Heparin

Determination with Ribonuclease

Nepomuk Zöllner and Bruno Lorenz

Heparin inhibits several enzyme reactions. It is most suitably determined by its inhibition of pancreatic ribonuclease. Like the coagulation of blood the inhibition of ribonuclease is sensitive to small changes in the heparin molecule. The method permits the estimation of small amounts of heparin and the kinetics of the inhibition can be measured (see below). Some "heparinoids", *e.g.* Thrombocide and sulphonated polysaccharides with heparin-like activity, are estimated as heparin. As all the other existing methods can only be used for the estimation of "heparin-like activity" this disadvantage is not very important. In contrast to the analytical methods employing the effect of heparin on blood coagulation, the use of ribonuclease inhibition has the advantage of simple experimental conditions with a precisely defined reaction mixture and a linear relationship between the values obtained experimentally and the heparin concentration. Proteins which bind heparin or high salt concentrations interfere with the determination.

Cyclic pyrimidine nucleotides are not suitable as substrates for the spectrophotometric determination of heparin. The change in optical density which occurs on cleavage of these nucleotides is too small.

Principle

Heparin inhibits the breakdown of ribonucleic acid[1,2] and cyclic pyrimidine nucleotides[3] by ribonuclease. This inhibition is probably competitive and can be represented by the formula:

$$\frac{v}{v_i} = 1 + K \frac{[I]}{K' + [S]} \quad (1)$$

where

v = rate of the uninhibited enzyme reaction
v_i = rate of the enzyme reaction inhibited by heparin
$[S]$ = substrate concentration
$[I]$ = inhibitor (heparin) concentration
K = Michaelis constant of the uninhibited reaction
K' = Michaelis constant of the inhibited reaction.

A. Spectrophotometric Method

Reagents

1. Sodium acetate, A. R., anhydrous
2. Acetic acid, A. R.
3. Ribonucleic acid
   from yeast, sodium salt; commercial preparation, see p. 1027.
4. Ribonuclease, RNase
   from pancreas, crystalline; commercial preparation, see p. 997.
5. Heparin*)

*) Biochemicum Roche or a standardized preparation suitable for injection, *e.g.* Heparin Novo or Liquemin from Hoffman-La Roche, Grenzach/Baden, Germany.

1) *N. Zöllner* and *J. Fellig*, Naturwissenschaften *39*, 523 [1952].
2) *N. Zöllner* and *J. Fellig*, Amer. J. Physiol. *173*, 223 [1953].
3) *G. Hobom*, M. D. Thesis, Universität München 1962.

Purity of the enzyme and the standard heparin preparation

The commercially available crystalline ribonuclease from pancreas need not be purified further. The activity of heparin as an inhibitor of ribonuclease parallels its activity as an anticoagulant of blood; therefore a heparin preparation which has been assayed for its anticoagulant activity should be used as a standard. The amount of heparin is given in International Units (IU). For many purposes a sufficiently accurate standard is obtained by weighing out the amounts of heparin; the concentration is then given in μg./ml. A fraction which is inactive with ribonuclease can be separated from impure heparin preparations by preparative electrophoresis[2].

Preparation of Solutions

Prepare all solutions with doubly glass distilled water.

I. Acetate buffer (0.2 M; pH 5.0):
Mix 70.5 vol. 0.2 M sodium acetate (8.204 g./500 ml.) and 29.5 vol. 0.2 M acetic acid (11.5 ml. acetic acid/1000 ml.).

II. Ribonucleate (0.2% w/v):
Dissolve 0.2 g. Na ribonucleate in 100 ml. acetate buffer (solution I).

III. Ribonuclease, RNase (50 μg. protein/ml.):
a) Dissolve 5 mg. crystalline ribonuclease from pancreas in 100 ml. doubly distilled water.
b) Just before use dilute this solution 1 : 5 with doubly distilled water.

IV. Heparin standard solution (10 μg./ml. or 1 IU/ml.):
Dilute commercial preparation (5000 IU/ml.) 1 : 5000 with doubly distilled water.

