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STUDIES ON THE BLOOD COAGULATION MECHANISMS
OF DIFFERENT SPECIES COMPARED TO MAN

Volume Two

by

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C O N T E N T S

APPENDIX I

THE PREPARATION OF REAGENTS

1. Acetic acid	page	2
2. Acetone		2
3. Adsorbing agents		2
A- Alumina		2
B- Barium sulphate		3
C- Tricalcium phosphate		4
4. Alizarin red indicator		4
5. Ammonium chloride standard solution		4
6. Ammonium sulphate saturated solution		4
7. Anticoagulants		4
A- Ammonium and potassium oxalate mixture		4
B- Potassium oxalate		5
C- Sequestrene		5
D- Sodium citrate		5
E- Sodium oxalate		5
8. Barbitone buffer for electrophoresis		5
9. Barium chloride standard solution		6
10. Calcium chloride solutions		6
11. Chloroform		6
12. Citrate - saline solution		6
13. De-ionised water		6
14. Folin and Ciocalteu's reagent		7
15. Formaldehyde solution		7
16. Formol - citrate solution		7

APPENDIX II

GENERAL TECHNIQUE AND EQUIPMENT

1. Collection of blood specimens	page 14
2. Centrifugation	15
3. Clotting experiments	16
4. Dialysis	16
5. pH measurements	16
6. Spectrophotometric measurements	16
7. Phase contrast microscopy and photomicrography	17
8. Micro-titration	18

APPENDIX III

PREPARATION OF COAGULATION FACTORS

1. Citrated platelet-rich plasma	20
2. Citrated platelet-poor plasma	21
3. Oxalated plasma	21
4. Adsorbed plasma	21
5. Serum	22
6. Fibrinogen: A. Phosphate precipitation method	23
B. Ammonium sulphate precipitation method..	25
7. Thrombin	27
8. Prothrombin	29
9. Factor V	30
10. Factor VII	31
11. Antihæmophilic globulin (factor VIII)..	32
12. Christmas factor (factor IX)..	32
13. Tissue extracts: I. Brain thromboplastin extract	33
II. Crustacean muscle extract	34

14. Cephalin	page	35
15. Platelet suspensions: A. Human, horse, and rabbit ..		36
B. Chicken thrombocyte suspension		37
C. Bovine platelet suspension ..		37
16. Crustacean blood cells' extract		37
17. Russell's viper venom reagent		38

APPENDIX IV

ASSAY METHODS AND SPECIAL TECHNIQUES

1. Platelet counts: A. Mammalian		41
B. Chicken thrombocytes' count		43
C. Lobster blood cells' count		44
2. Fibrinogen estimation		
I. micro-Kjeldahl method		45
II. modified Greenberg method		49
III. Stirland's turbidimetric method		51
3. Whole blood clotting time		52
4. Clot retraction		52
5. Plasma recalcification time		53
6. Quick's one-stage prothrombin time		53
7. Thrombin-fibrinogen dilution curve		54
8. Thrombin-fibrinogen reaction of the plasma		56
9. Effect of various reagents on the thrombin-fibrinogen reaction		56
10. Testing for the species specificity of the thrombin- -fibrinogen reaction in a purified system		57
11. Measurement of antithrombin		
A. Method of Douglas and Biggs, 1953		57
B. Modified method of Astrup and Darling, 1942d ..		58

12. Measurement of prothrombin	
A. One-stage method of Koller et al, 1951	page 60
B. Globulin fraction method of Douglas and Biggs, 1953	62
C. Two-stage area method of Biggs and Douglas, 1953a	63
13. Prothrombin consumption test	
A. Method of Biggs and Macfarlane. 1957	64
B. Method of Douglas and Biggs, 1953	66
14. Measurement of factor V	67
15. Measurement of factor VII	
A. By correction of phenindione plasma	69
B. By correction of Seitz-filtered bovine plasma ..	70
16. Brain thromboplastin - factor V specificity	71
17. Brain thromboplastin - factors VII and X specificity ..	72
18. Measurement of factor X	73
19. R.V.V.-cephalin clotting time of plasma	76
20. Thromboplastin generation test	76
A. Intrinsic thromboplastin dilution curve	78
21. Measurement of antihæmophilic globulin	81
22. Measurement of Christmas factor	84
23. Demonstration of inhibitors of human intrinsic thromboplastin in thromboplastin generation mixtures	87
24. Measurement of Hageman factor	
I. Method of Ratnoff and Rosenblum, 1958	89
II. By correction of Hageman factor deficient plasma ..	91
25. Measurement of the esteratic activity of thrombins ..	93

APPENDIX V

RESULTS OF EXPERIMENTS

Concentration of the coagulation factors in the control human plasma used as standard page	97
Appendix V, A. Results of experiments on fowl blood	100
Appendix V, B. Results of experiments on bovine blood	155
Appendix V, C. Results of experiments on horse blood	197
Appendix V, D. Results of experiments on rabbit blood	238
Appendix V, E. Results of experiments on lobster blood	280
References	309

LIST OF ILLUSTRATIONS

1. Reference thrombin-fibrinogen dilution curve	page 55
2. Human intrinsic thromboplastin dilution curve	80
3. Thrombin-fibrinogen reaction of chicken plasma	106
4. Antithrombin assay in chicken plasma	108
5. Factor V assay in chicken plasma	114
6. Measurement of prothrombin in chicken plasma	119
7. Chicken active intrinsic thromboplastin dilution curve	128
8. Antihæmophilic globulin activity of chicken adsorbed plasma in the T.G.T., using human platelets	135
9. Antihæmophilic globulin activity of chicken adsorbed plasma in the T.G.T., using human cephalin	136
10. Antihæmophilic globulin activity of chicken adsorbed plasma in the T.G.T., using chicken thrombocytes ..	137
11. Chicken factor V specificity in modified T.G.T.	141
12. Christmas factor activity of chicken serum in the T.G.T., using human platelets	142
13. Christmas factor activity of chicken serum in the T.G.T., using chicken thrombocytes	143
14. Chicken blood thromboplastin generation	145
15. Chicken plasma substrate reactivity in the T.G.T., using human platelets	146
16. Chicken plasma substrate reactivity in the T.G.T., using chicken thrombocytes	147
17. Chicken plasma inhibitors of human intrinsic thromboplastin	150
18. Chicken serum inhibitors of human intrinsic thromboplastin	153
19. Thrombin-fibrinogen reaction of bovine plasma	159
20. Antithrombin assay in bovine plasma by the method of Astrup and Darling, 1942d	161

21. Antithrombin assay in bovine plasma by the method of Douglas and Biggs, 1953	page 163
22. Bovine plasma factor V assay	167
23. Bovine plasma factor VII assay	170
24. Bovine plasma prothrombin assay by the one-stage method of Koller and associates, 1951	173
25. Bovine plasma prothrombin assay by the two-stage method of Biggs and Douglas, 1953a	175
26. Bovine plasma activity in the T.G.T.	184
27. Bovine plasma A.H.G. activity in the T.G.T.	185
28. A.H.G. assay in bovine plasma	187
29. Activity of bovine serum in the T.G.T.	188
30. Bovine serum Christmas factor activity in the T.G.T.	189
31. Christmas factor assay in bovine serum	191
32. Thromboplastic activity of bovine platelets in the T.G.T.	192
33. Bovine plasma substrate reactivity in the T.G.T.	195
34. Bovine plasma substrate reactivity in the T.G.T.	196
35. Thrombin-fibrinogen reaction of horse plasma	201
36. Antithrombin assay in horse plasma by the method of Douglas and Biggs, 1953	204
37. Antithrombin assay in horse plasma by the method of Astrup and Darling, 1942d	206
38. Factor V assay in horse plasma	209
39. Horse plasma factors VII and X activity	212
40. Measurement of horse plasma prothrombin	215
41. Horse plasma Hageman factor assay	219
42. Horse plasma activity in the T.G.T.	222
43. A.H.G. assay in horse plasma	224
44. Horse serum activity in the T.G.T.	225
45. Christmas factor assay in horse serum	227

	Page
46. Thromboplastic activity of horse platelets in the T.G.T.	228
47. Horse plasma substrate reactivity in the T.G.T.	232
48. Horse plasma substrate reactivity in the T.G.T.	233
49. Horse plasma inhibitors of human intrinsic thromboplastin	236
50. Horse serum inhibitors of human intrinsic thromboplastin	237
51. Thrombin-fibrinogen reaction of rabbit plasma	242
52. Antithrombin assay in rabbit plasma by the method of Douglas and Biggs, 1953	245
53. Antithrombin assay in rabbit plasma by the method of Astrup and Darling, 1942d	247
54. Rabbit plasma factor V assay	251
55. Rabbit plasma factors VII and X activity	254
56. Measurement of rabbit plasma prothrombin	257
57. Hageman factor assay in rabbit plasma	262
58. Rabbit plasma activity in the T.G.T.	265
59. A.H.G. assay in rabbit plasma	267
60. Rabbit serum activity in the T.G.T.	268
61. Christmas factor assay in rabbit serum	270
62. Thromboplastic activity of rabbit platelets in the T.G.T.	271
63. Rabbit plasma substrate reactivity in the T.G.T. ..	275
64. Rabbit plasma substrate reactivity in the T.G.T. ..	276
65. Rabbit plasma inhibitors of human intrinsic thromboplastin	278
66. Rabbit serum inhibitors of human intrinsic thromboplastin	279
67. Phase contrast photomicrograph of lobster blood, one minute after withdrawal, showing process of cell explosion ..	290
68. Phase contrast photomicrograph of the same field as above, two minutes later, showing the dense coagulation islands around the explosive cells	290
69. Phase contrast photomicrograph of lobster blood several minutes after withdrawal, showing two well developed coagulation islands	291

70. Electrophoresis pattern and its densitometric scanning of lobster plasma and serum	page 293
71. Electrophoresis pattern and its densitometric scannings of lobster plasma and serum	294
72. Electrophoresis patterns and their densitometric scannings of human plasma, serum, and purified fibrinogen	295
73. Electrophoresis patterns and their densitometric scannings of lobster blood cells' extract, lobster plasma, and lobster plasma defibrinated with lobster cell extract	296
74. Electrophoresis patterns and their densitometric scannings of purified lobster fibrinogen preparations	298

LIST OF TABLES

i. The concentration of coagulation factors in the control human plasma	page 98
ii. Thrombin-fibrinogen dilution curves	99
1. Chicken plasma fibrinogen estimation by the micro-Kjeldahl method	102
2. Chicken plasma fibrinogen estimation by Stirland's method	104
3. Thrombin-fibrinogen reaction of chicken plasma	105
4. Antithrombin assay in chicken plasma, experiment 1 ..	107
5. Antithrombin assay in chicken plasma, experiments 2 and 3	109
6. Average antithrombin concentrations in chicken plasma	109
7. Species specificity of the thrombin-fibrinogen reaction	110
8. Thrombin clotting times of chicken plasma	110
9. Quick's one-stage prothrombin time of chicken plasma ..	111
10. Chicken factor V-brain thromboplastin species specificity	112
11. Factor V assay in chicken plasma	113
12. Chicken factors VII and X-brain thromboplastin species specificity	115
13. Stuart-Prower factor (factor X) assay in chicken plasma	116
14. R.V.V.-cephalin clotting time of chicken plasma	117
15. Measurement of prothrombin in chicken plasma by the two- stage area method of Biggs and Douglas, 1953a	118
16. Measurement of prothrombin in chicken plasma by the globulin fraction method of Douglas and Biggs, 1953 ..	120
17. Thrombocyte count in chicken blood	120
18. Chicken whole blood clotting time	121
19. Recalcification time of chicken plasma	121
20. Hageman factor and P.T.A. activity of chicken plasma ..	122
21. Thromboplastin generation test, using human platelets	123

22. Thromboplastin generation test, compilation of data presented in table 21	page 125
23. Thromboplastin generation test, using human cephalin ..	126
24. Thromboplastin generation test, using chicken thrombocytes	129
25. Chicken plasma substrate reactivity in the T.G.T., using human platelets	132
26. Chicken plasma substrate reactivity in the T.G.T., using human cephalin	133
27. Chicken plasma substrate reactivity in the T.G.T., using chicken thrombocytes	134
28. Chicken plasma A.H.G. assay	138
29. Chicken A.H.G. specificity in modified T.G.T.	139
30. Chicken factor V specificity in modified T.G.T.	140
31. Christmas factor assay in chicken serum	144
32. Demonstration of inhibitors against human intrinsic thrombo- plastin in chicken adsorbed plasma	148
33. Investigation for inhibitors against human intrinsic thromboplastin in chicken serum	151
34. Chicken clot retraction	154
35. Chicken prothrombin consumption index	154
36. Measurement of fibrinogen in bovine plasma	157
37. Thrombin-fibrinogen reaction of bovine plasma	158
38. Antithrombin assay in bovine plasma by the method of Astrup and Darling, 1942d	160
39. Antithrombin assay in bovine plasma by the method of Douglas and Biggs, 1953	162
40. Quick's one-stage prothrombin time test of bovine plasma	164
41. Bovine factor V-brain thromboplastin species specificity	165
42. Assay of bovine plasma factor V activity	166
43. Bovine factors VII and X - brain thromboplastin species specificity	168
44. Bovine plasma factor VII assay	169

