity in these cases, numbering four coronary patients, three older controls and 75 younger controls would have been later than shown in the figure 3. Because of these factors it is impossible to calculate the true mean values for the time of onset of turbidity in the three groups studied.

(B) Final Turbidity Readings. In table 6 we have compared the range of turbidity readings, mean values and standard deviations of final turbidity values in the three groups studied. There is a significant difference ($p = 0.05$) between the coronary and the older control group. There is a much greater difference ($p > .001$) between the young control group, and the two other groups.

When a turbidity reading of 60 units is selected (a value which corresponds roughly to about 250 mg. per 100 cc. cholesterol), 72 per cent of the coronary group, 54 per cent of the older control group, and 13 per cent of the young controls exceed this value.

(C) Relationship of Time of Onset of Tur-
bidity to Final Turbidity Readings. In figure 4 the relationship of the final turbidity readings to the time of onset of turbidity (using only the cases where the latter value was precisely established) is shown. Since many cases were not followed beyond 4.5 hours the figure is heavily weighted by those cases in which turbidity occurred early. There is a very wide range of turbidity readings for each time interval, but there appears to be a linear relationship between time of onset of initial turbidity and final turbidity values in the time zone between 3 and 5.5 hours. Beyond 5.5 hours the data suggest that the relationship no longer holds.

**Table 6.—Summary Table of Final Turbidity Values**

<table>
<thead>
<tr>
<th></th>
<th>Coronary Group</th>
<th>Old Controls</th>
<th>Young Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>67.55</td>
<td>60.99</td>
<td>50.23</td>
</tr>
<tr>
<td>S.D.</td>
<td>±13.15</td>
<td>±12.00</td>
<td>±10.28</td>
</tr>
<tr>
<td>S.E.</td>
<td>1.88</td>
<td>1.71</td>
<td>0.90</td>
</tr>
<tr>
<td>(p^*)</td>
<td>0.05</td>
<td>&gt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

* Probability of significant difference from coronary group.

**Table 7.—Correlation of Serum Lipid Fractions with Final Turbidity Reading**

<table>
<thead>
<tr>
<th></th>
<th>Coronary Group</th>
<th>Old Control Group</th>
<th>Young Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(r)</td>
<td>(r)</td>
<td>(r)</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>0.61</td>
<td>0.61</td>
<td>0.58</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>0.36</td>
<td>0.75</td>
<td>0.39</td>
</tr>
<tr>
<td>Ester cholesterol</td>
<td>0.50</td>
<td>0.57</td>
<td>0.41</td>
</tr>
<tr>
<td>Lipid phosphorus</td>
<td>0.75</td>
<td>0.78</td>
<td>0.75</td>
</tr>
<tr>
<td>Fatty acids of neutral fat</td>
<td>0.51</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>C/P molar ratio</td>
<td>-0.05</td>
<td>0.16</td>
<td></td>
</tr>
</tbody>
</table>

(D) Relationship of Final Turbidity to Serum Lipid Fractions. The mean values and statistical analysis of the serum lipid fractions in the three groups studied are shown in table 10. Comparing the coronary and older control groups, it is apparent that there is a statistically significant difference in the free and total cholesterol and lipid phosphorus. All the lipid fractions are significantly lower in the young control group as compared with the other groups.

Correlations between the final turbidity and the individual lipid fractions have been calculated for the three groups and are shown in table 7. Similar values were obtained for “cholesterol” in all three groups (\(r = 0.61, 0.61, 0.58\)). High correlation values were also obtained for lipid phosphorus in the coronary and older control groups (\(r = 0.75\) and 0.78). In the older control group, the free cholesterol showed a higher degree of correlation (0.75) than in any of the other groups.

5. Discriminatory Ability of the Lecithinase Test

In table 8 the data for the three groups studied have been arranged to show the percentage of cases with early development of turbidity and high final turbidity in each group. It is noted that both of these factors coincide in a much greater percentage of cases in the coronary group as compared to the older control group (64 per cent against 14 per cent). Using both criteria, the degree of discrimination is better than that for either one alone.