Stability of the solutions

Store all solutions in a refrigerator at 2 to 4° C. The substrate solution II keeps for about 2 months in a refrigerator when a thymol crystal is added. The ribonuclease solution (IIIa) is stable for a similar period. A slight loss of activity is not important since standards are included in each series of measurements.

Procedure

Experimental material

Since large amounts of protein interfere with the determination[4] and the evaluation of the experimental results requires known salt concentrations[5], heparin must be separated from these substances before the determination. By simple means satisfactory yields of heparin can only be obtained from a few tissues[6]; the presence of much mucopolysaccharide makes the separation difficult[7]. A quantitative separation of heparin from serum cannot be obtained with the methods so far described[8,9]. Heparin can be obtained from plasma in good yield as follows[10]: dilute 1 ml. oxalated plasma with 2 ml. 0.05 M acetate buffer (pH 5.9) and add 0.6 ml. of a 0.4% (w/v) 5-aminoacridine hydrochloride solution. Shake thoroughly and centrifuge at high speed. Suck off the supernatant. Dissolve the precipitate

4) *B. Lorenz, R. Lorenz* and *N. Zöllner*, Z. exp. Med. *133*, 144 [1960].
5) *B. Lorenz, R. Lorenz* and *N. Zöllner*, Z. Naturforsch. *15* b, 62 [1960].
6) *J. D. H. Homan* and *J. Lens*, Biochim. biophysica Acta *2*, 333 [1948].
7) *E. Buddecke*, Hoppe-Seylers Z. physiol. Chem. *310*, 171 [1958].
8) *L. B. Jaques, F. C. Monkhouse* and *M. Stewart*, J. physiol. *109*, 41 [1949].
9) *F. C. Monkhouse* and *L. B. Jaques*, J. Lab. clin. Med. *36*, 782 [1950].
10) *N. Zöllner, C. Burger* and *R. Braun*, Hoppe-Seylers Z. physiol. Chem., in press.

(heparin and some protein) in 4 drops 0.2 N NaOH, dilute with 1 ml. water and extract several times with ether. Use the aqueous, alkaline solution of heparin for the determination.

Spectrophotometric measurements

Preliminary remarks: As a rate is to be measured the enzyme solution must be pipetted very accurately. It is sufficient to work in a room with a constant temperature. The solutions are equilibrated by placing in a water bath.

Each series of measurements should contain at least two standards and a heparin-free control. It is recommended that the control determination is carried out several times and that the results are averaged. With high concentrations of inhibitor the initial decrease in the optical density is slower than that on the main part of the curve; this flatter part of the curve can also be seen with low enzyme concentrations in the absence of inhibitor[11] and is therefore not an inhibitor effect.

By changing the ribonucleate concentration the sensitivity of the method can be altered[2]; the ribonucleate concentration given is for the estimation of very small amounts of heparin. The analysis is more sensitive and less time consuming when a recording spectrophotometer equipped for the spreading of extinction differences is used. This makes possible the evaluation of the initial part of the reaction curve.

Method: Wavelength: 300 mμ; light path: 1 cm.; final volume: 2.0 ml. Measure against water.

Pipette into test tubes:

	Experimental	*Standard*	*Control*
sample (containing 1 to 5 μg. heparin)	0.1—1 ml.	—	—
heparin standard solution (IV)	—	0.1—0.5 ml.	—
doubly distilled water	to 1 ml.	to 1 ml.	1 ml.
RNase solution (IIIb)	0.5 ml.	0.5 ml.	0.5 ml.
ribonucleate solution (II)	0.5 ml.	0.5 ml.	0.5 ml.

Shake and immediately pour the mixtures into cuvettes. Read the optical density at minute intervals until a total decrease in optical density of 0.060 is obtained. Plot the optical density (ordinate) against the time (abscissa).

