THE CONSTRUCTION AND CLINICAL SIGNIFICANCE OF THE COAGULOHEPATOGRAM*

BY

W. RIEBEN

From the Laboratory of the Division of Surgery, University of Lausanne, Switzerland

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The term coagulohepatogram is proposed for curves obtained with a simple two-stage prothrombin method by successive determinations of thrombin formed from prothrombin during a 15-minute period from the start of activation with thromboplastin-calcium. The three liver specific factors which comprise the coagulohepatogram are (1) accelerator, (2) prothrombin, and (3) the natural plasma antithrombin, and these can be simultaneously measured by using three dilutions of plasma 1:25, 1:50, and 1:75.

Technique for the Determination of Plasma Prothrombin by Two-stage Method

For the determination of plasma prothrombin levels, two main types of methods have been chiefly employed. Quick (1938, 1945) introduced the first satisfactory one-stage procedure. Within a short time after its original publication Warner, Brinkhous, and Smith (1936) described a more complicated procedure in which the whole clotting process is separated into two phases.

In recent years many observations have been made that indicate that the two types of methods do not necessarily measure one and the same plasma components. Such discrepancies are found in liver diseases (Ziffren, Owen, Warner, and Peterson, 1942), pernicious anaemia (Warner and Owen, 1942), in the behaviour of plasma of newborns and infants (Brinkhous, Smith, and Warner, 1937), and of banked blood and lyophilize plasma. Differences in appraising the effect of methylxanthines have also been described (Rieben, 1946a).

The original two-stage method of Warner et al. (1936) is based on serial dilutions of plasma before transformation of the prothrombin into thrombin by means of thromboplastin and calcium. Greater accuracy has been claimed for it than for the one-stage procedures, but it is too elaborate for clinical use.

The present contribution concerns an adaptation of the two-stage method of Herbert (1940) which is capable of great accuracy and which has proved to be extremely satisfactory for experimental purposes and routine use on a large scale. As many as 25 plasmas can be analysed by one person in a day. The procedure is based on readily available preparations and is much simpler than the Herbert technique. As a rule only one plasma dilution is tested, except for coagulohepatograms, where three different dilutions are recommended.

Variations in the concentration of accelerator factors, termed "accelerator" in this paper, seriously affect the values obtained with one-stage procedures.

This technique was devised in the Department of Medicine, New York Hospital, and Cornell University Medical College, New York.

Reagents.—The following are needed.

Thromboplastin.—Two solutions are prepared: solution 1, 0.9% NaCl; solution 2, 0.25 g. anhydrous calcium chloride and 4.28 g. of NaCl are dissolved in distilled water and made up to 500 ml. To 150 mg. of dried rabbit brain ("bacto" thromboplastin†) in a 50 ml. conical centrifuge tube are gradually added 15 ml. of saline with continuous grinding in order to obtain a smooth emulsion. The tube is then transferred to a beaker containing water at 54° C., and the contents are stirred for at least 10 minutes with a glass rod to destroy prothrombin and antithrombin. After cooling to room temperature 15 ml. of the calcium-containing saline (solution 2) are added. After continuous stirring for at least five minutes, the contents of the tube are centrifuged for five minutes at 2,000 r.p.m. The supernatant fluid is pipetted off. It can be diluted up to one half of its quantity with a solution made up from identical amounts of solution 1 and solution 2.


† Manufactured by Difco Laboratories, Detroit, Mich., U.S.A.
Fibrinogen.—It was found advantageous to use a stable dried fraction of beef plasma commercially obtainable from the Armour Laboratories, Chicago, Illinois. To obtain 50 ml of a buffered fibrinogen solution, 0.5 mg of fibrinogen is weighed out and transferred through a wide-mouth funnel to a seamless cellulose dialysis tube of 30 mm. diameter. Then 50 ml of buffered oxalated saline are pipetted into the sac and the contents are dialysed for one hour against 2 litres of the same buffered oxalated saline in a 2-litre graduated cylinder. The fibrinogen sack is then transferred to a similar cylinder containing buffered saline only. Dialysis is continued for another hour. The buffered fibrinogen is then placed in 100 x 13 mm. pyrex tubes and kept frozen in the freezing compartment of a refrigerator. Before use the amount needed for each series is thawed out at 4-7°C. Preparations stored in the frozen state keep for at least two or three weeks. They are standardized simultaneously against one or more plasma of normal prothrombin activity, or against a thrombin standard as indicated later.