In table 9 the diagnostic findings in the cases in the younger and older control groups which showed both early onset of turbidity and high final turbidity have been listed. In
TABLE 8.—Summary of Turbidity Test Results in All Cases

<table>
<thead>
<tr>
<th>Group</th>
<th>No. in Group</th>
<th>Time of onset of turbidity &lt; 4.5 hrs.</th>
<th>Final turbidity &gt; 60 units</th>
<th>Both criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary...</td>
<td>50</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Older controls...</td>
<td>50</td>
<td>22</td>
<td>54</td>
<td>14</td>
</tr>
<tr>
<td>Young controls...</td>
<td>131</td>
<td>21</td>
<td>13</td>
<td>6</td>
</tr>
</tbody>
</table>

TABLE 9.—Clinical Features of Cases in Both Control Series Showing High Indexes

<table>
<thead>
<tr>
<th>Name</th>
<th>Sex</th>
<th>Age</th>
<th>Clinical Features</th>
<th>LT</th>
<th>T. O. T.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tre</td>
<td>M</td>
<td>44</td>
<td>Foot deformity, BP 140/100</td>
<td>62.9</td>
<td>4.0</td>
</tr>
<tr>
<td>Ker</td>
<td>M</td>
<td>61</td>
<td>G.L. investig., phycosis, BP 150/100</td>
<td>77.6</td>
<td>3.0</td>
</tr>
<tr>
<td>Kel</td>
<td>F</td>
<td>37</td>
<td>Para I. periodic examination</td>
<td>63.8</td>
<td>&lt;4.0</td>
</tr>
<tr>
<td>Sml</td>
<td>M</td>
<td>47</td>
<td>Periodic examination</td>
<td>68.8</td>
<td>4.0</td>
</tr>
<tr>
<td>Dav</td>
<td>M</td>
<td>43</td>
<td>Periodic examination</td>
<td>87.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Hay</td>
<td>F</td>
<td>39</td>
<td>Periodic examination</td>
<td>66.3</td>
<td>3.5</td>
</tr>
<tr>
<td>Cha</td>
<td>M</td>
<td>69</td>
<td>Hemorrhoids</td>
<td>85.4</td>
<td>4.0</td>
</tr>
<tr>
<td>Ros</td>
<td>M</td>
<td>22</td>
<td>Postop. appendicitis</td>
<td>61.1</td>
<td>4.0</td>
</tr>
<tr>
<td>Eag</td>
<td>M</td>
<td>33</td>
<td>Periodic examination</td>
<td>85.4</td>
<td>4.0</td>
</tr>
<tr>
<td>Len</td>
<td>F</td>
<td>19</td>
<td>Obesity</td>
<td>60.2</td>
<td>4.0</td>
</tr>
<tr>
<td>Von</td>
<td>M</td>
<td>26</td>
<td>Obesity (+22 lbs)</td>
<td>67.3</td>
<td>3.5</td>
</tr>
<tr>
<td>Ben</td>
<td>M</td>
<td>20</td>
<td>Obesity (+5 lbs)</td>
<td>68.3</td>
<td>3.5</td>
</tr>
<tr>
<td>Duf</td>
<td>F</td>
<td>22</td>
<td>Normal—pre-employment</td>
<td>65.8</td>
<td>3.5</td>
</tr>
<tr>
<td>Bed</td>
<td>M</td>
<td>26</td>
<td>Obesity (+10 lbs)</td>
<td>75.7</td>
<td>4.0</td>
</tr>
<tr>
<td>Buj</td>
<td>M</td>
<td>32</td>
<td>Avitaminosis, ex P.O.W.</td>
<td>72.0</td>
<td>3.5</td>
</tr>
</tbody>
</table>

The older control group, two of the cases had borderline diastolic pressures (100 mm. Hg), and of the remaining five, four were clinically normal, and one was hospitalized for hemorrhoidectomy. In the young control group, only two individuals were unequivocally normal, four suffered from varying degrees of obesity, one had been a prisoner of war and had suffered from avitaminosis, and one was recovering from appendicitis. In the normal series there were 16 individuals who were classified as obese. Four fulfilled both criteria of the test, three showed high final turbidity only and one early onset of turbidity only. Thus the obese young people gave more positive tests than did those of normal weight, on a relative basis. Whereas the young normal series was evenly divided on the basis of sex, six out of the eight cases which fulfilled both criteria were males. Failure of 36 per cent of the coronary patients to give a positive test may be due to the following:

1. In a very small proportion of cases of myocardial infarction etiologic factors other than atherosclerosis are responsible.