Calculations

From the graph for the standards obtain the times Δt_i required for an optical density decrease ΔE of 0.040. From the graph for the control obtain the Δt for the same decrease in optical density. Plot the quotients $\frac{\Delta t_i}{\Delta t}$ against the heparin concentration of the standards (standard curve, which should be a straight line, crossing the ordinate at 1.0). Similarly, for each sample calculate the quotient $\frac{\Delta t_i}{\Delta t}$ (Δt_i = time for $\Delta E = 0.040$ in the experimental tube and Δt = time for $\Delta E = 0.040$ in the control) and read off the corresponding heparin concentration from the standard curve.

Specificity

Many sulphonated, macromolecular compounds inhibit ribonuclease, but of the mucopolysaccharides isolated from biological material only heparin exhibits inhibitor activity (β-heparin has not been

[11] *N. Zöllner*, Habilitationsschrift, Universität München 1954.

tested). One possibility for differentiating heparin from these other substances is its sensitivity to acid; 30 min. hydrolysis in 0.03 N HCl at 80° C virtually destroys its inhibitor activity [2].

B. Titrimetric Method [12]

The titrimetric method is especially suitable for the determination of small amounts of heparin. For the rapid measurement of numerous samples it is better to use the spectrophotometric method. Proteins interfere less with the titrimetric method. In both methods, changes of salt concentration affect the inhibitory activity of heparin to about the same extent.

Principle

Ribonuclease cleaves cyclic 2′,3′-pyrimidine nucleotides to 3′-pyrimidine nucleotides. The amount of acid liberated is determined by the amount of alkali required to keep the pH constant. The measurements are carried out at pH 5.6. Approximately half of the hydrolysed ester bonds titrate [3], but this is not important for the determination of heparin.

Reagents

1. Cyclic 2′,3′-cytidine phosphate (or cyclic 2′,3′-uridine phosphate) barium salt; commercial preparation, see p. 1009.
2. Ethylene-diamine-tetra-acetic acid, EDTA disodium salt, EDTA-$Na_2H_2 \cdot 2H_2O$ (*e.g.* Komplexon III)
3. Sodium chloride, A. R.
4. Hydrochloric acid, A. R., 0.1 N
5. Sodium hydroxide, A. R., 0.1 N
6. Sodium hydroxide, A. R., 0.005 N
7. Ribonuclease, RNase (see p. 79)
8. Heparin (see p. 79)

Preparation of Solutions

Singly distilled water is sufficient for the preparation of solutions I—III and V, but IV must be prepared with doubly distilled water.

I. Cyclic nucleotide (*ca.* 0.015 M):
Dissolve 56 mg. of the barium salt in 10 ml. distilled water.

II. Ethylene-diamine-tetra-acetate, EDTA (3.3×10^{-4} M):
Dissolve 12.5 mg. EDTA-$Na_2H_2 \cdot 2H_2O$ in 100 ml. distilled water.

III. Sodium chloride (0.1 M):
Dissolve 5.845 g. NaCl in distilled water and make up to 1000 ml.

IV. Ribonuclease, RNase (100 μg. protein/ml.):
Dissolve 5 mg. ribonuclease and 0.5 mg. EDTA-$Na_2H_2 \cdot 2H_2O$ in 50 ml. doubly distilled water.

V. Heparin standard solution (see p. 80).

Stability of the solutions

See page 80.

12) *N. Zöllner* and *G. Hobom*, unpublished.

Procedure

Preliminary remarks: see under "Ribonuclease, titrimetric determination", p. 798.

Separation of heparin

See page 80.

Reaction mixture

Final volume: 3 ml.; room temperature (constant). Other details, see p. 81.

Each series of measurements should contain at least two standards and a heparin-free control.

Pipette successively into small beakers:

	Experimental	*Standard*	*Control*
nucleotide solution (I)	1.00 ml.	1.00 ml.	1.00 ml.
EDTA solution (II)	0.20 ml.	0.20 ml.	0.20 ml.
NaCl solution (III)	0.25 ml. *)	0.25 ml.	0.25 ml.
sample solution (containing 1—5 μg. heparin = 0.1—0.5 IU)	0.10—1.00 ml.	—	—
heparin standard solution (V)	—	0.10—0.50 ml.	—
doubly distilled water	to 2.90 ml.	to 2.90 ml.	to 2.90 ml.