Veronal Buffer Solution.—Instead of imidazol, M/10 sodium veronal-N/10 HCl buffer is recommended. The buffer mixture described here tends to crystallize when stored for prolonged periods of time. For this reason it has to be made up at frequent intervals from stock solutions of M/10 sodium-diethyl-barbiturate and N/10 HCl. In order to obtain 100 ml of this buffer giving a pH of 7.6, 61.5 ml of M/10 sodium veronal and 38.5 ml of N/10 HCl are mixed.

Buffered Oxalated Saline.—To 40 ml of pH 7.6 veronal buffer are added 40 ml of 1.85% potassium oxalate and the mixture diluted to 2 litres with 0.9% saline.

Buffered Saline.—To 40 ml of pH 7.6 veronal buffer isotonic saline is added to make a final volume of 2 litres.

Veronal Buffer for Daily Determinations.—By adding 3.85 ml of N/10 HCl to 6.15 ml of M/10 sodium veronal a buffer of pH 7.6 is made which is used in preparing the activation mixture with equal amounts of (1:50) plasma dilutions with saline and double the amount of thromboplastin calcium mixture.

Procedure.—This is as follows:

Blood Collection.—Into a sterilized and dry 2 ml syringe are drawn up 0.2 ml of sterilized 1.85% potassium oxalate (M/10). From the cubital vein enough blood is now withdrawn to bring it up to the 2 ml level in the syringe. After mixing, the needle is removed and the contents gently transferred to a 15 ml. pyrex centrifuge tube which is then centrifuged for seven or eight minutes at 2,500 r.p.m. The plasma is separated and at once transferred to a 100 x 13 mm. pyrex tube, and 0.1 ml portions diluted with 5.0 ml portion of saline (for coagulohematogram dilute also with 2.5 and 7.5 ml saline respectively). The diluted plasma is stored at 4-7°C. It should be analysed within two to five hours.

Conversion of Prothrombin into Thrombin.—During the first stage of the determination the dilute plasma is converted to yield thrombin by addition of thromboplastin and calcium. Of the freshly prepared pH 7.6 veronal buffer, 0.5 ml is added to 0.5 ml of the 1:50 plasma dilution and 1 ml of thromboplastin-calcium mixture then added. Using 1:50 dilutions, prothrombin times between 24 and 40 seconds were obtained for normal plasmas. For plasmas of very low prothrombin activity 1:25 dilutions can be used both for unknown and control plasma.

Determination of Thrombin Activity.—In the second stage fibrinogen is added to an aliquot of the activation mixture and the activity of the thrombin formed is determined by measuring the time required to form a fibrin clot. At room temperature a point close to maximum thrombin activity is normally reached in two to five minutes. During incubation the tube is taken out after the first two minutes and the open end held close to an indirect light source (to recognize the Tyndall phenomenon). The contents are gently shaken at half-minute intervals by swinging the lower end of the tube in a circle until a fine fibrin web appears, indicating a point close to maximum thrombin activity. At this moment 0.2 ml of the mixture is pipetted into a preheated tube (37°C.). It is then left at 37°C. for 30 to 40 seconds and 0.1 ml of fibrinogen solution is added from a 0.2 ml pipette. This is best performed by resting the tube on the button of a stopwatch holder. The very instant the fibrinogen is blown out, the stopwatch is started by simultaneously exerting downward pressure with the thumb.

After a five-second interval used for mixing the tube is again immersed in the 37°C. constant temperature water bath and the clotting time is accurately determined. This can be done by placing it on the horizontal glass portion of a Fisher Kahn viewer* under the magnifying lens.

After determining the clotting time for the first sample, two additional 0.2 ml aliquots are tested in a similar fashion. The average of the three readings is used for the calculation of the clotting time.