2. Lipid levels in individuals with coronary disease are known to be much more variable than in normals. The episodic character of the serum lipoprotein changes may explain the failure of positive tests to be obtained in some cases, and the variability of some of the cases on retest.

3. The etiology of atherosclerosis is as yet far from certain. We must admit the possibility of etiologic factors other than lipid instability in atherosclerosis, in the present state of our knowledge.

DISCUSSION

The lecithinase of Cl. welchii toxin alters the lipoproteins of serum, breaking up the complex molecules of lipid and protein, and releasing the lipid. In the course of this reaction new classes of lipoproteins appear which differ in mobility from the pre-existing ones. The work of Petermann suggested that the β globulin lipoproteins were selectively attacked by lecithinase. The point of attack is most likely the lecithin in this complex molecule, and the breakdown of the lecithin may loosen the bonds holding the complex molecule together, resulting in changes in molecular shape and volume. Such changes, together with the coalescence of free lipid droplets may be responsible for the resultant turbidity. At any rate, the appearance of turbidity may be taken as an indication of profound changes in the physicochemical structure of the serum lipoproteins. It is contended that the early occurrence of such changes in some sera after exposure to lecithinase, is an index to the fact that the lipoproteins are unstable in vivo. Where the lipoproteins are more stable, longer
periods of exposure to the enzyme are required to break up the lipoprotein complex. In figure 1 it has been shown that this is not related to the rate of breakdown of the lecithin (as measured by release of phosphorus). Despite relatively similar rates of hydrolysis of lecithin, one serum became turbid in half of the time required for the second. There must therefore be other forces at work, which resist the disruptive tendencies of the lecithinase. It is also possible that only certain classes of lipoproteins can be attacked by the enzyme, that is, there may be some molecules where the lecithin is exposed by virtue of the internal structure of the molecule. The latter possibility suggests that the early appearance of turbidity may be related to the presence of unusually large amounts of such lipoprotein classes.

The fact that the early development of turbidity occurred in 72 per cent of a group with coronary arteriosclerosis as compared with 22 per cent and 21 per cent of the older and younger control groups respectively would suggest that in the coronary group, the lipoproteins are most amenable to disruption by lecithinase and possibly less stable than in the control groups. It is of interest that there was no real difference in the percentage of individuals with early onset of turbidity between the older and younger control groups despite a considerable difference in mean age of the two groups. It is well known that the atherogenic process begins early in life and progresses for many years. It is not surprising, therefore, that a relatively high percentage of young people show evidence of “unstable lipoproteins.”

Early development of turbidity appears to be related in part to the degree of final turbidity which will develop in the serum after 72 hours. Since final turbidity correlates well with the levels of the serum lipids determined chemically, early turbidity is sometimes an index to grossly disturbed serum lipid levels. This is not always true, and in the present series there are many instances where turbidity developed early in the presence of normal serum lipid levels. The relationship may merely be due to the fact that the lipids are being carried in certain lipoprotein classes which are especially amenable to attack by the enzyme.

The relationship between final turbidity and the serum lipid fractions have been shown in table 7. The only conclusions that can be drawn from these data are that the final turbidity correlates in a general way with serum lipid levels, and can be used as an index in gross disturbances of serum lipid levels. Our data on the serum lipid fractions are in general agreement with those published by other investigators.6, 7, 8 The difference in the total cholesterol values between the coronary and control groups is largely accounted for by the free cholesterol. This is not in agreement with most workers who have found the difference mainly in the ester fraction.6, 7, 8 We were unable to discern any difference in the cholesterol-phospholipid molar ratio between the two groups, and between them and the young control group.

The two criteria discussed above, (early onset of turbidity and high final turbidity) have been applied to a study of normal individuals and individuals with coronary disease. The author is fully aware of the fallacy of considering any group of older individuals as “normal” with respect to atherogenesis, and for that reason it was decided to study both young and old “normal” individuals. As was noted above, both normal groups showed the same incidence of early development of turbidity, but there was a marked difference in mean final turbidity levels. This latter finding is probably a reflection of higher lipid levels in the older age groups. Simultaneous presence of both early turbidity and high final turbidity in the same serum was encountered in 64 per cent of the coronary group, 14 per cent of the older control group and 6 per cent of the young controls. It is apparent, therefore, that while either of the indices used permits some degree of discrimination between normal individuals and those with coronary atherosclerosis, the simultaneous presence of both indices is highly in favor of the presence of coronary atherosclerosis.