Adjust to pH 5.60 (glass electrode) with 0.1 N HCl using a magnetic stirrer; check the stability of the pH over a period of several minutes. Pipette

0.10 ml. RNase solution (IV)

into all mixtures. Add sufficient 0.005 N NaOH, at intervals of 40—80 sec. over a period of 10 min., so that the solution, which becomes acid due to action of the enzyme, is maintained at just above pH 5.60. For further details, see p. 798 under "Ribonuclease, titrimetric determination". The ml. or μequiv. NaOH required per min. are used for the calculations.

Calculations

As described under "Ribonuclease, titrimetric determination" (see p. 799). Average the μequiv. NaOH/min. for the experimental, control and standard reaction mixtures. Plot the quotients of [μequiv. NaOH/min. (control)] : [μequiv. NaOH/min. (standard)] on the ordinate against amounts of heparin (μg. or IU) in the standard reaction mixtures (abscissa). Calculate the quotients for the experimental reaction mixture [μequiv. NaOH/min. (control)] : [μequiv. NaOH/min. (experimental)] and read off the corresponding amounts of heparin from the standard curve.

The experimental values for μequiv. NaOH/min. are reproducible over several determinations; with some practice, 0.2—0.3 μg. or 0.02—0.03 IU of heparin can be determined with cyclic cytidine phosphate as substrate.

Sources of Error

The conditions chosen for the determination of heparin make it very sensitive, but also very liable to interference. By increasing the salt concentration or the pH to 6 the method is made less sensitive. Even under these conditions with cyclic uridine phosphate as substrate 0.35 μg. heparin are still detectable.

Specificity

See page 81.

*) If necessary, allow for the ionic strength of the heparin sample.

Determination with Pyruvic Kinase

Hans-Dieter Horn

Heparin is estimated quantitatively by determining the plasma protamine titre, the delay in blood coagulation, the antithrombin activity or the inhibition of prothrombin activation. In addition, there are methods which depend on the metachromatic effect of heparin reacting with basic dyes. The accuracy and sensitivity of the methods differ, the reaction mixtures are often complicated, and the specificity of certain methods, particularly in the examination of pathological sera, may not be sufficiently high. The method described here does not have these disadvantages. However, it has only so far been applied to pure solutions and not to biological material.

Principle

Heparin inhibits certain enzymes: hyaluronidase[1–3], ribonuclease[4–7], acid and alkaline phosphatases[8], adenyl deaminase[9], trypsin[10,11], pyruvic kinase[12], fumarase[13], glutathione reductase[14,15], glucose-6-phosphate dehydrogenase, glutamic dehydrogenase and alcohol dehydrogenase[12].

The liver alcohol dehydrogenase and muscle pyruvic kinase are especially suitable for the determination of heparin. The assay with pyruvic kinase is preferable to that with alcohol dehydrogenase, since the scatter of the values is smaller. The heparin solution to be analysed is allowed to act on the enzyme (5–10 min. at room temperature is sufficient in the assay with pyruvic kinase) and then the enzyme activity is compared to that of the untreated enzyme. The heparin concentration corresponding to the inhibition is read off from a standard curve.

Reagents

1. Tris-hydroxymethyl-aminomethane, tris
2. Hydrochloric acid, 1 N, A. R.
3. Magnesium chloride, $MgCl_2 \cdot 6\ H_2O$, A. R.
4. Potassium chloride, A. R.
5. Phosphoenolpyruvate, PEP
   crystalline tricyclohexylammonium salt, commercial preparation, see p. 1024.
6. Reduced diphosphopyridine nucleotide, DPNH
   sodium salt, DPNH-Na_3; commercial preparation, see p. 1011.
7. Adenosine diphosphate, ADP
   sodium salt, ADP-Na_3; commercial preparation, see p. 1004.
8. Pyruvic kinase, PK
   crystalline, from rabbit skeletal muscle; commercial preparation, see p. 997.