Calculation.—The prothrombin activity of an unknown is obtained by dividing the average prothrombin time of a series of normal plasmas by the prothrombin time of the unknown and multiplying by 100. For example, 1:50 plasma dilutions of five different normal persons yielded prothrombin times of 31.3, 27.7, 34.3, 35.7, and 39.2 seconds respectively, the average being 33.6 seconds. The prothrombin time of a 1:50 dilution of an unknown was found to be 47.8 seconds. Its prothrombin activity is therefore 70.3%.

Error of the Method.—Mean and standard deviation, \( \sqrt{\Sigma (N-I)} \), were determined independently by two observers on 0.1 ml plasma aliquots of 47 different plasmas. The mean errors at various levels were all related to a prothrombin activity of 100%. A mean deviation of ±1.55% and a standard deviation of ±1.88% were found in this large series.

Technique for Obtaining Coagulohepatograms

This has been devised for the simultaneous determination of accelerator and of prothrombin and antithrombin activity of blood plasma.

For the determination of coagulohepatograms a temperature of 37° C. is chosen. For the curves for the determination of accelerator activity a plasma dilution of 1: 75 is used. In this instance clotting times are determined every minute. The accurate determination of the interval from the start of activation to the moment of maximum activity is decisive, and for it I propose the term "accelerator time." Accelerator time is recorded in minutes and fractions of minutes and is compared with that of plasma of known accelerator activity.

For the determination of prothrombin activity the use of 1: 50 plasma dilutions is recommended and the procedure described above followed with one difference. Instead of carrying out the activation at room temperature, the tests are carried out at 37° C.

For the determination of antithrombin activity a plasma dilution of 1: 25 is chosen. Working at 37° C. a strong antithrombin effect can be observed and the thrombin units destroyed in 15 minutes can be read from the 1: 25 curve (Fig. 1).

It has been shown in a previous paper (Rieben, 1950) that for the accurate determination of antithrombin activity in low prothrombin plasmas, 1.5 units of thrombin in veronal-HCl-buffer of pH 7.6 is necessary in order to obtain a sufficiently long descending limb on the activation curve for determination of the antithrombin effect.

For the determination of factor V methods have been described by Owren (1947), who used different dilutions of a standard plasma for comparison, and showed that "the velocity of thrombin formation rises with increasing concentration of factor V up to a certain limit. Further increase of factor V beyond this limit is without influence. No thrombin formation takes place without factor V."

Using 1: 75 dilutions of plasma, factor V present is sufficiently diluted to avoid falling into the range where further increase of accelerator does not any longer shorten the period of maximum activation.

The method of Ware and Seegers (1948) for the determination of Ac-globulin is based on a standardization procedure with known amounts of purified Ac-globulin. The authors admit that this method, though excellent theoretically, is very cumbersome, and is hardly applicable to clinical use. There is no reason why results obtained by either of the two procedures mentioned should not be useful when incorporated into coagulohepatograms.

The new method for accelerator activity determinations is the simplest clinical method known so far. It has the great advantage of making use of the same technique, the same reagents, and the same apparatus as the above described two-stage prothrombin method used by the author since 1945.

Reagents.—For coagulohepatograms the use of a thrombin standard is recommended. It permits quantitative appraisal of antithrombin activity and comparison of results obtained in different laboratories.

Fibrinogen.—While fibrinogen powder is readily available in Great Britain and the United States, it has only fairly recently become available on the Continent. When fresh it does not need dialysis and is ready for use by the addition of distilled water.

Thromboplastin.—"Bacto" thromboplastin has been used throughout. For the determination of accelerator activity variations of thromboplastin are of great importance. With a given prothrombin and accelerator activity the time used to obtain maximum thrombin depends on it. For this reason analysis of a plasma of known accelerator activity is simultaneously carried out with each series.

Thrombin.—Standardized crystallized thrombin (Hoffmann-La Roche, Bâle) was used throughout these experiments. The commercial preparation is available in 100 mg. amounts containing 60 international thrombin units (t.u.) per mg.