45. Bovine plasma factor X assay	page 171
46. Bovine plasma prothrombin assay by the one-stage method of Koller et al, 1951	172
47. Bovine plasma prothrombin assay by the two-stage method of Biggs and Douglas, 1953a	174
48. Bovine plasma prothrombin assay by the globulin fraction method of Douglas and Biggs, 1953	176
49. Bovine prothrombin consumption index	176
50. R.V.V.-cephalin clotting times of bovine plasma	177
51. Bovine blood platelets' count	177
52. Bovine whole blood clotting times	178
53. Recalcification time of bovine plasma	178
54. Hageman factor and P.T.A. activity in bovine plasma	179
55. Thromboplastin generation test, using human platelets.. .. .	180
56. Thromboplastin generation test, using bovine platelets	183
57. A.H.G. assay in bovine plasma	186
58. Christmas factor assay in bovine serum	190
59. Bovine plasma substrate reactivity in the T.G.T., using human platelets	193
60. Bovine plasma substrate reactivity in the T.G.T., using bovine platelets	194
61. Fibrinogen estimation in horse plasma	199
62. Thrombin-fibrinogen reaction of horse plasma	200
63. Species specificity of the thrombin-fibrinogen reaction	202
64. Antithrombin assay in horse plasma by the method of Douglas and Biggs, 1953	203
65. Antithrombin assay in horse plasma by the method of Astrup and Darling, 1942d	205
66. Quick's one-stage prothrombin time of horse plasma	207
67. Assay of factor V activity in horse plasma and serum	208

68. Horse factors VII and X - brain thromboplastin species specificity	page 210
69. Horse plasma factor VII assay	211
70. Horse plasma factor X assay	213
71. Horse plasma prothrombin assay by the two-stage method of Biggs and Douglas, 1953a	214
72. Horse plasma prothrombin assay by the globulin fraction method of Douglas and Biggs, 1953	216
73. Horse prothrombin consumption index	216
74. Horse blood platelets' count	217
75. Horse whole blood clotting times	217
76. Recalcification clotting times of horse plasma	218
77. Horse plasma Hageman factor assay	218
78. Thromboplastin generation test, using human platelets..	220
79. Thromboplastin generation test, using horse platelets..	221
80. A.H.G. assay in horse plasma	223
81. Christmas factor assay in horse serum	226
82. Clot retraction of horse blood	229
83. Horse plasma substrate reactivity in the T.G.T., using human platelets	230
84. Horse plasma substrate reactivity in the T.G.T., using horse platelets	231
85. Investigation of inhibitors against human intrinsic thromboplastin in horse plasma and serum in the T.G.T.	234
86. Fibrinogen estimation in rabbit plasma	240
87. Thrombin-fibrinogen reaction of rabbit plasma	241
88. Species specificity of the thrombin-fibrinogen reaction	243
89. Antithrombin assay in rabbit plasma by the method of Douglas and Biggs, 1953	244
90. Antithrombin assay in rabbit plasma by the method of Astrup and Darling, 1942d	246

91. Quick's one-stage prothrombin time of rabbit plasma ..	page 248
92. Rabbit factor V - brain thromboplastin species specificity	249
93. Rabbit plasma factor V assay	250
94. Rabbit factors VII and X - brain thromboplastin species specificity	252
95. Rabbit plasma factor VII assay	253
96. Rabbit plasma factor X assay	255
97. Rabbit plasma prothrombin assay by the two-stage method of Biggs and Douglas, 1953a	256
98. Rabbit plasma prothrombin assay by the globulin fraction method of Douglas and Biggs, 1953	258
99. Rabbit prothrombin consumption index	259
100. Rabbit blood platelets' count	259
101. Rabbit whole blood clotting times	260
102. Recalcification clotting time of rabbit plasma	260
103. Rabbit plasma Hageman factor assay	261
104. Thromboplastin generation test, using human platelets	263
105. Thromboplastin generation test, using rabbit platelets	264
106. A.H.G. assay in rabbit plasma	266
107. Christmas factor assay in rabbit serum	269
108. Clot retraction of rabbit blood	272
109. Rabbit plasma substrate reactivity in the T.G.T., using human platelets	273
110. Rabbit plasma substrate reactivity in the T.G.T., using rabbit platelets	274
111. Investigation of inhibitors against human intrinsic thromboplastin in rabbit plasma and serum in the T.G.T.	277
112. Activity of lobster muscle extract	282
113. Species specificity of lobster muscle extract	283
114. Factor V activity in lobster plasma	284

115. Factor VII activity in lobster serum	page 284
116. Factor X assay in lobster plasma	285
117. R.V.V.-cephalin clotting time of lobster plasma ..	286
118. Thrombin clotting time of lobster plasma	286
119. Antithrombin assay in lobster plasma	287
120. Lobster whole blood clotting times	288
121. Hageman factor and P.T.A. activity in lobster plasma..	289
122. Hageman factor assay in lobster plasma	289
123. Lobster blood cells' count	292
124. Fibrinogen estimation in lobster plasma	297
125. Activity of lobster blood cells' extract	299
126. Measurement of the esteratic activity of lobster blood cells' extract	301
127. Storage lability of the lobster blood cells' extract..	301
128. Effect of heparin on the activity of the lobster blood cells' extract	302
129. Lobster blood cells' extract - fibrinogen reaction ..	303
130. Antihaemophilic globulin activity in lobster plasma ..	304
131. Christmas factor activity in lobster serum	305
132. Development of clotting activity in native lobster whole blood	306
133. Clot retraction of lobster whole blood	307
134. General comparison of the levels of the blood coagulation factors of the investigated species	308

APPENDIX I

THE PREPARATION OF REAGENTS

A P P E N D I X I

THE PREPARATION OF REAGENTS

1. Acetic acid

1 %, 2 %, and 5 % (v/v) solutions of Glacial acetic acid A.R. (Sp.G. 1.060) are made in distilled water.

2. Acetone A.R. (B.D.H.)

3. Adsorbing agents

A- Alumina (Aluminium hydroxide gel) (Method of Bertho and Grassman, 1938).

Solution 1	:	Ammonia (Sp.G. 0.880)	25 mls.
		Distilled water	25 mls.
Solution 2	:	Ammonium sulphate	11 gms.
		Distilled water	300 mls. (in a litre flask)

Solution 3 : 38.35 gms. of ammonium alum (Aluminium ammonium sulphate) are dissolved in 500 mls. distilled water. The solution is then heated to 58°C., at which temperature it is maintained over a pilot flame.

Solution 1 is added to solution 2, the mixture shaken thoroughly and heated to 58°C. Solution 3 is then added while stirring and the mixture kept at 58°C. for 10 minutes. The following washings are then carried out on the precipitate

obtained by centrifugation of the preparation at 2,500 r.p.m. for 10 minutes, the washings similarly separated :-

- a) 750 mls. distilled water + 0.22 ml of solution 1
- b) 750 mls. distilled water + 0.44 ml of solution 1
- c) 750 mls. distilled water,
- d) 750 mls. distilled water,
- e) 750 mls. distilled water.

The supernatant should be clear after each spinning. The precipitate is then suspended in the least amount of water that is required to make a gelatinous suspension that can be pipetted without undue difficulty. The volume of water usually used in this work varied from 85 to 100 mls. The preparation keeps for about six weeks.

B- Barium sulphate (Biggs and Macfarlane, 1957).

1 lb. of x-ray $BaSO_4$ is suspended in about 4 litres of 0.005 M trisodium citrate and allowed to stand overnight. The milky supernatant is drawn off and discarded. The sediment is resuspended in a further 4 litres of citrate solution and again allowed to settle. The sediment is then collected on a large filter paper and dried in a hot air oven at $100^{\circ}C$. The dried material is then powdered. This washing is required because the $BaSO_4$ contains some very fine material which is not removed from the plasma by centrifugation at 2000 r.p.m.

C- Tricalcium phosphate

i. 0.2 M stock solution : 6.66 gms. of anhydrous CaCl_2 are dissolved in 100 mls. of water and added to a solution containing 15.8 gms. trisodium phosphate in 100 mls. water, while thoroughly stirring. The pH is adjusted to 7 and the precipitated $\text{Ca}_3(\text{PO}_4)_2$ is washed repeatedly by decantation till the NaCl is removed. The volume is made up to 200 mls. with water.

ii. 0.016 M working solution : dilute 8 mls. of the stock solution up to 100 mls. with water. Each ml. of this suspension contains 50 mg. tricalcium phosphate. For use, 1 ml. of this 0.016 M suspension is centrifuged, the supernatant discarded, the tube drained, and 1 ml. of the plasma added and mixed with it.

4. Alizarin red indicator (Alizarin mono-sodium sulfonate)

0.1 gm is dissolved in 100 mls. of water and a trace of thymol added to inhibit moulds.

5. Ammonium chloride standard

0.771 gm NH_4Cl is dissolved in 1 litre of water. (0.2 mg N/ml).

6. Ammonium sulphate saturated solution

100 gms. $(\text{NH}_4)_2\text{SO}_4$ are dissolved in 130 mls. of water with the aid of heat, leave to cool and decant.

7. Anticoagulants

A- Ammonium and potassium oxalate mixture

1.2 gms. of ammonium oxalate and 0.8 gm. of potassium oxalate are dissolved in 100 mls. water. Half millilitre volumes (each

containing 10 mgms. of the mixed salts) are delivered into small tubes marked at the 5 ml level, which are then dried in an oven at a temperature not exceeding 60°C. Smaller and larger volumes can be used, adjusting the volume of blood so that 2 mgms. of the dry salts are used for each 1 ml of blood.

B- Potassium oxalate

i. 1.85 gm % (w/v) in water.

ii. 2.5 gm % (w/v) in water.

C- Sequestrene (di-sodium ethylenediamine tetra-acetic acid)

A 10 % (w/v) solution is made in distilled water. 0.1 ml volumes are delivered by pipette into small bottles or tubes marked at the 5 ml level. The solution is then allowed to dry at room temperature.

D- Sodium citrate

3.8 gms. of trisodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2 \text{H}_2\text{O}$) are dissolved in 100 mls. of distilled water.

E- Sodium oxalate (0.1 M)

1.34 gms. are dissolved in 100 mls. of distilled water.

8. Barbitone buffer for electrophoresis (Flynn and DeMayo, 1951)

1.84 gms. barbitone, and 10.3 gms. sodium diethyl barbitone are dissolved in distilled water and the volume made up to one litre. This buffer has an ionic strength (μ) of 0.05 and a pH of 8.6

9. Barium chloride standard solution (Shank and Hoagland, 1946)

0.0962 N solution is made by dissolving 1.175 gms of barium chloride ($\text{BaCl}_2 \cdot 2 \text{H}_2\text{O}$) in 100 mls of distilled water. Three millilitres of this suspension when diluted up to 100 mls with 0.2 N - H_2SO_4 give a turbidity equivalent to 20 units.

10. Calcium chloride solution

A) 0.1 M solution is made by dissolving 11.099 gms of anhydrous CaCl_2 in 1 litre of distilled water. The solution is standardised by titrating it against standard potassium thiocyanate solution. From this stock solution, 0.05 M (M/20) and 0.025 M (M/40) solutions are made by dilution with water.

B) 0.25 M solution is made by dilution from a stock 1 M solution prepared by dissolving 11.099 gms of anhydrous CaCl_2 in 100 mls of distilled water.

C) 3 % solution is made by dissolving 3 gms anhydrous CaCl_2 in 100 mls distilled water.

11. Chloroform A.R. (B.D.H.)

12. Citrate - saline solution

30.6 gms NaCl and 15.2 gms trisodium citrate are dissolved in 4 litres of distilled water.

13. De-ionised water (called distilled water throughout this work)

Water de-ionised in an Elgastat portable Deioniser, Type B 102 (Elga Products Ltd.) was used throughout this work instead of distilled water. It had a resistance of more than 4,000,000 ohms,

which means it is purer than water that has passed three distillations in glass whose resistance is usually about 800,000 ohms.