1) *D. McClean*, J. Pathol. Bacteriol. *54*, 284 [1942].
2) *K. Meyer*, Physiol. Rev. *27*, 335 [1947].
3) *M. B. Mathews* and *A. Dorfman*, Physiol. Rev. *35*, 381 [1955].
4) *N. Zöllner* and *J. Fellig*, Naturwissenschaften *39*, 523 [1952]; Amer. J. Physiol. *173*, 233 [1953].
5) *J. S. Roth*, Arch. Biochem. Biophysics *44*, 265 [1953].
6) *G. De Lamirande*, *G. Weber* and *A. Cantero*, Amer. J. Physiol. *184*, 415 [1956].
7) *L. Vandendriessche*, Arch. Biochem. Biophysics *65*, 347 [1956].
8) *L. M. Buruiana*, Naturwissenschaften *44*, 306 [1957].
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12) *H.-D. Horn* and *F. H. Bruns*, Verh. dtsch. Ges. inn. Med. *65*, 604 [1959].
13) *A. Fischer* and *H. Herrmann*, Enzymologia *3*, 180 [1937].
14) *H.-D. Horn*, Verh. dtsch. Ges. inn. Med. *64*, 315 [1958].
15) *H.-D. Horn* and *F. H. Bruns*, Biochem. Z. *331*, 58 [1958].

9. Lactic dehydrogenase, LDH
   crystalline, from rabbit skeletal muscle; commercial preparation, see p. 986.
10. Heparin
    e.g. as an aqueous solution*) containing 5000 units/ml. A unit = 7.8 μg. heparin.

Preparation of Solutions

I. Tris buffer (0.1 M; pH 7.4):
   Dissolve 12.11 g. tris-hydroxymethyl-aminomethane in *ca.* 84 ml. 1 N HCl, adjust to pH 7.4 with 1 N HCl and dilute to 1000 ml. with distilled water.

II. Mg^{2+}-K^+ solution (0.03 M Mg^{2+}; 0.01 M K^+):
   Dissolve 600 mg. $MgCl_2 \cdot 6\ H_2O$ and 75 mg. KCl in a small amount of tris buffer (solution I) and dilute to 100 ml. with distilled water.

III. Phosphoenolpyruvate (600 μg. PEP/ml.):
   Dissolve 18 mg. phosphoenolpyruvate (tricyclohexylammonium salt) in tris buffer (solution I) and make up to 100 ml.

IV. Adenosine diphosphate (*ca.* 8.5 mg. ADP/ml.):
   Dissolve 100 mg. ADP-Na_3 in 10 ml. tris buffer (solution I) and adjust to pH 7.4 with 0.1 N NaOH.

V. Pyruvic kinase, PK (0.17 mg. protein/ml.):
   Dilute 1 mg. protein (crystalline suspension in ammonium sulphate solution) to 5 ml. with distilled water, dialyse for 8—12 hours at 0°C against 5000 ml. distilled water, and after the dialysis dilute to 6 ml. with distilled water.

VI. Lactic dehydrogenase, LDH (*ca.* 0.4 mg. protein/ml.):
   Dilute 2 mg. protein (crystalline suspension in ammonium sulphate solution) to 5 ml. with distilled water and dialyse as for pyruvic kinase. The exact volume after dialysis is unimportant, since the enzyme is added in excess.

VII. Reduced diphosphopyridine nucleotide (*ca.* 6 mg. β-DPNH/ml.):
   Dissolve 70 mg. DPNH-Na_2 in 10 ml. tris buffer (solution I).