In order to standardize a daily series of coagulohepatograms, 50 mg. of crystallized thrombin are accurately weighed out and placed in a 50 ml. Erlenmeyer, and
immediately before use 18.75 ml. of chilled veronal HCl buffer are added. Of this, 1.0 ml. is rapidly transferred to a 15 ml. centrifuge tube containing 4 ml. of veronal-HCl-buffer. Of this final solution, 0.5 ml., representing 1.60 t.u., is pipetted into a 100 × 13 mm. pyrex tube kept in the constant temperature water bath at 37° C. This tube is then treated as those containing plasma dilution for prothrombin determinations.

Contrary to common belief, a diluted thrombin solution is sufficiently stable for 15 minutes in the presence of thromboplastin-calcium to make it an ideal standard for coagulohepatograms. The amount of thrombin per tube (1.60 t.u.) closely corresponds to a 1:50 dilution of plasma of 100% prothrombin activity (Table I).

**Table I**

<table>
<thead>
<tr>
<th>Factor and Plasma Dilution</th>
<th>Maximum Thrombin Activity (Average)</th>
<th>Time Required for Maximum Activity* (min.)</th>
<th>Range of Activity (% of Average Normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:25 plasma: Antithrombin</td>
<td>1.0 t.u.</td>
<td>0.975</td>
<td>1:5:1.0–2.0:65:130</td>
</tr>
<tr>
<td>1:50 plasma: Prothrombin...</td>
<td>1.60</td>
<td>3.0</td>
<td>2:5–3:5:70:130</td>
</tr>
<tr>
<td>1:75 plasma: Accelerator</td>
<td>1.10</td>
<td>5</td>
<td>4:6:60–150</td>
</tr>
</tbody>
</table>

* Depends on activity of thromboplastin preparations, which vary from one laboratory to another.

**Procedure.**—This is as follows.

**Blood Samples.**—Due to the instability of accelerator, plasma separated immediately after venesection is kept at a temperature close to freezing. Instead of potassium oxalate suitable for prothrombin determination the use of 3.2% trisacibic sodium citrate is recommended for coagulohepatograms.

**Technique for Coagulohepatograms.**—Plasma, 0.1 ml., is measured into three 10 or 15 ml. centrifuge tubes. By adding 2.5 ml., 5.0 ml., and 7.5 ml. of 0.9% saline, dilutions of 1:25, 1:50, and 1:75 are obtained. After mixing 0.5 ml. of each plasma dilution is placed in 100 × 13 mm. pyrex tubes in the 37° C. constant temperature water bath. After one minute 1.0 ml. of thromboplastin-calcium kept at 37° C. is added from a 1 ml. Mohr pipette. A first stopwatch is simultaneously started to measure activation time. At intervals of one to two minutes clotting times are determined on 0.2 ml. plasma aliquots by blowing 0.1 ml. of fibrinogen solution into the tubes and simultaneously starting a second stopwatch kept in a stopwatch holder. The procedure closely resembles that of the two-stage method for prothrombin determination described above. Instead of awaiting the appearance of a fibrin web, serial determinations are carried out during a 15-minute period to yield values for the whole curve.

Once the curve of the 1:50 plasma dilution is plotted, the prothrombin activity of the unknown plasma is read from its maximum.

In order to determine accelerator activity, the whole procedure is repeated with 1:75 plasma in a similar fashion. It is important to note the one minute values. The curve obtained by plotting thrombin units corresponding to each clotting time against activation time reaches a point where a maximum amount of prothrombin is converted to thrombin. The interval from the start of activation to maximum thrombin activity serves to calculate accelerator activity.

In order to determine antithrombin activity 1:25 dilutions of plasma are used. On account of the rapid transformation of prothrombin to thrombin in 1:25 plasma, the maximum of the curve is very rapidly reached (cf. Fig. 1). Special care has to be taken that the first one minute value as well as the 15-minute value are accurately measured. At one minute intervals further aliquots of 0.2 ml. are determined until it is clear where the maximum of the curve is situated. The clotting times are compared with a thrombin standard as described above. The corresponding thrombin activity of each sample is plotted and the 1:25 curve thus obtained (cf. Fig. 1).

**Calculations for Compiling Coagulohepatograms**

**Accelerator Activity.**—The accelerator activity of an unknown plasma is obtained by dividing the average time used to obtain maximum thrombin activity of a normal plasma in 1:75 dilution by the time used by the unknown plasma under similar conditions, and multiplying by 100. Under the conditions used in my two-stage method, but working at 37° C., maximum activity is reached between four and six minutes in 1:75 dilution of normal plasma using Difco thromboplastin.