14. Folin and Ciocalteu's reagent (B.D.H.).

15. Formaldehyde solution (37 %, pH 8)

The pH is adjusted by adding NaOH solution till phenolphthalein indicator (0.04 % in 95 % alcohol) becomes faintly pink.

16. Formol - citrate solution

3 gms trisodium citrate are dissolved in water, and 1 ml of 40 % formalin is added, and the final volume made up to 100 mls. Filter before use.

17. Glass powder

Pyrex glass powder of porosity 3, mesh 200, is vigorously stirred with distilled water (1.5 litres/100 gms powder). Stand for 12 hours, and decant the turbid supernatant. Repeat till the supernatant is clear, and dry the powder on filter paper in the hot air oven.

18. Glycerol A.R. (B.D.H.)

19. Heparin

Pularin vials (Evans Medical Ltd., Liverpool) containing 5,000 units/ml were used.

20. Imidazole (Glyoxaline) buffer (Mertz and Owen, 1940)

Base solution : 680 mg imidazole dissolved in 50 mls water.

Buffer at pH 7.3 : 2.5 parts of base solution + 1.86 parts

N/10 HCl + 5.64 parts of distilled water.

21. Hydrochloric acid

Normal solution : 86 mls of concentrated HCl (Sp.G. 1.18) are made up to 1 litre with water.

N/6 and N/10 solutions : are made from the above by dilution.

22. Kjeldahl catalyst tablets (B.D.H.)

Each tablet contains 1 gm Na_2SO_4 and the equivalent of 0.1 gm of mercury.

23. Leishman's stain

0.15 gm of Leishman powder is dissolved in 100 mls of methyl alcohol by grinding in a mortar. The stain is left to mature in a clear glass bottle for few months. Filter before use.

24. Phenol red indicator (B.D.H.)

0.01 gm of phenol red powder is dissolved in 100 mls distilled water to make a 0.01 % solution.

25. Phosphate buffers

A) For fibrinogen preparation (Biggs and Macfarlane, 1957, as modified from Jaques, 1943). A 2 molar phosphate buffer at pH 6.6 is made by dissolving 817 gms of anhydrous KH_2PO_4 in one litre of distilled water to which has been added 750 mls of 4 N KOH solution (containing 168 gms KOH). After warming to dissolve, the volume is made up to 3 litres and the solution filtered. Molar and M/4 solutions are made from this stock solution by dilution.

B) For elution of prothrombin (Biggs and Macfarlane, 1957)

Solution 1 : 11.88 gms $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$ are dissolved in 1 litre of distilled water to make an M/15 solution.

Solution 2 : 9.1 gms KH_2PO_4 are dissolved in 1 litre of distilled water to make an M/15 solution.

To make a buffer at pH 8, 9.7 parts of solution 1 are added to 0.3 parts of solution 2.

26. Polybrene (Hexadimethrine bromide)

Polybrene is the brand name of hexadimethrine bromide which is manufactured by Abbott Laboratories, North Chicago, Illinois. The contents of one ampoule (100 mg dissolved in 10 mls isotonic aqueous solution) were used as such.

27. Ponceau S stain

0.2 gm % solution of the powder is made in 3 % aqueous trichloroacetic acid solution.

28. Pyrocatechol solution

1 gm of o-dihydroxy-benzene (B.D.H.) is dissolved in 100 mls of saline.

29. Saline solution

A sterile solution containing 0.877 gms NaCl/100 mls distilled water was used.

30. Siliconed glassware (Dacie, 1956)

A 10 % (v/v) solution of silicone M 441 (I.C.I.) is made in petroleum ether. The clean glassware is immersed in it for five

minutes, allowed to drain dry, then soaked overnight in distilled water, rinsed in a change of distilled water, and finally dried in an incubator.

31. Sodium carbonate

0.5 %, 2 %, and 20 % solutions are made by dissolving the respective weights of anhydrous Na_2CO_3 in 100 ml amounts of water. Filter the concentrated solution before use.

32. Sodium citrate in saline (as eluate)

5 gms of trisodium citrate are dissolved in 100 mls of saline.

33. Sodium chloride

A 1 % (w/v) solution in distilled water was used in the turbidimetric method of fibrinogen estimation.

34. Sodium hydroxide

A) Normal solution : 40 gms of NaOH are dissolved in 1 litre of freshly boiled distilled water (CO_3 - free).

B) N/2 , N/6 , N/20 , N/70 , and N/100 solutions : these are made by dilution from the above normal solution, the N/70 solution is standardised by titration against N/50 sulphuric acid before using it in the micro-Kjeldahl method for protein estimation.

C) 10 % and 40 % solutions : dissolve the respective weights of NaOH in 100 ml volumes of distilled water. Filter before use.

35. Sterimats (T.B.Ford Ltd., High Wycombe, Bucks.)

Two kinds of Sterimat asbestos filter pads of 14 cm. diameter

were used, one containing 20 % asbestos (FCB), and the other 30 % asbestos (S/FC/2).

36. Sulphuric acid

A) Concentrated A.R.: Sp.G. 1.84

B) Normal solution : 27.8 mls of the A.R. acid are added slowly to water and the volume made up to 1 litre.

C) N/5 and N/50 solutions : are made from the above by dilution.

37. TAME substrate (L.Light & Co. Ltd., Colnbrook, England.)

0.746 gm of TAME powder (p-toluene sulphonyl-L-arginine methyl ester) is thoroughly dissolved in 10 mls of distilled water to make a 0.2 molar solution.

38. Toluidine blue

i. 25 mg toluidine blue powder dissolved in 100 mls saline.

ii. 50 mg toluidine blue powder dissolved in 100 mls saline.

39. Tris buffer

60.57 gms of Tris powder (2-amino-2-(hydroxy-methyl)-1:3-propanediol) Tris-(hydroxy-methyl)-amino-methane, (B.D.H.) are dissolved in about 800 mls of distilled water to make a 0.5 molar solution. The pH is adjusted to 9 by adding 2 N- HCl, finally the volume is made up to one litre with water.

40. Tyrosine

A) Stock standard solution : 100 mg of DL-Tyrosine (B.D.H.) are dissolved in 100 mls of N/10 HCl.

B) Working standard solution : dilute the stock solution 1 in 10 in water to make a solution containing 0.1 mg/ml.

41. Veronal buffer (Owren, 1949).

5.878 gms of sodium diethyl barbiturate, and 7.335 gms of sodium chloride are dissolved in a mixture of 500 mls water and 215 mls of N/10 HCl. After complete solution, the volume is made up to 1 litre with water and the pH checked. This buffer has an ionic strength (μ) of 0.15 and its pH should be 7.33

42. Zinc powder A.R. (B.D.H.)

This powder is virtually NH_3 -free. The maximum limit of impurity for nitrogen is 0.02 %.

APPENDIX II

GENERAL TECHNIQUE AND EQUIPMENT

A P P E N D I X I I

GENERAL TECHNIQUE AND EQUIPMENT

The following specifications of general technique have been applied throughout this work.

1. Collection of blood specimens

Blood was obtained by the use of siliconed syringes and needles (appendix I, 30) by applying the least suction possible to avoid frothing. The blood was immediately delivered into siliconed tubes containing the anticoagulant and the contents mixed by gentle inversion of the tube twice. Samples suspect of frothing or incipient clotting were abandoned. All specimens were used fresh as soon after collection as possible, keeping them meanwhile at 4°C.

The various sites for withdrawal of the blood varied according to the species concerned as follows :-

<u>Species</u>	<u>Site of withdrawal</u>	<u>Needle (S.W.G.)</u>	<u>Amount</u>
i. Human	: antecubital veins	2	5 - 10 ml.
ii. Chicken	: wing veins	12	2 - 5 ml.
iii. Lobster	: claw joints	1	2 - 5 ml.
iv. Horse	: external jugular vein	1	10 - 20 ml.
v. Rabbit	: ear marginal veins	cut by sharp razor	5 - 10 ml.
vi. Cattle	: bovine blood was obtained from the slaughter-house		

where the cows were killed by the captive bolt, and while the animal was bled from the neck the midstream blood was collected in a siliconed plastic beaker and immediately delivered in the appropriate siliconed tubes. The time between the slaughter of the animal and blood mixing with the anticoagulant was about one minute. The plasma was separated within half an hour of the collection of the blood.

2. Centrifugation

All centrifugations were carried out in a refrigerated M.S.E. Magnum centrifuge (model S 51) set at 4°C., unless otherwise indicated. The following are the relative centrifugal forces (RCF) or g values for the various speeds used with each particular head :-

<u>Speed</u> (r.p.m.)	<u>16-place head for tubes</u> (up to 50 ml.)	<u>6-place head for cups</u> (50 - 250 ml. each)
500 :	72 g	65 g
1,000 :	290 g	257 g
1,500 :	651 g	578 g
2,000 :	1,160 g	1,029 g
2,500 :	1,810 g	1,610 g
2,800 :	2,270 g	---
	<u>Super speed 6-place angle head (up to 7 ml.)</u>	
5,000 :	1,930 g	
10,000 :	7,724 g	
15,000 :	17,380 g	
20,000 :	30,900 g	
25,000 :	48,280 g	

3. Clotting experiments

These were carried out in glass tubes of uniform diameter (10 x 75 mms.), which were placed shortly before use in an electric water-bath, type S.B.2 (Grant Instruments (Cambridge) Ltd.) which was thermostatically controlled at $37^{\circ}\text{C.} \pm 0.25$ (unless otherwise stated) and fitted with a propeller circulator which ensures virtually no temperature gradient throughout the bath, even when fully loaded with test-tubes.

Samples were delivered using blood pipettes of 0.1 ml and 0.2 ml ± 0.002 ml, (Elliott Ltd., E-Mil Works, Treforest, Glam.)

4. Dialysis

Dialysis was performed at 4°C. in Visking casings, (H.M.C.).

5. pH measurements

During the first year of this work (1961) the pH was measured by the E.I.L. direct reading pH meter, model 23 A (Electronic Instruments Ltd.). Thereafter by a Beckman 9600-9602 Zeromatic direct reading pH meter.

6. Spectrophotometric measurements

Were performed using a Unicam S.P.500 spectrophotometer, (Unicam Instruments Ltd.) using cuvettes of 1 cm. diameter.

7. Electrophoresis

Electrophoretic runs were performed in a horizontal Shandon tank, using cellulose acetate strips (Oxoid) measuring 5 x 20 cms. The current was supplied through a constant voltage power supply

unit, type 2523 (Shandon Scientific Co. Ltd., London) giving a volt gradient of 7.5 V/cm., and 0.7m.amp./cm. width of the strip. Duration of the run was 2 hours. A barbitone buffer of pH 8.6 was used (appendix I, 8). The strips were stained with Ponceau S stain (appendix I, 27) for 5 - 10 minutes, and washed in three successive trays containing 5 % aqueous acetic acid. Finally, they were cleared in paraffin oil.

Densitometric scanning of the electrophoresis strips was carried out in an electric double-beam recording and integrating reflectance densitometer, Chromoscan J 118 (Joyce, Loebel, and Co. Ltd., England) using the blue filter, and the slit aperture. With the gain control set at 5, the gear ratio 1 : 1 was selected to allow direct matching between the strips and their tracings. Cam 5-034/B was used for all the tracings.

8. Phase contrast microscopy and photomicrography

An Ortholux microscope (Leitz) was used, fitted with built-in illumination, Heine condenser, using the oculars x8 and x12 in combination with the objectives Pv achromatic 20/0.45 and Pv n apo 40/0.70. The photomicrographs were taken with a Leica 35 mm camera (Leitz Instruments Ltd., London) fitted with a visoflex attachment, on Ektachrome high-speed artificial light film. Prints from the transparencies were made commercially by Kodak Ltd., London.

9. Micro-titration

This was carried out using the syringe type microburette (model SB2) fitted with ST buret tip, (Micro-Metric Instruments Co., Cleveland, Ohio.). This outfit delivers accurately volumes of 0.001 ml which registers as one division on the counter dial.

A P P E N D I X I I I

P R E P A R A T I O N O F C O A G U L A T I O N F A C T O R S

A P P E N D I X I I I

P R E P A R A T I O N O F C O A G U L A T I O N F A C T O R S

1. Preparation of citrated platelet-rich plasma

Whole blood is collected in a graduated centrifuge tube and mixed with 3.8 % trisodium citrate in the proportion of 9 parts blood to 1 part citrate. The mixture is centrifuged for 5' at 1,500 r.p.m., and the supernatant plasma pipetted off into a clean lusteroid tube.