VIII. Heparin standard solutions (0.05—2.5 units or 0.39—19.5 μg./0.5 ml.):
   Dilute 1 ml. heparin solution containing 5000 units/ml. (= 39 mg. heparin/ml.) to 1000 ml. with dist. water. Prepare further dilutions of this solution with dist. water:
   a) standard solution (2.5 units/0.5 ml. or 19.5 μg./0.5 ml.)
   b) 20 ml. standard solution + 30 ml. dist. water (1 unit/0.5 ml. or 7.8 μg./0.5 ml.)
   c) 10 ml. standard solution + 40 ml. dist. water (0.5 units/0.5 ml. or 3.9 μg./0.5 ml.)
   d) 5 ml. standard solution + 45 ml. dist. water (0.25 units/0.5 ml. or 1.95 μg./0.5 ml.)
   e) 2 ml. standard solution + 48 ml. dist. water (0.1 units/0.5 ml. or 0.78 μg./0.5 ml.)
   f) 1 ml. standard solution + 49 ml. dist. water (0.05 units/0.5 ml. or 0.39 μg./0.5 ml.)

Procedure

Experimental material

So far, the method has only been used to determine the content of heparin preparations where special preliminary treatment of the sample is unnecessary. How far interfering substances in biological material must be removed before the determination of heparin has not been studied (refer to p. 80).

*) Liquemin, Hofmann-La Roche, Grenzach/Baden, Germ.; Eleparon, Luitpold-Werke, Munich, Germ.

Spectrophotometric measurements

For each series of measurements a standard curve is prepared with standard solutions VIIIa–f. The enzyme activity (mean values of duplicate determinations) is expressed as $\Delta E/30$ sec. The time between the start of the enzyme reaction and the start of the optical density measurements need not be determined exactly, since the reaction is linear with time.

Wavelength: 366 mμ (or 340 mμ); light path: 1 cm.; measure against a blank cuvette containing water; room temperature (constant during a series of measurements) or better still constant at 25°C.

Pipette successively into all the cuvettes:

0.2 ml. PK solution (V).

Add to cuvette no. 1:

0.5 ml. distilled water.

Add to cuvettes no. 2–7:

0.5 ml. standard solution (VIIIa, b, c, d, e, f)
(corresponding to 0.05–2.5 units heparin).

Add to the experimental cuvette:

0.5 ml. sample (containing 0.05 to 2.0 units heparin).

Mix and allow to stand for 10 min. at room temperature (*ca.* 25°C). Pipette into all the cuvettes:

0.2 ml. ADP solution (IV)
0.2 ml. LDH solution (VI)
0.2 ml. Mg^{2+}-K^+ solution (II)
0.1 ml. DPNH solution (VII)
1.5 ml. tris buffer (solution I).

Mix, place cuvette no. 1 in the light path of the spectrophotometer and start the reaction by mixing in

0.1 ml. PEP solution (III).

Measure the decrease in the optical density at 10 to 20 sec. intervals (at least three) and calculate the $\Delta E/30$ sec. Proceed in a similar manner with the other cuvettes. $(\Delta E/30 \text{ sec.})_1$ to $(\Delta E/30 \text{ sec.})_7$ are the values for the standard curve, $(\Delta E/30 \text{ sec.})_{sample}$ is the unknown value. Repeat the whole series of measurements and average the values.

Evaluation

$(\Delta E/30 \text{ sec.})_1$ is a measure of the rate of the uninhibited pyruvic kinase reaction and $(\Delta E/30 \text{ sec.})_{2-7}$ measures the rate of the PK reaction after addition of heparin. Plot $(\Delta E/30 \text{ sec.})_{1-7}$ (ordinate) against the amounts of heparin (abscissa) in the cuvettes 1–7. Obtain the amount of heparin in the sample from the standard curve.

Notes

α-Heparin of Deutsche Hofmann La Roche, Grenzach/Baden, Germany, and β-heparin of Luitpold-Werke, Munich, Germany, were tested. The inhibition curve with β-heparin does not completely agree with that of α-heparin over the range of lower concentrations. Whether this is due to the different numbers of sulphate residues in the molecule, to steric differences or to the use of different units for concentration by the two firms, remains an open question.

The assay with ADH can be carried out in a similar manner. However, the assay with pyruvic kinase is recommended.