**Prothrombin Activity.**—The prothrombin activity of the coagulohepatogram is calculated by comparison with a standard thrombin solution containing 1.60 t.u. per tube. This value corresponds closely to a plasma of 100% prothrombin activity in 1:50 dilution. It is also possible, as I have shown in a previous publication, to use lyophile plasma or normal plasma obtained the same day for comparison. This has the disadvantage of variations corresponding to the normal range of prothrombin activities.

\[
\text{% prothrombin activity} = \frac{t_a}{t} \times 100
\]

where \( t_a \) is the prothrombin time of a plasma of 100% activity and \( t \) the prothrombin time of the unknown.
Natural Antithrombin Activity.—This can be obtained from the 1:25 curve of the coagulohepatogram (cf. Fig. 1). The difference between the highest value of the curve and the 15-minute value gives the antithrombin activity of a given tube. When highest accuracy is required, a correction can be made for the thrombin destroyed at 37°C during the activation period of 15 minutes. For example, maximum thrombin activity of a 1:25 plasma dilution was found to be 2.61 t.u. and the 15-minute value was 1.04 t.u. In the same period the standard thrombin solution showed a decrease of activity from 1.60 t.u. to 1.25 t.u., i.e., by 0.35 units. The antithrombin effect of this tube was therefore 2.61−1.04−0.35 t.u. = 1.22 antithrombin (a.t.u.). The average normal being 1.0 a.t.u. (more accurately 0.975±0.32 a.t.u. (1950a), antithrombin activity of the unknown was therefore 1.22×100 = 122% of average normal.

Details of interpretation of antithrombin values have been given in a previous publication (1950). The natural antithrombin activity measured by the method presented here is quite different from heparin. Clinical evidence of the appearance of heparin in blood plasma is very rare. Significant increases of heparin have been reported in anaphylactic shock and mast cell hyperplasia (Jorpes, personal communication). The presence of heparin is not considered in the antithrombin method here described. Its effect can be quantitatively neutralized by adding 0.25 ml. of a 1:2,000 buffered solution of protamine (Hoffmann-La Roche). In the absence of heparin such an addition in no way affects the coagulohepatogram.

Normal variations of the coagulohepatogram are given in Table I in relation to its three components. The accuracy of accelerator activity determinations does not equal that of the prothrombin and antithrombin methods described in the present publication. The error of the prothrombin method is less than ±2%.

A practical graphic presentation of the three different activities of the coagulohepatogram is obtained by representing them in three adjoining longitudinal columns; the first one corresponds to accelerator activity, the second to prothrombin activity, and the third to natural antithrombin activity. Examples of this type of graphic illustration can be found in Figs. 3, 4, 5A, and 5B. It has the great advantage of giving an excellent
visual comprehension and quantitative comparison of the three clotting factors read from a coagulohepatogram.

Applications of the Coagulohepatogram

The new concept of the coagulohepatogram was first applied to experimental studies of a dicoumarol derivative of lesser toxicity, tromexan (ethyl-ether of bis 3,3'-(4-oxycoumarinyl acetic acid). By simultaneous use of Quick's one-stage and my own two-stage procedure for prothrombin determinations, significant discrepancies of the curves were observed. The more rapid fall of values with the one-stage method (cf. Fig. 3) can be explained by the fact that following tromexan administration accelerator activity of plasma decreases. Experiments with my two-stage method have shown that results depend little on accelerator variations when working with 1:50 plasma dilutions. In Fig. 2 the effect of tromexan on liver function during an 18-day period is demonstrated. On the sixth and seventh day tromexan administration was stopped or greatly reduced.

As can be seen from Fig. 2, accelerator drops and returns to normal more rapidly than prothrombin measured by the two-stage technique. The practical value of coagulohepatograms is evident when one follows their changes during tromexan treatment as is illustrated in Fig. 3.