N.B. i. Chicken plasma treated as above contains virtually no platelets, as they are nucleated cells and rapidly sediment with the other cells. To obtain platelet-rich chicken plasma it was necessary to spin the blood at 500 r.p.m.; the plasma in such circumstances contains also some white blood cells, mainly the lymphocytes which are of about the same size as chicken thrombocytes.

ii. Bovine blood had to be spun at 2,500 r.p.m. (at least) to effect as good a separation as with human blood. Such plasma was used for all assays except those necessitating the presence of the platelets. To obtain platelet-rich bovine plasma the blood was spun several times (for 5 minutes each) starting with

very slow speeds, and after pipetting off the supernatant few millilitres, the blood is spun again at a higher speed, and so on. The first two thirds only of the plasma is used as the platelet-rich fraction.

2. Preparation of citrated platelet-poor plasma

(High-spun plasma)

The platelet-rich plasma prepared as above is centrifuged at 15,000 r.p.m. for 5 minutes, the supernatant decanted into lusteroid tubes and kept at -20°C . The plug of sedimented platelets is used for the preparation of platelet suspensions.

3. Preparation of oxalated plasma

Nine parts of blood were mixed with one part of 1.34 % sodium oxalate (unless otherwise mentioned) and centrifuged for 5 minutes at 1,500 r.p.m.

N.B. Crustacean blood (haemolymph) was constantly obtained over 2.5 % potassium oxalate in the proportion of 9 to 1, and immediately centrifuged for 5 minutes at 2,000 r.p.m.

4. Preparation of adsorbed plasma

A) Alumina-adsorbed plasma (Alumina plasma)

1 ml citrated plasma is mixed with 0.1 ml alumina (appendix I, 3A),

and the mixture allowed to stand at 37°C. for 3', then centrifuged for 5' at 1,500 r.p.m. to remove the alumina. For use, such treated plasma is usually diluted 1 in 5 with saline. Throughout the text, by "adsorbed plasma" is meant alumina-treated plasma.

B) Barium sulphate - adsorbed plasma

1 ml oxalated plasma is mixed with 100 mg BaSO₄ (appendix I, 3B), the mixture incubated at room temperature for 15' with occasional stirring, then centrifuged at 2,000 r.p.m. for 15' to remove the BaSO₄. Other variations are indicated where necessary.

C) Ca₃(PO₄)₂ - adsorbed plasma

1 ml oxalated plasma is mixed with 50 mg tricalcium phosphate (appendix I, 3C), the mixture allowed to stand at room temperature for 10' with occasional stirring, and the deposit removed by centrifuging.

5. Preparation of serum

Whole blood is collected in a dry tube and allowed to stand for 4 hours at 37°C., the serum is then separated by centrifugation. Such serum when prepared from human blood has been shown not to contain any appreciable amounts of prothrombin, factor V, or A.H.G. (Douglas and Biggs, 1953). For lobster serum, whole lobster blood was allowed to clot spontaneously in ordinary glass tubes, then shaken with ballotini glass beads, centrifuged, and

the supernatant separated for use.

6. Preparation of fibrinogen

A) Phosphate precipitation method (Biggs and Macfarlane, 1957)

To 1 litre of fresh citrated bank plasma is added 20 ml alumina (appendix I, 3A), and the mixture placed at 37°C. for 15', then centrifuged at 2,000 r.p.m. for 15'. The supernatant is tested by Quick's one-stage test; the clotting time should be more than 90" to ensure proper adsorption, or else the plasma will need re-adsorption. The supernatant is poured into a measuring cylinder, cooled to 4°C., and the volume noted, volume X. The following steps are then carried out :-

- To X mls plasma are added X mls 2M phosphate buffer previously cooled to 4°C. (appendix I, 25A). The mixture is stood for 15' then centrifuged at 2,000 r.p.m. for 15' at 4°C. Discard the supernatant.
- The precipitate is washed in 300 mls M phosphate buffer, and centrifuged at 2,000 r.p.m. for 15'.
- Dissolve the precipitate in 250 mls M/4 phosphate buffer ...:
volume Y.
- Y mls precipitate solution + Y mls 2M phosphate buffer, centrifuge for 15'.
- Wash ppt. in 200 mls M phosphate buffer and centrifuge for 15'.

- Dissolve ppt. in 130 mls M/4 phosphate buffer: volume Z.
- Z mls ppt. solution + Z mls 2M phosphate buffer, centrifuge.
- Wash ppt. in 120 mls M phosphate buffer, and centrifuge.

The final precipitate is dissolved in 100 mls citrate-saline solution (appendix I, 12) then dialysed against 4 litres of citrate-saline solution in the cold, overnight. The solution is then centrifuged at 4°C. for 15' at 2,000 r.p.m. to make it free from pro-fibrin (Apitz, 1938; Owren, 1947), then delivered in 5 ml amounts in screw-capped bijou bottles, and stored at -20°C.

For use, this fibrinogen solution was diluted 1 in 2 in saline. Fibrinogen prepared by this method is known to be free from other known coagulation factors except A.H.G. and possibly plasminogen. The relative purity of the different batches was also ascertained by clotting doublefolding dilutions of the fibrinogen in saline with a standard amount of thrombin, and plotting the clotting times in seconds against the fibrinogen concentrations, which should give a straight line relationship. Also by clotting the same amount of fibrinogen with doublefolding dilutions of thrombin, when a pure preparation should give a straight line on plotting the clotting times in seconds against the reciprocal of the thrombin concentrations (Astrup and Darling, 1941).

Before using a newly prepared fibrinogen solution, a thrombin-fibrinogen dilution curve was constructed for it using the stock

standard thrombin, as described in appendix IV, 7, and illustrated in figure 1. If this curve was found to conform to the original thrombin-fibrinogen dilution curve, the fibrinogen is suitable for use, otherwise it was discarded.

Fibrinogen prepared and tested as above was used as substrate in all test systems whenever required, unless otherwise stated.

Fibrinogen solutions of other species were prepared by the same method and under the same conditions as the human control, only using proportionately smaller quantities all through the method.

B) Ammonium sulphate precipitation method (modified from Duchâteau and Florkin, 1954). This method was used only to prepare crustacean fibrinogen.

- The lobster haemolymph was obtained (as described in appendix II, 1-iii) over 2.5 % potassium oxalate monohydrate, in the proportion of 9 to 1 respectively, and immediately centrifuged at 1,500 r.p.m. for 5'.

- The pH of the haemolymph is adjusted to 6.8 by the addition of 1 % acetic acid.

- To a volume of the supernatant "plasma" is added drop by drop a half volume of saturated ammonium sulphate solution while the mixture is stirred by a magnetic stirrer.

- Leave the mixture to precipitate for 12 hours (at least), then

centrifuge at 3,000 r.p.m. for 15'. Discard the supernatant and drain the tubes well, then dissolve the rose-orange ppt. in a volume of saline equal to three quarters the original plasma volume: ppt. 1 solution.

- To ppt. 1 solution is added dropwise saturated ammonium sulphate solution till its concentration attains 31 %, stand for 2 hours, then centrifuge. The ppt. is dissolved in saline equal to one quarter the original plasma volume: ppt. 2 solution.

The supernatant is kept.

- To the supernatant which is slightly opalescent is added more ammonium sulphate saturated solution to attain a concentration of 45 %, stand for 12 hours at least, then centrifuge at 3,000 r.p.m. for 15', and dissolve the ppt. in a volume of saline equal to one quarter the original plasma volume: ppt. 3 solution.

Filter to eliminate the insoluble residue.

- The different precipitate solutions (1, 2, and 3) were dialysed against saline in the cold, overnight, as soon as each preparation was made. The fibrinogen solutions were kept in 5 ml amounts at -20°C .

In the original authors' method, the blood was left after the spinning down of the cells in the second step, in the cold for 12 - 24 hours. This step was dispensed with, as in most instances the blood was found to have clotted during that period.

Again, they dissolved the precipitates obtained in distilled water, and no dialysis was done. Solution in saline was thought to be more physiological, and the dialysis introduced to get rid of the excess sulphate ions.

In their hands ppt.3 was the purest form, being electrophoretically homogeneous, while ppt.1 was of moderate purity (contaminated with haemocyanin) and ppt.2 of no importance at all as it clotted in 12 minutes. As explained in the text in more detail, using the above modifications, ppt.1 was obtained as of moderate purity, while ppt.2 was the purest, most reactive, and electrophoretically homogeneous fibrinogen preparation (figure 74), while ppt.3 was very minimal and contained no fibrinogen at all, being non-clottable.

7. Preparation of thrombin

A) Thrombin Topical (Parke, Davis & Co.)

This is a bovine thrombin preparation, commercially processed from bovine plasma by the addition of traces of tissue thromboplastin and calcium chloride to activate the prothrombin. It was used throughout this study for the various assays, unless otherwise stated, as in the species specificity tests of the thrombin and fibrinogen solutions. The contents of 1 vial were dissolved in 5 mls of glycerol-saline mixture (equal parts) giving a concentration of 1,000 N.I.H.units/ml. This is kept at -20°C ., and before use

dilutions were made in saline. Each batch was cross-matched against the standardised fibrinogen by the thrombin-fibrinogen dilution curve procedure. If it was found to conform to the original dilution curve, which invariably occurred except in one batch that was discarded, denoting an identical concentration as the original thrombin solution, it was released for use in the various assays, and also kept as reference standard for standardising future fibrinogen preparations, within one month of its preparation.

Such concentrated thrombin solutions kept at -20°C . for one month were found to have the same potency as when fresh, as judged by the similar thrombin-fibrinogen dilution curves obtained with the same batch of fibrinogen.

B) Thrombins of other species (Biggs and Macfarlane, 1957)

400 mls of citrated fresh plasma is made up to 4 litres with distilled water, and the pH adjusted to 5.3 with 2 % acetic acid. Centrifuge and discard the supernatant. Dissolve the ppt. in 100 mls saline and readjust the pH to 7 with 2 % sodium carbonate. To this solution is added 12 mls $\text{M}/4 \text{CaCl}_2$ and the coagulated fibrin removed as it forms, this is performed using one's hand and squeezing the fibrin tightly before its removal. Stand the solution for 2 hours to allow for maximum thrombin generation. Add an equal amount of acetone to the remaining solution, and centrifuge. The thrombin is extracted from the precipitate by adding saline (100 mls) and stirring with a glass rod to break

up the precipitate. Centrifuge and discard the precipitate. The thrombin solution is dialysed against distilled water in the cold, overnight, then stored in 2 ml amounts in the deep-freeze.

Usually the various thrombins were prepared from proportionately smaller amounts alongside the human control, under the same experimental conditions.

8. Preparation of prothrombin

(method of Biggs and Macfarlane, 1957)

To 1 litre of fresh normal citrated platelet-poor plasma is added 25 mls alumina and the mixture incubated for 15' at 37°C., then centrifuged and the supernatant tested by Quick's one-stage prothrombin time. The clotting time should be longer than 90", else the plasma is to be reabsorbed. The supernatant is either discarded or used for the preparation of A.H.G. or fibrinogen if required. The precipitate is washed twice with cold distilled water. The prothrombin is then eluted from the ppt. by mixing it with 25 mls of phosphate buffer at pH 8 (appendix I, 25,B) and standing the mixture at 37°C. for 20 - 60 minutes, then centrifuged. The supernatant which contains the prothrombin is retained, and the ppt. is again eluted with a further 25 mls of phosphate buffer, the second supernatant is then added to the first, and the pH adjusted to 7 by adding 2 % acetic acid. This

final solution is dialysed against 4 litres of citrate-saline in the cold, overnight, then stored in 5 ml amounts in the deep-freeze. Such prothrombin preparations were free from A.H.G., factor V, fibrinogen, antithrombin, and plasmin.

For preparing prothrombin of other species proportionately smaller amounts of reagents were used, depending on the quantity of plasma available.

9. Preparation of factor V

(method of Douglas and Biggs, 1953)

Two mls. of fresh citrated plasma are treated with 0.2 ml of alumina for 3' at 37°C. and the alumina removed by centrifuging. To the supernatant in a high-spinning tube is added 1 ml of saturated ammonium sulphate solution, mixed well, then centrifuged at 15,000 r.p.m. for 5'. The supernatant is poured into another clean high-spinning tube, 1 ml of saturated ammonium sulphate solution is added with thorough mixing, the mixture centrifuged again at 15,000 r.p.m. for 5' and the supernatant discarded. The precipitate which contains the factor V is dissolved in 2 mls citrate-saline and dialysed against 4 litres of citrate-saline in the cold, overnight. The solution is then diluted to 10 mls with saline and tested as follows :-

0.3 ml amounts of prothrombin (appendix III, 8), factor V

solution under test (different dilutions), and brain thromboplastin (appendix III, 13) are delivered into a clotting tube placed in a water-bath at 37°C. and the mixture recalcified with 0.3 ml M/40 calcium chloride solution, simultaneously starting a stop watch. After 5 and 6 minute intervals, 0.1 ml amounts of the incubation mixture are pipetted off onto 0.4 ml amounts of fibrinogen solution previously warmed to 37°C. and the clotting times noted. The highest dilution giving a reasonably short clotting time is noted and the factor V preparation diluted accordingly, as it may be very potent, then stored in 2 ml amounts at -20°C. Such a factor V preparation has been shown to be free from prothrombin, factor VII, Christmas factor, Stuart-Prower factor, and most of the A.H.G., fibrinogen, and antithrombin, (Douglas, 1957). When prepared from bovine plasma, the factor V substance usually contains some prothrombin (Biggs and Macfarlane, 1962).