Eight out of 131 normal young individuals (equally divided as to sex) showed both early development of turbidity and high final turbidity. Six were males and four suffered from obesity. Thus both maleness and obesity were
Lipid Ester which Fatty Total C/P disproportionately Free protein and cases. ing thinase, more understandard the factors bidity after lecithin, appear to occurred to depend on other factors which govern lipoprotein stability. In 50 individuals with coronary disease, turbidity occurred early (less than 4.5 hours) in 72 per cent, while a similar phenomenon was seen in only 22 per cent of a control group of normal subjects of similar age, and in 21 per cent of a group of young normal individuals.

3. The degree of final turbidity correlates moderately well with the serum lipids as measured quantitatively. Final turbidity was higher in the coronary group (mean, 67.55) than in the older control (mean, 60.99) and young control groups (mean, 50.23).

Final turbidity estimation may be used as a screening technique for detecting individuals with abnormal serum lipid levels.

4. Early onset of turbidity, and high final turbidity reading were present simultaneously in 64 per cent of the coronary cases, 14 per cent of the older controls and 6 per cent of the young controls. The application of these criteria permits separation of individuals with coronary atherosclerosis from a control population.

5. The lecithinase test technic has been described, and the variability introduced by the enzyme and serum factors investigated. The test is not affected by storage of serum for periods of 7 to 10 days. Solutions of enzyme do not lose their potency for at least one month. Relatively stable values are obtained when individuals are retested for periods varying up to six months.

**ACKNOWLEDGMENTS**

I wish to acknowledge the support and advice generously given to me by Dr. K. A. Evelyn, of the Department of Biophysics, and Dr. G. L. Duff, of the Department of Pathology, McGill University.

**SUMARIO ESPAÑOL**

1. La toxina alpha de *C. welchii*, una potente lecithinasa, desorganiza las lipoproteínas del suero, liberando los lípidos de su enlace con las proteínas y causando turbidez intensa en el suero. Hay evidencia que sugiere que la enzima puede mas específicamente atacar las lipoproteínas menos estables.

2. El tiempo tomado para la turbidez aparecer, luego de haberse incubado la enzima y el
suero bajo condiciones uniformes, varió grandemente en el suero de diferentes personas. En este estudio se demuestra que este fenómeno no aparece depender en la velocidad de la hidrolisis de la lecitina, pero es aparentemente dependiente en otros factores que gobiernan la estabilidad de la lipoproteína. En 50 individuos con enfermedad coronaria, la turbidez apareció bastante temprano (menos de 4.5 horas) en 72 por ciento, mientras que un fenómeno similar se vio en 22 por ciento de un grupo control de sujetos normales de similar edad y en 21 por ciento de un grupo normal de individuos jóvenes.

3. El grado de turbidez final correlaciona moderadamente bien con los lípidos del suero cuando se miden cuantitativamente. La turbidez final fue más alta en el grupo coronario (promedio 67,35) que en el grupo de controles viejos (promedio 66,99) y que en el grupo de controles jóvenes (promedio 50,23). La estimación de la turbidez final puede ser usada como una técnica encubridora para descubrir individuos con niveles anormales de lípidos del suero.

4. Aparición temprana de turbidez y turbidez alta final estuvieron presentes simultáneamente en 64 por ciento de los casos coronarios, 14 por ciento de los controles viejos y 6 por ciento de los controles jóvenes. La aplicación de estos criterios permite la separación de individuos con aterosclerosis coronaria de la prolaación control.

5. La técnica de la prueba de lecitinasasa se ha descrito y la variabilidad introducida por la enzima y los factores del suero se ha investigado. La prueba no es afectada al almacenar el suero por 7 o 10 días. Soluciones de la enzima no pierden su potencia por lo menos por un mes. Valores relativamente estables se obtienen cuando los individuos son probados una vez más por períodos variando hasta seis meses.

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Serum Lipoprotein Stability in Atherosclerosis

LOUIS HORLICK

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