![Graph showing average normal activity of various factors before and after tromexan treatment.](http://jcp.bmj.com/)
The three factors of the coagulohepatogram have already undergone changes after one day of treatment. Low accelerator activity on the third day contrasts with higher values for prothrombin and antithrombin. At this moment there is a distinct dissociation of accelerator and prothrombin.

The value of the coagulohepatogram being recognized, an extended study of various normal and pathological plasmas and a screening of liver disorders and vascular diseases were undertaken. These studies will be described in detail elsewhere. Some preliminary examples are given here concerning the realm in which the coagulohepatogram seems to be of particular value, namely, in vascular and liver disorders.

Vascular Diseases.—Of 20 cases of vascular diseases and thrombo-embolic conditions studied with the new technique, the great majority showed strikingly abnormal coagulohepatograms. The findings are summarized in Fig. 4. Of particular importance are increased antithrombin values in cases of endarteritis obliterans, anaphylactoid purpura, and two representative cases of myocardial infarction.

Disorders of the Liver and Bile Ducts.—Pathological values of accelerator activity, i.e., values below 60%, were frequently observed in parenchymatous liver diseases. As could be expected, low values were found in cases treated with dicoumarol or tromexan and also in lyophile plasma. Measured with the author’s two-stage procedure, the prothrombin activity of the latter is normal.

In Figs. 5A and 5B coagulohepatograms of representative cases of a variety of liver diseases and diseases of the bile ducts are given under seven different headings.

In cholelithiasis normal coagulohepatograms were obtained in a few instances. In several cases abnormal coagulohepatograms became normal following cholecystectomy. Depression of accelerator is frequently a distinct sign of secondary liver involvement in these cases, due to bad biliary flow or to congestion. Biliary obstruction and jaundice of obstructive type were frequently accompanied by high antithrombin values. This is of definite diagnostic importance. In biliary
obstruction high accelerator values have recently been reported by Owren (1949), who uses a more elaborate method. In several cases of jaundice of short duration (Fig. 5B) values of 60% were obtained.

Abnormal coagulohepatograms are important in a large proportion of cases of jaundice, and last but not least in various early and late phases of liver cirrhosis. This is borne out by Fig. 5B. The striking behaviour of the three factors involved in the coagulohepatogram in neoplastic liver diseases should also be mentioned (Fig. 5B). In early involvement of liver parenchyma, accelerator activity may be decreased. In more advanced cases of subacute and chronic hepatitis, accelerator is most often depressed. Attention was drawn to the fact that early parenchymatous liver disease and early liver damage can be revealed by the accurate two-stage prothrombin method (Rieben, 1946b).

In previous communication (1950), in confirmation of Volkert (1942), an initial fall of antithrombin was described in liver damage due to obstructive jaundice, followed in most cases by a distinct rise above normal. In neoplastic liver disease, be it primary or secondary, the degree of obstruction can often be estimated by a glimpse at the coagulohepatogram which shows a rise of antithrombin activity.
CLINICAL SIGNIFICANCE OF THE COAGULOHEPATOGRAM

Summary and Conclusions

The coagulohepatogram is a new method of appraisal of three vital partial functions of the liver quite distinct from non-specific liver function tests. The factors surveyed by this simple, easily remembered and visually very comprehensive method show dissociations in various vascular, haemorrhagic, thrombo-embolic, and liver disorders. Experimentally, changes of the coagulohepatogram following tromexan treatment are a striking example.

The clinical significance of the coagulohepatogram is greatest in conditions where any of the three liver functions on which it is based may undergo changes. Accelerator and prothrombin activity show dissociation following tromexan and in acute and chronic liver disease. Thus the coagulohepatogram gives us insight into a most delicate triad of liver functions by permitting simultaneous appraisal of three specific liver substances known in isolated form.

Studies are now focused on a survey of various stages of acute, subacute, and chronic liver disorders, eclampsia, and diseases of the newborn. To the clinician the coagulohepatogram is a new diagnostic tool valuable in the differential diagnosis of liver diseases. It helps him to obtain a survey of three sensitive partial functions of the liver. The coagulohepatogram thus fulfils the criteria proposed for modern liver function tests, namely, specificity and quantitative appraisal.

REFERENCES

Jorpes, Karolinska Institutet, Stockholm. Personal communication.