10. Preparation of factor VII

(Biggs and Macfarlane, 1957)

Ten mls. of serum (appendix III, 5) are adsorbed with barium sulphate powder (100 mg/ml) at 37°C. for 15'. The mixture is centrifuged at 2,000 r.p.m. for 10' and the ppt. washed twice with cold distilled water. The deposit is treated with 5 mls of 5 % trisodium citrate in saline (appendix I, 32) for 15' at 37°C.

The inactive BaSO₄ is removed by centrifugation, and the eluate dialysed against 5 litres citrate-saline in the cold overnight, finally the volume is made up to 10 mls with saline.

This preparation contains factors VII, IX, and X.

11. Preparation of anti-haemophilic globulin (factor VIII)

(Biggs and Macfarlane, 1957)

The alumina adsorbed plasma which remains after step 2 for prothrombin preparation is used. The supernatant which contains little prothrombin, factor VII, or factor IX, is precipitated with 33 % saturation of ammonium sulphate, i.e. to each 10 mls adsorbed plasma is added 5 mls saturated ammonium sulphate solution.

The mixture is centrifuged and the ppt. dissolved in a volume of saline equal to half the original plasma volume, i.e. 5 mls.

This solution is dialysed against 5 litres of citrate-saline in the cold, overnight, and the final volume is made up with saline to 10 mls (the original adsorbed plasma volume).

This A.H.G. preparation was always used fresh for the various tests.

12. Preparation of Christmas factor (factor IX)

(White, Aggeler, and Glendening, 1953)

Whole blood is allowed to clot and stand for 24 hours at room

temperature. The serum is separated after centrifuging, and to 9 mls of serum is added 1 ml of 3.2 % trisodium citrate and the mixture stood for 30' at 37°C. to allow for the neutralization of any remaining thrombin. The pH is adjusted to 2.9 by the dropwise addition of N-HCl, and allow to stand for 2 hours at 37°C., to destroy factor VII activity. The pH is readjusted to 7.3 with N-NaOH. The serum is then adsorbed with BaSO₄ (100 mg per ml) for 15' at 37°C. The mixture is centrifuged at 2,000 r.p.m. for 30', and the ppt. washed twice with cold distilled water. The ppt. is then eluted with 5 mls of 5 % trisodium citrate in saline for 15' at 37°C., and the eluate dialysed against 5 litres of citrate-saline, in the cold overnight. The volume of the Christmas factor solution is then made up with saline to the original serum volume.

13. Preparation of tissue extracts

I. Preparation of brain thromboplastin extract (Owren, 1949)

A fresh whole normal brain is freed from all blood vessels and membranes and washed well with water. The brain matter is emulsified in an equal volume of saline warmed to 37°C. for one minute in an electric homogeniser. The emulsion is centrifuged at 2,000 r.p.m. for 30', the supernatant separated and the deposit discarded. Doublefolding dilutions of the supernatant are made

in saline and tested by the one-stage prothrombin time using normal homologous plasma and M/40 CaCl_2 . The dilution which gives the shortest clotting time (13 - 16 seconds) is noted, and the whole supernatant diluted accordingly. To this extract is added a one tenth volume of Owren's buffer (appendix I, 41). The final extract is distributed in 5 ml amounts in small bijou bottles and stored at -20°C . A fresh bottle is opened at a time, and once used should not be re-frozen for later use.

For the preparation of chicken brain extract it was necessary to use from 6 to 10 whole chicken brains for one preparation, so as to supply enough material of the same potency for as large a battery of tests as possible.

II. Preparation of crustacean muscle extract

A) By the method of Loeb, 1905

One part of fresh lobster muscle is washed well with water, then ground with one part sand and one part distilled water in a mortar. The homogenate is stood overnight in the refrigerator, then centrifuged at 3,000 r.p.m. for 10 minutes. The supernatant is delivered into small bottles and kept at -20°C .

B) By the method of Owren, 1949

The same procedure is used as for the preparation of the brain thromboplastin extract, except for substituting the lobster muscle instead of the brain substance.

14. Preparation of crude cephalin

(Bell and Alton, 1954)

Preparation of acetone dried brain (Biggs and Macfarlane, 1957)

A fresh human brain is obtained, freed from all blood vessels and meninges, then macerated with 3 - 4 times its volume of acetone in a mortar. The acetone is changed 4 times and the granular powder is dried on a suction filter (200 gms wet weight of brain yield about 30 gms dry powder).

Extraction of the cephalin

The acetone dried brain is further washed with acetone (20 mls per gm of brain powder) till the washings are cholesterol-free as judged by a negative Liebermann-Burchard reaction. After removal of the supernatant acetone, the brain powder is again dried on a suction filter. This powder is extracted with chloroform A.R. (50 mls/gm powder) at room temperature for 2 hours with occasional shakings. Filter to remove the ppt. which is discarded, and the filtrate evaporated under vacuum. This usually yields about 300 mg of gummy active material for every gram of dried brain used. The residue is finely homogenised in saline (50 mls/gm dried brain) to give a concentration of about 0.6 gm cephalin per cent, then stored in small amounts at -20°C . When frozen, this stock solution is stable for 12 months at least and can be thawed and refrozen

repeatedly without loss of activity. The exact dilution of each batch for maximal potency should be determined.

For use, the stock solution is diluted 1 in 100 in saline or in veronal buffer. This preparation would contain about 0.006 gm cephalin per cent and is designated as Cephalin 1/100 throughout this study. Other dilutions were sometimes used and are mentioned where appropriate.

This cephalin preparation has proved relatively pure as the clotting times of recalcified plasma with various dilutions of cephalin increased with higher dilutions (i.e. decreased activity); had it been a crude preparation the clotting times should have been shorter with the initial dilutions due to dilution of inhibitors. Also it has been shown to possess no thromboplastic lipoprotein contaminants i.e. no free thromboplastic activity.

15. Preparation of platelet suspensions

A) Human platelet suspension (Biggs and Macfarlane, 1957)

Fresh platelet-rich human plasma is spun at 15,000 r.p.m. for 5' and the clear supernatant removed (used as high-spun plasma). The button of platelets is washed twice in saline after thoroughly resuspending the platelets in it. The final plug of platelets is suspended in saline in a volume equal to half that of the original plasma. The suspension is stored at -20°C . in small amounts,

each sufficient for one day's use. For use in the T.G.T. the suspension is diluted in saline (1 : 2).

The same procedure was used for the preparation of horse and rabbit platelet suspensions.

B) Chicken thrombocyte suspension

Fresh platelet-rich chicken plasma prepared as described in appendix III, 1, i, is treated as above. This preparation also contains some lymphocytes.

C) Bovine platelet suspension

Fresh citrated platelet-rich bovine plasma obtained by the stepwise centrifugation method described in appendix III, 1, ii, is treated as above.

16. Preparation of crustacean blood cells' extract

Crustacean blood (haemolymph) is obtained from a joint space by the use of siliconed needles and syringes. Nine parts of the blood are mixed with one part of 2.5 % potassium oxalate solution in a siliconed centrifuge tube and is immediately spun at 4°C. for five minutes at 2,000 r.p.m. The supernatant blue plasma is decanted and the tube drained well and rinsed with saline without dislodging the precipitate. This cell sediment is suspended in a volume of saline equal to one third the original blood volume, and is thoroughly broken up using wooden applicator sticks, for

one to two minutes. The suspension is centrifuged again for five minutes at 2,000 r.p.m., the clear supernatant is decanted into a clean lusteroid tube and used as fresh as possible.

A potent preparation should clot an aliquot of oxalated lobster plasma in the presence of calcium chloride solution (3%) in about six seconds. It also should clot an aliquot of the purified lobster fibrinogen in the presence of calcium chloride solution in about the same time.

On keeping, this preparation was found to lose all activity even if kept frozen at -20°C . for several hours only.

17. Preparation of Russell's viper venom reagent

A) R.V.V. in saline

The contents of each vial of Styppen (Burroughs Wellcome & Co.) are dissolved in 1 ml saline solution and stored frozen at -20°C . The concentration of this solution is 0.1 mg/ml and usually expressed as 1/10,000 (i.e. 1 gm/10,000 mls). Further dilutions in saline are made before use as required.

B) R.V.V. in cephalin

The contents of each vial of Styppen (0.1 mg) are dissolved in 1 ml of 1/10 cephalin suspension. The latter is made by dilution of the stock cephalin suspension (appendix III, 14) 1 : 10 in veronal buffer (appendix I, 41). The concentration of R.V.V.

in this solution is 1/10,000, and the concentration of cephalin is 0.06 gm % approximately. This is kept frozen at -20°C . in 0.5 ml volumes. Before use, further dilutions are made in cephalin suspension (1/10) to give final concentrations of R.V.V. of 1/40,000, 1/80,000, and 1/200,000 as required.

APPENDIX IV

ASSAY METHODS AND SPECIAL TECHNIQUES

A P P E N D I X I V

ASSAY METHODS AND SPECIAL TECHNIQUES

Throughout this work fresh blood or reagents prepared therefrom, obtained from the same subject (the author) were used to represent the normal human control materials. They were ascribed 100 per cent values so that the results obtained for the various species can be assessed on a relatively uniform basis.

Fresh test samples of plasma or serum from each species were pooled from at least three animals each time to obviate individual variations in the coagulation factors' levels from affecting the results as much as possible.

1. Platelet and thrombocyte counts

A) Platelet count (Dacie, 1956)

The following method was used for counting human, bovine, equine, and rabbit platelets.

Ten mls. of filtered formol-citrate solution (appendix I, 16) are delivered into a clean screw-capped bottle. A small volume of freshly withdrawn blood is delivered into a waxed watch-glass (after removal of the syringe needle) and the blood immediately

diluted 1 in 100 in the formol-citrate solution by using a 0.1 and 0.2 ml blood pipette, first drawing the diluting fluid up to the 0.1 mark, then the blood is taken up into the pipette till the upper level of the fluid reaches the 0.2 mark. The contents of the pipette are washed into the 9.9 mls of diluting fluid remaining in the bottle. The suspension of blood is well mixed by inversion several times. The platelets are well preserved in this fluid for several hours. The contents of the bottle are again well shaken for 2 minutes before a Neubauer counting chamber is filled with the suspension, which is placed in a moist chamber and left undisturbed for 20 minutes to allow the platelets to settle. The preparation is then examined under the microscope. Racking down the condenser renders the platelets easily distinguished, appearing as highly refractile particles. They should be well separated from each other, the presence of many platelet clumps invalidates the count in which case it should be repeated. The number of platelets in one square millimetre are counted, and the procedure repeated on the other side of the chamber, taking the average of the total counts.

Calculation Total platelet count /c.mm. whole blood =
average count/sq.mm. x 10 x 100 (dilution factor).

N.B. i. For human platelet counts, fresh venous blood was obtained by a clean venepuncture using siliconed syringes.

ii. For bovine platelet counts, blood was collected at the slaughter house in siliconed plastic containers as described in appendix II, 1, vi, and immediately delivered into a 5 ml bijou bottle containing the appropriate amount of dried sequestrene (appendix I, 7, C), well shaken, and the count carried out within 1 hour of collection.

B) Chicken thrombocyte counts

Since chicken platelets are nucleated cells it was necessary to count the total white blood corpuscles first, then make a differential white cell count from a blood film in which is included the thrombocytes. From these data the total thrombocyte counts were calculated.

i. Preparation and staining of the blood film: a drop of fresh blood is obtained by pricking the wing vein and immediately spread on a glass slide and allowed to dry in the air. The slide is flooded with Leishman's stain (appendix I, 23) for 2 - 3', then double the volume of distilled water is added and well mixed by blowing through a pasteur pipette. A shiny scale forms if the dilution is properly done. After 5 - 7' the slide is washed in a stream of distilled water till the film differentiates into a rose-pink colour. The film is then allowed to dry in the air.

ii. Differential count: thrombocytes are apt to be mistaken for lymphocytes unless great care is paid to detail. The former

are of similar size as the small lymphocytes, occur either singly or in groups of two or three, have a rather abundant light violet cytoplasm which may contain few azurophilic granules and may appear vacuolated, while their nuclei are small, usually oval, and concentric.

iii. Total leucocyte count: using a bulb diluting pipette the blood is diluted 1 in 20 in 2 % acetic acid solution. After thorough mixing a counting chamber is filled (both sides) and the white cells contained in 1 square mm. area are counted on each side and the average calculated. It should be noted that the red cells of the chicken are also nucleated and are not haemolysed by the acetic acid solution, but it is very easy to distinguish them from the white cells because of their oval shape and haemoglobin content.

Calculation Total leucocyte count/c.mm. whole blood =
average count/sq.mm. x 10 x 20 (dilution factor).

C) Lobster blood cells' count

i. Total cell count : is carried out as an ordinary total leucocyte count as described above.

ii. Differential cell count : since lobster cells concerned with the coagulation process cannot be differentiated easily by cytology because of their rapid disintegration, they were counted by phase contrast microscopy immediately after withdrawal of the blood from the animal. Thus visualised, the "explosive cells"

can be seen to produce rounded vacuoles which explode, clotting the surrounding plasma film which then appears as a finely granular area of higher density. Such cells were counted in each field as compared to the total cells in the same field. Several fields are thus examined covering at least 1000 cells for each individual count. The percentage of the explosive cells is then easily calculated, and from the total count their absolute number is derived accordingly.

2. Fibrinogen estimation

I. micro-Kjeldahl method (Cullen and van Slyke, 1920)

The fibrinogen after being converted into fibrin is digested with concentrated sulphuric acid, the protein nitrogen is driven off by the action of excess concentrated sodium hydroxide in the form of ammonia which is collected volumetrically in standard sulphuric acid solution. The excess sulphuric acid is back titrated with sodium hydroxide to calculate the amount neutralized by the ammonia. The amount of protein present is thus calculated on the assumption that 16 % of the dry weight of protein is nitrogen. This is quite arbitrary since each protein has a different nitrogen concentration. If this factor is used, the weight of nitrogen found as ammonia is multiplied by the factor 6.25 to obtain the amount of protein present in milligrams.

Apparatus

- Digestion tubes and rack.
- Glass beads, these should be boiled in concentrated sodium hydroxide before use to drive off any ammonia in the dust which may contaminate them.
- micro-Kjeldahl distillation apparatus.

Reagents

- Na_2SO_4 catalyst tablets, (appendix I, 22)
- Zinc dust, (appendix I, 42)
- H_2SO_4 , A.R., (appendix I, 36A)
- NaOH 40 % (appendix I, 34C)
- N/50 H_2SO_4 (appendix I, 36C)
- N/70 NaOH (appendix I, 34B)
- Alizarin red indicator, (appendix I, 4)
- Standard NH_4Cl solution, (appendix I, 5).

Method

1. Preparation of standard : 5 mls standard NH_4Cl solution are delivered in a digestion tube + 1 catalyst tablet + 1 ml conc. H_2SO_4 + 3 glass beads.
2. Preparation of the blank : 5 mls of water are substituted for the standard solution.
3. Preparation of the test specimen : should not contain more than 2 mg nitrogen. In case of fibrinogen, that from 1 to 2 mls

of fresh plasma would be an adequate sample. One ml of the plasma is added to 9 mls saline and after mixing 1 ml of thrombin is added and the tube placed for 15' at 37°C. The formed clot is collected on a glass rod and washed in two changes of saline then squeezed down the rod into the digestion tube. One catalyst tablet is added together with 5 mls water, 1 ml conc. H₂SO₄, and 3 glass beads.

N.B. a) For chicken fibrinogen 1 ml M/40 CaCl₂ solution had to be added to the diluted plasma thrombin mixture to bring about fibrinogen conversion.

b) For lobster fibrinogen: as this was very difficult to collect on the glass rod, after clotting the diluted plasma with 0.5 ml lobster cell extract in the presence of 1 ml of 3 % CaCl₂ solution, the fibrin is collected on ballotini glass beads, washed twice with saline with centrifuging after each wash. The glass beads with the adsorbed fibrin are transferred into the digestion tube to which is added the rest of the reagents as above.

4. Boil the tubes very gently till the excess water is boiled off, then as the solutions turn brown the flame is increased and the mixtures boiled vigorously till they become clear, and thereafter gently for 30 minutes.
5. While the digestion is in progress the N/70 NaOH solution is

standardised against N/50 H₂SO₄ solution.

6. Prepare several 250 ml conical flasks each containing 10 mls of N/50 H₂SO₄ and 0.8 ml alizarin red indicator.
7. Dilute the digested samples with distilled water to not more than 4 mls total volume. This is dangerous and should be done over a sink as the tubes may explode if the water is added too quickly.
8. Transfer the contents of one digestion tube to the distillation flask, the apparatus having been first steamed up for about one hour. Place one of the conical flasks under the delivery tube of the distillation plant. Rinse the distillation flask three times with 2 ml volumes of distilled water, adding about 0.2 gm zinc dust before the last rinse.
9. Add at least 5 mls conc. NaOH solution and immediately clip the funnel to prevent any escape of ammonia to the outside.
10. Distill off about 50 mls into the conical flask.
11. The distillation flask is then washed with distilled water twice, and the procedure repeated for the next digestion tube and so on.
12. Back titrate the contents of each flask against standardised N/70 NaOH.

Calculation mg protein/c.c.plasma = $\frac{10 \times 0.28 \times 6.25 (B - T)}{A}$

B = mls N/70 NaOH required in titration of the blank.

T = mls N/70 NaOH required in titration of test specimen.

A = mls N/70 NaOH required in titration of 10 mls N/50 H_2SO_4

N.B. If the blood was obtained with a fluid anticoagulant, correction for the dilution of the plasma is made taking the haematocrit value in consideration for each particular sample.

II. Modified Greenberg method (Tyrosine method)

Reagents

- NaOH 10% (appendix I, 34, C).
- Na_2CO_3 20% (appendix I, 31).
- Folin and Ciocalteu's phenol reagent.
- Standard tyrosine (appendix I, 40, A & B).
- Thrombin Topical (Parke, Davis & Co.).

Method

1. Preparation of test specimen: 0.5 ml plasma is added to 9 ml saline in a test-tube containing 0.5 ml ballotini glass beads. The solution is mixed gently and 0.2 ml thrombin solution (50 units/ml) is added and the tube mixed rapidly and left at 37°C. for 10-15', after which time the fibrin is collected on the beads by brisk shaking without inversion, finally centrifuging. The deposit is washed twice with saline which should almost fill the test-tube to wash off all soluble proteins, and the washing removed after centrifugation. One ml of NaOH 10% solution is delivered down the sides of the tube and heated in a boiling water-bath for approximately 2 minutes, then

cooled under the tap. To the mixture is added 6 mls of water, together with 3 mls of 20% Na_2CO_3 and 1 ml of Folin phenol reagent. Stand for 30 minutes, then add 1 ml to 3 mls of distilled water and mix well. The extinction reading of the mixture is measured at 650 μ .

2. Preparation of the blank: substitute water for the plasma and proceed as above.
3. Preparation of the standard calibration curve: amounts of the working standard tyrosine solution (appendix I, 40, B) ranging from 0.05 to 4 mls are made up to 6 mls with water, then add to each tube 1 ml of 10% NaOH + 3 mls 20% Na_2CO_3 + 1 ml Folin phenol reagent. Stand for 30 minutes for maximum colour development, dilute an aliquot of each 1 in 4 with water and read at 650 μ then draw the curve relating the extinction readings to the equivalent tyrosine concentration in milligrams.

Calculation

The tyrosine content of the test plasma is read off from the calibration curve and converted into fibrinogen as follows:-
mg fibrinogen/100 mls plasma = mg tyrosine x 11.7 (conversion factor) x 200 (dilution factor).

N.B. For the lobster fibrinogen estimation, lobster cell extract and 3% CaCl_2 solutions were used instead of the thrombin solution

which does not clot lobster plasma. The final result was corrected for the plasma dilution as it was collected over fluid potassium oxalate in the ratio of 9 to 1.

III. Turbidimetric method (Stirland, 1956)

Blood is obtained over dry oxalate or sequestrene (2 mg/ml) and the plasma separated. 0.2 ml plasma is mixed thoroughly with 3 mls of 1% NaCl solution in each of two half inch diameter test-tubes, (dilution 1 in 16). One of the tubes is placed in a hot water bath at 56°C. for 15 minutes. The extinction reading at 650 m μ is taken using the unheated tube as the blank.

Preparation of the standard curve: (Shank and Hoagland, 1946, and Kunkel, 1947). A solution of 3 mls of 0.0962 N-BaCl₂.2H₂O (appendix I, 9) is diluted up to 100 mls with 0.2 N-H₂SO₄ at 10°C. This solution is equivalent to 20 Kunkel units. Dilutions are made from this with distilled water to represent 5, 10, and 15 Kunkel units, and the solutions read at 650 m μ immediately. The curve is drawn relating the extinction readings to the equivalent turbidity units. One Kunkel unit equals 42.8 mg fibrinogen per 100 mls plasma (or 26.8 μ gm fibrinogen/ml diluted plasma solution).

N.B. If the fibrinogen is higher than 850 mg % it requires further dilution of the test plasma, e.g. 0.1 ml plasma diluted in 3.1 ml NaCl solution (dilution 1/32); while if the fibrinogen is very low (faint turbidity) use a dilution of 1 in 8.

3. Whole blood clotting time

(method of Lee and White, 1913)

Whole venous blood obtained by a clean venepuncture (appendix II, 1) is delivered in 1 ml volumes in each of four small test-tubes (10 x 75 mm) placed in a water bath at 37°C. The tubes are tilted in turn at half minute intervals till each tube can be inverted without spilling the blood. A stop watch is started as the blood enters the syringe, and the clotting time recorded when the blood has solidified. The average of the four clotting tubes is calculated. Whole blood clotting times were estimated in ordinary glass tubes and in siliconed glass tubes.

For lobster haemolymph clotting time, the test was carried out both at 37°C. and at 15°C. which is nearer their normal habitat temperature.

4. Clot retraction

(method of Macfarlane, 1938, as modified by Aggeler, Lucia, and Hamlin, 1942).

Apparatus - siliconed centrifuge tubes graduated in 0.1 ml divisions.

- spiral made of wire whose diameter is 1-1.5 mm.

Method Freshly withdrawn venous blood is immediately placed

into the siliconed tube up to the 5 ml mark exactly. The wire spiral is then placed in the tube which is put in a water bath at 37°C. The tube is examined from time to time until the blood has clotted. It is then left undisturbed in the water bath for a further hour at the end of which the wire around which the clot should have retracted is carefully lifted up and allowed to drain for about one minute and then is removed. The volume of the expressed serum and red cells is read off directly from the tube graduations. This volume is expressed as a percentage of the 5 mls of blood, thus giving the percentage retraction of the clot in one hour.

5. Plasma recalcification time

Into a tube in a water bath is delivered 0.1 ml of citrated platelet-rich plasma and 0.1 ml of saline. Simultaneously with the addition of 0.1 ml M/40 CaCl₂ solution a stop watch is started and the clotting time recorded.

6. Quick's one-stage prothrombin time

0.1 ml of citrated plasma and 0.1 ml of tissue extract (appendix III, 13) are mixed in a clotting tube placed in a water bath at 37°C. for 30", and a stop watch is started at the moment of the addition of 0.1 ml M/40 CaCl₂ and the tube tilted almost

horizontally every few seconds until a firm clot is observed, and the time recorded. The test is done in duplicate and the average reported.

7. Thrombin-fibrinogen dilution curve

(Biggs and Macfarlane, 1957)

Thrombin Topical (Parke, Davis & Co.) solution containing 1000 N.I.H. units/ml (appendix III, 7) is diluted with saline to contain 50, 40, 30, 20, 15, 10, 8, 5, 4, 3, 2, and 1 units of thrombin per ml. (occasionally double-folding dilutions were made to contain 50, 25, 12.5, 6.2, 3.1, and 1.5 units/ml for the sake of simplicity). Dilutions were always made in siliconed tubes to obviate the adsorption of the thrombin on the glass surface (Waugh and Livingstone, 1951, Seegers et al, 1952) especially in solutions of low concentration.

A series of tubes containing 0.4 ml amounts of fibrinogen are placed in the water bath at 37^oC. and to each is added 0.1 ml of each of the thrombin solutions and the clotting times recorded.

The clotting times are plotted against the thrombin concentrations on double logarithmic paper when a straight line should be obtained (figure 1). From this curve the clotting times of 0.4 ml amounts of fibrinogen can be read in terms of thrombin units. These units have no absolute significance but are used for comparing the

relative amounts of thrombin formed in the various test systems.

Plotting the clotting times in seconds against the reciprocal of the thrombin concentrations gives a straight line which almost passes through the origin, because however strong the concentration of thrombin is the fibrinogen still takes some time to clot.

New batches of fibrinogen or thrombin were always assayed by this method, only those conforming to the original curve shown in figure 1 were used.

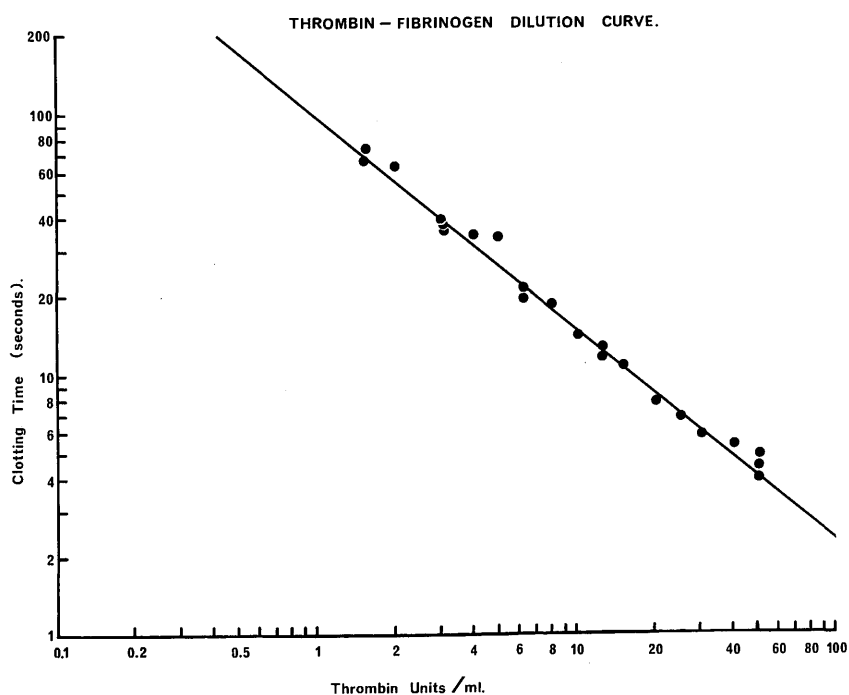


Fig. 1 Reference thrombin-fibrinogen dilution curve.

8. Thrombin-fibrinogen reaction of the plasma

Serial dilutions of thrombin are made ranging from 1/1 to 1/32 in saline in siliconed tubes. Six clotting tubes are placed in the water bath, each containing 0.1 ml of the citrated plasma together with 0.1 ml of saline. 0.1 ml of each thrombin dilution is added to each and the clotting times recorded. A curve is drawn by plotting the clotting times in seconds against the thrombin concentrations in units. Usually the most concentrated solution of thrombin (1/1) contained 50 units/ml.

Sometimes, double the amounts were used so as to be able to test the effect of other reagents on the reaction by substituting them for the saline, as described in the next test.

9. Effect of various reagents on the thrombin-fibrinogen reaction

Essentially the same procedure as in the thrombin-fibrinogen reaction of the plasma except for using 0.2 ml amounts of each constituent. The effect of adding toluidine blue (appendix I, 38), pyrocatechol (appendix I, 28), heparin (appendix I, 19), hexadimethrine bromide (appendix I, 26), and M/40 CaCl₂ solutions either singly or in combination, was tested by substituting 0.1 ml of the required reagent instead of 0.1 of the saline solution or 0.1 ml of each reagent instead of the 0.2 ml of saline.

10. Testing for the species specificity of the thrombin-fibrinogen reaction in a purified system

Purified thrombin (appendix III, 7, B) and purified fibrinogen (appendix III, 6, A) prepared from the various species were used. 0.4 ml of each fibrinogen solution mixed with 0.1 ml saline was clotted with 0.1 ml of each thrombin solution at 37°C. and the clotting time recorded. The results are compared with that of a homologous human system. The effect of pyrocatechol was tested by adding 0.1 ml of the pyrocatechol solution (appendix I, 28) instead of the 0.1 ml of saline, to the fibrinogen solution under investigation, then clotting the mixture with the thrombin solutions.

N.B. Both thrombin and fibrinogen preparations were processed at the same time under identical conditions and tested immediately after processing. Stored batches were not used for this test.

11. Measurement of antithrombin

A. Method of Douglas and Biggs, 1953

To 0.5 ml fresh citrated plasma is added 0.5 ml thrombin solution (50 units/ml) and the clot immediately removed by winding it on a wooden swab stick. At intervals of $\frac{1}{2}$, 1, 2, 3, 4, 5, and 6 minutes from the addition of the thrombin, 0.1 ml amounts are removed from this incubation mixture and added to 0.4 ml amounts

of fibrinogen previously prepared in clotting tubes placed in the water bath at 37°C., and the clotting times recorded. The equivalent thrombin units are read from the thrombin-fibrinogen dilution curve (figure 1) and a curve relating the logarithm of the thrombin in units to the incubation time in minutes is drawn.

The test plasma is similarly treated as the control plasma. The distance from the zero point at which the curve crosses the abscissa is a measure of the antithrombin activity.

Calculation Antithrombin activity per cent = $\frac{C}{T} \times 100$

where C = distance of control human plasma (considered 100 %)

and T = distance of test plasma.

B. Modified method of Astrup and Darling (1942d) (Biggs and Macfarlane, 1957)

Citrated plasma is diluted 1 in 5 in saline and mixtures of this diluted plasma and saline are made as follows:-

Diluted plasma in ml.	0.0	0.05	0.10	0.15	0.2	0.3
Saline in ml.	0.3	0.25	0.20	0.15	0.1	0.0

To each of these mixtures is added 1 ml of thrombin solution, and the mixtures incubated for 15 minutes at 37°C. after which time the tubes are removed and placed in water at the temperature of melting ice to stop the neutralization of the thrombin. From each tube 0.1 ml volume of solution is delivered onto 1 ml amounts of fibrinogen previously distributed in clotting tubes kept at 37°C. and the clotting times recorded.

The concentration of the thrombin solution is so adjusted that 0.1 ml from the control mixture containing no plasma clots the fibrinogen in 10-15 seconds. The concentration of the test plasma is also so adjusted that the fibrinogen clotting time of the highest concentration of plasma does not exceed 60 seconds.

Calculation Antithrombin concentration = $\frac{a}{n} (1 - t_o / t_n)$

where a = the concentration of thrombin in units/ml.,

n = the amount of undiluted plasma in each mixture,

t_o = the fibrinogen clotting time of the control with no plasma,

t_n = the fibrinogen clotting times with the various plasma concentrations.

The values t_o/t_n are plotted against the concentration of plasma on ordinary graph paper and the best line is drawn through the points. From this line is read the corrected values for t_o/t_n which are used for the calculation of the antithrombin concentration that is expressed in units/ml plasma.

Though this method gives a direct estimate of the antithrombin activity in terms of units/ml without reference to a control normal plasma, it was thought better to include testing the control human plasma under the same conditions and express the result as a percentage of the control value.

12. Measurement of prothrombin

A. One-stage method of Koller, Loeliger, and Duckert, 1951

Adsorbed oxalated bovine plasma is used as substrate to supply constant amounts of factor V and of fibrinogen. It is prepared by obtaining bovine blood over sodium oxalate (1.34%) in the ratio of 9 to 1 respectively. The plasma is separated and adsorbed with tricalcium phosphate (50 mg/ml plasma) for 10 minutes at room temperature with occasional stirring, the plasma finally centrifuged to separate the precipitate. This plasma was prepared fresh for each assay. Prothrombin-free oxalated human serum (or purified factor VII prepared therefrom) is used to supply factor VII. It is prepared as described in appendix III, 5, then tested for its prothrombin content as follows:- 0.1 ml volumes of adsorbed human or bovine plasma, undiluted serum (under test), and brain thromboplastin are placed in a tube in a water bath at 37°C. and after 20 seconds 0.1 ml of M/40 CaCl₂ solution is added and the clotting time recorded. It should be more than 90 seconds, otherwise the serum is not suitable for this assay system.

Test procedure

In a clotting tube in a water bath at 37°C. is placed 0.1 ml of the test plasma diluted 1 in 10 in veronal buffer, 0.05 ml of

the adsorbed bovine plasma (substrate), 0.05 ml of the prothrombin-free oxalated serum (oxalated 1 in 10 with 1.34% sodium oxalate), and 0.1 ml of brain thromboplastin. After 20 seconds 0.1 ml of M/40 CaCl_2 solution is added and the clotting time of the whole mixture recorded.

A normal standard dilution curve is made by substituting the test plasma by various dilutions of normal plasma made with veronal buffer ranging from 1 in 10 (100%) to 1 in 640 (1.5%). The standard curve gives a straight line when the prothrombin concentration in percentage is plotted against the clotting times in seconds on double logarithmic paper.

In the original assay the authors used acetone-dried human brain, also they used human oxalated serum (or factor VII purified from human serum) in the test mixtures. Experiments mentioned in this work using this technique were carried out using a saline brain extract (appendix III, 13, I) instead of the acetone-dried preparation. Also combinations of human and bovine brain extracts with human and bovine prothrombin-free sera were used in testing the same samples of pooled plasmas in the same day, all other reagents being kept constant.

The concentration of prothrombin of the test plasma was read off from the standard dilution curve plotted using the same kind of brain thromboplastin and serum in the mixture, the result is

expressed in percentage, designating the 100% value to the 1/10 dilution of the normal plasma.

B. Globulin fraction method of Douglas and Biggs, 1953

This is a method for the estimation of prothrombin in a system free of antithrombin.

To 1 ml of citrated plasma is added 9 mls distilled water, and the pH adjusted to 5 by the addition of 1% acetic acid. The mixture is centrifuged and the supernatant discarded. The ppt. is washed twice with distilled water whose pH has previously been adjusted to 5. The ppt. is then dissolved in 2 mls of citrate saline (appendix I, 12) and the pH readjusted to 7 by adding 0.5% Na_2CO_3 solution. The prothrombin content of this globulin fraction is estimated as follows:-

In a clotting tube is placed 0.3 ml amounts of the test solution, brain thromboplastin, and M/40 CaCl_2 and the mixture incubated at 37°C . for 30 minutes, then 0.1 ml amounts are transferred into 0.4 ml amounts of fibrinogen solution previously pipetted in clotting tubes in the water bath, and the clotting times recorded. Two or three readings are taken for each mixture and the average calculated. The same procedure is made for the standard normal plasma, also for the citrated sera. The results are expressed in terms of thrombin units as read from

the thrombin-fibrinogen dilution curve, figure 1.

The prothrombin concentration of the test plasma is then expressed in percentage as compared with the standard plasma, using the following equation:-

$$\frac{\text{Thrombin units of test mixture}}{\text{Thrombin units of the standard}} \times 100$$

C. Two-stage area method of Biggs and Douglas, 1953a

To a mixture of 0.4 ml citrated plasma and 0.4 ml brain extract at 37°C. is added 0.4 ml M/40 CaCl₂ and a stop watch is started. At 15, 30, 45, 60 seconds, and thereafter at one minute intervals 0.1 ml samples are removed from this incubation mixture and added to 0.4 ml volumes of fibrinogen solution warmed to 37°C. in the water bath. The clotting times are recorded and the test continued until these exceed 3 minutes. Clotting usually occurs in the incubation mixture itself, the clot should then be removed by winding it on a wooden swab stick. The clotting times of the fibrinogen samples are converted into thrombin units as read from the thrombin-fibrinogen dilution curve, figure 1. A curve of thrombin generation and disappearance can then be drawn on ordinary graph paper and the area enclosed under the curve measured either by counting the squares on the graph paper or by weighing the cut-out area under the curve. The same procedure is carried

out for the normal standard plasma and the test plasma. The area obtained with test plasma is expressed as a percentage of the normal plasma.

As this test system is affected by the antithrombin content of the plasma in the mixture, it is an essential prerequisite that the antithrombin content of both test and normal plasmas are of similar magnitude. The antithrombin content of the plasmas was therefore measured by the method of Douglas and Biggs (1953) as described in appendix IV, 11, A, or by the modified method of Astrup and Darling (1942d) described in appendix IV, 11, B.

Biggs and Douglas (1953a) have shown that variation in the potency of the brain thromboplastin does not affect the results.

This method was carried out using both the human brain preparation as well as brain preparations from other species, all other reagents being constant. The species of the brain extract utilised is mentioned with the results of each experiment.

13. Prothrombin consumption test

A. Method of Biggs and Macfarlane, 1957

The test serum is prepared by allowing the blood used for the estimation of the coagulation time by the Lee and White method (1913) in ordinary glass tubes to stand for 50 minutes at 37°C. after

the coagulation time. The clots are then gently freed from the sides of the tubes with a wooden swab stick and the serum pooled in one tube after centrifugation at 1,500 r.p.m. for 5 minutes. At exactly one hour after coagulation has occurred a prothrombin consumption test is carried out, as follows:-

Four tubes are prepared in the water bath at 37°C., each containing 0.4 ml of fibrinogen solution. In another clotting tube in the water bath is delivered 0.2 ml test serum, 0.2 ml of brain thromboplastin, 0.2 ml saline, and immediately on the addition of 0.2 ml M/40 CaCl₂ a stop watch is started, the contents rapidly mixed and the tube replaced in the water bath. At exactly 45 seconds after recalcification, 0.1 ml of the incubation mixture is transferred into one of the tubes containing fibrinogen and the clotting time recorded by another stop watch. At 90 seconds a further 0.1 ml of the incubation mixture is transferred onto another fibrinogen tube and its clotting time observed.

The whole procedure is repeated using test citrated plasma instead of the test serum. In this case, shortly after the recalcification the incubation mixture itself forms a clot which should be rapidly removed on a wooden applicator stick while carefully squeezing out all fluid trapped in it. The clotting times of subsamples at 45 and 90 seconds' intervals are recorded as previously. It should be noted that in original test the

intervals for subsampling were 30 and 60 seconds with which it was sometimes difficult to subsample in case of plasma as by the time the clot was removed the 30 seconds' interval has elapsed. Using the slightly longer interval of 45 seconds was quite adequate.

Calculation

$$\text{Prothrombin consumption index (P.C.I.)} = \frac{\text{mean clotting time of plasma}}{\text{mean clotting time of serum}} \times 100$$

This test suffers from the fact that low values represent the normal, while high values are abnormal. The normal range for human blood is from 0 to 40%. The normal serum time should be more than 30 seconds.

B. Method of Douglas and Biggs, 1953

This method estimates the prothrombin content of the test plasma and serum by the globulin fraction technique described in appendix IV, 12, B.

After the preparation of the serum as in the method of Biggs and Macfarlane (1957) described above, at exactly one hour from the clotting of the blood the serum is citrated by adding one part of 3.8% sodium citrate solution to four parts of serum, thus preventing further conversion or utilization of prothrombin into thrombin, and the preparation of the globulin fraction can then be done at leisure.

$$\text{Calculation P.C.I.} = \frac{\text{thrombin units in serum}}{\text{thrombin units in plasma}} \times 100$$

14. Measurement of factor V

Essentially the same method as described by Biggs and Macfarlane (1957) was used, testing the ability of the test plasma to correct the clotting time of plasma artificially made factor V deficient, as compared to the standard normal plasma. However, since Katz and Ducci (1957) have shown that various factors are affected when the exact amount of plasma (to be rendered factor V deficient) and the size of the container are not strictly adhered to as described by them, their method for the preparation of the factor V deficient plasma was used. The dilution of test plasma in the substrate factor V deficient plasma was also modified from that described by Biggs and Macfarlane (1957), designating the 100% value to the 1 in 10 dilution of the test or standard plasma.

Preparation of factor V deficient plasma (Katz and Ducci, 1957)

Five millilitres of human plasma (oxalated in the proportion of 9 parts blood to 1 part 1.85% potassium oxalate solution) are placed in a test-tube measuring 13 x 100 mm. (or 55 mls oxalated plasma in a 50 ml conical flask). The tube is stood in a water bath at 37°C. for 24 hours exactly. Plasma samples thus treated which give clotting times around 30 seconds (by Quick's one-stage test) are then stored in the refrigerator at 4°C. in 5 ml amounts. The reactivity of the plasma is checked daily by Quick's one-stage

test, the clotting time should be between 45 and 90 seconds. If it is less than 45" the plasma should be stored further, while if it is longer than 90" the plasma becomes unsuitable and should be discarded. Such prepared plasma was shown by the authors to behave similar to plasma stored at 4^oC. for 15-25 days, giving good clots with no granulation. They also found that the different behaviour of this deficient plasma on the addition of normal plasma is not explained by changes in prothrombin or factor VII as their concentrations were normal in all these plasmas, and could only be related to changes in factor V concentrations.

Test procedure

Various dilutions of the normal plasma in the factor V deficient plasma are made by mixing 0.1 ml of the normal plasma with 0.9 ml of the deficient plasma to constitute the 100% value for factor V. From this mixture 0.3 ml is transferred into a tube containing 0.3 ml deficient plasma to make a solution with 50% of factor V concentration, and after thorough mixing further double-folding dilutions are made in the deficient plasma to represent factor V concentrations of 25, 12.5, 6.2, 3.1, 1.5, and 0%. Each of these dilutions is then tested by Quick's one-stage test in duplicate and the average clotting times noted and plotted against the factor V concentrations in percentage on double logarithmic paper when an approximate straight line is obtained.

Dilutions of the test plasma in the deficient plasma corresponding to 1/10, 1/20, and 1/40 are made and tested as above. The factor V content of each dilution is read from the standard dilution curve and multiplied by the dilution factor, considering the 1/10 dilution equivalent to 100%, and the average of the readings reported. If the concentration of factor V in the test plasma is much greater than that in the normal human plasma, further dilutions are made as required e.g. 1/80, so that their clotting times fall on the part of the curve between 100 and 10% which is the most exact part of the curve.

15. Measurement of factor VII

A. By correction of phenindione plasma

Essentially the same procedure as that for factor V estimation described above, except for substituting phenindione human plasma instead of the stored plasma. The phenindione plasma was obtained from patients under anticoagulant therapy during the first few days only, at the time when the factor VII content is at a very low level, while prothrombin and factor X are very slightly reduced (Douglas, 1962). Plasmas having clotting times twice the control plasma are suitable. The ability of the test plasma to correct the factor VII deficiency is compared with that of the control by reading the test result from the control dilution curve.

B. By correction of Seitz-filtered bovine plasma (method of Koller, Loeliger, and Duckert, 1951).

Preparation of the Seitz-filtered plasma: bovine blood oxalated in the proportion of 9 parts blood to 1 part of M/10 sodium oxalate is spun and the plasma separated. The plasma is filtered at room temperature through an asbestos filter pad containing 20% asbestos, then through another pad containing 30% asbestos (appendix I, 35). The filtration speed is maintained at approximately one drop per second, so as to effect the proper separation of factor VII. The filtrate is collected in aliquots, and the prothrombin content of each estimated by the method of the same authors as described in appendix IV, 12, A. Batches of filtrate with prothrombin values above 15% are suitable for use in this test.

Test procedure

0.1 ml of the test oxalated plasma (appendix III, 3) diluted 1 in 10 in veronal buffer, is added to 0.1 ml of the Seitz-filtered bovine plasma and 0.1 ml of brain thromboplastin. After 20 seconds, 0.1 ml M/40 CaCl_2 is added and the clotting time recorded. Further dilutions of the test plasma in veronal buffer are made as required so that the clotting times can be read on the part of the standard dilution curve between 100 and 10%.

The standard dilution curve is made by testing dilutions of normal oxalated human plasma ranging from 1/10 to 1/640 (equivalent to 100% and 1.5% respectively) and testing them as above. The

average of the clotting times (made in duplicate) in seconds are plotted against percentage concentrations of factor VII on double logarithmic paper, when an approximate straight line is obtained, designating the 100% value to the 1/10 dilution.

In the original authors' method human acetone-dried brain was used. In view of the fact that the use of human brain to measure the factor VII content of plasmas from various animals gave artificially fallacious results due to the species specificity of their interaction, the test was also carried out using brain extracts of other species prepared in the same way as the human.

It should also be noted that this method is sensitive to, and therefore a measure of, factor X in the test plasmas, since the Seitz-filtered plasma is virtually free from both factors VII and X.

16. Brain thromboplastin - factor V specificity

The method used to investigate the species specificity of the brain thromboplastin - factor V interaction was a two-stage procedure in which the brain preparation was incubated at 37°C. with the adsorbed plasma diluted 1 in 5 in saline (appendix III, 4, A), together with M/40 CaCl₂ solution, in equal amounts. At one minute intervals after recalcification, 0.1 ml amounts are transferred from the incubation mixture and added together with

0.1 ml M/40 CaCl_2 to 0.1 ml of the substrate and the clotting time recorded. The substrate was either fresh human plasma, fresh plasma from the species under investigation, or human plasma artificially rendered factor V deficient by storage (appendix IV, 14).

In some tests the adsorbed plasmas were substituted by the purified factor V prepared as described in appendix III, 9, and diluted so that the factor V concentration would be equivalent to that in adsorbed plasma diluted 1 in 5 in saline.

Brain preparations were made by Owren's method (appendix III, 13, and further diluted 1 in 2 or 1 in 4 in saline according to their potency.

The results of the mixtures containing the test plasma or factor V preparation were compared with those of mixtures containing the control human plasma or factor V preparation. Saline control mixtures were also included in which saline was substituted for the adsorbed plasma or the purified factor V.

17. Brain thromboplastin - factors VII and X specificity

Essentially the same procedure was used as for the brain thromboplastin - factor V specificity, except for substituting the human or test serum diluted 1 in 10 in saline for the adsorbed plasma or the purified factor V. Sometimes a purified factor VII preparation (appendix III, 10) was used instead of the serum.

The substrate was either fresh human citrated plasma, fresh citrated plasma of the species under investigation, or human phenindione plasma. The results of the heterologous mixtures were compared to those of homologous mixtures. Saline control mixtures were also tested in which saline was used instead of the serum or factor VII preparation.

18. Measurement of factor X

(method of Bachmann, Duckert, and Koller, 1958)

Reagents

1. Oxalated control and test plasma: 9 parts blood added to one part M/10 sodium oxalate and centrifuged at 2,500 r.p.m. for 5'.
2. Veronal buffer, (appendix I, 41).
3. M/40 CaCl_2 solution, (appendix I, 10, A).
4. Cephalin: in the original authors' paper, crude cephalin prepared from human brain by ether extraction was used. In this work, Bell and Alton's acetone-insoluble chloroform-soluble cephalin extract of human brain was used (appendix III, 14). It was dissolved in veronal buffer so as to contain approximately 0.06 gm active material per cent. (Authors used cephalin suspension containing about 0.047 gm %).
5. Russell's viper venom - cephalin: (R.V.V.-cephalin) R.V.V. of a concentration of 1/200,000 in cephalin (appendix III, 17, B) was used. After thawing at 37°C., it is shaken vigorously to

obtain a good suspension yielding maximum activity.

6. Seitz-filtered oxalated bovine plasma: (substrate) should be prepared very carefully since small amounts of Stuart-Prower factor (factor X) in it render the dilution curve very flat. In a two litre original Seitz filter apparatus with 14 cm diameter, place two asbestos filter pads, the lower with 30% asbestos, and the upper with 20% asbestos (Sterimats, appendix I, 35). About 1,500 mls oxalated bovine plasma are poured in the filter apparatus and filtration carried out at room temperature. The filtration speed is maintained at 1 drop per second by occasional increase of the air pressure. Speeds higher than 1-2 drops per second cause inefficient separation of the prothrombin from the Stuart-Prower factor and factor VII, and the yield will be poor. The first 200 mls of the filtrate are discarded, then fractions of 50 mls each are collected and assayed for their prothrombin content by the method of Koller et al (1951) which if above 25%, the zero value of such suitable fractions is determined by the mixing of 0.1 ml filtered plasma fraction with 0.1 ml veronal buffer and 0.1 ml of R.V.V.-cephalin suspension. The mixture is stood for 30 seconds at 37°C. then 0.1 ml M/40 CaCl_2 solution is added and the clotting time recorded. Each fraction which has a zero value more than 80-90 seconds is suitable for use as substrate for the test. Suitable fractions are distributed in

small amounts sufficient for one assay and one dilution curve done in duplicate (usually 2.5 mls) and kept at -20°C .

Test procedure

The tubes containing the R.V.V.-cephalin reagent and the CaCl_2 solutions are placed in the water bath. In another two clotting tubes (duplicate) is delivered the following: 0.1 ml substrate Seitz-filtered bovine plasma, 0.1 ml test plasma or serum diluted 1/10 in veronal buffer, and 0.1 ml R.V.V.-cephalin solution. After 30 seconds incubation at 37°C ., 0.1 ml of M/40 CaCl_2 solution is added and the clotting time recorded. The average of the two tubes is reported. Further dilutions of the test plasma or serum are tested similarly if required.

Construction of the normal dilution curve

Various dilutions of the normal plasma or serum in veronal buffer ranging from 1 in 10 (designated 100%) to 1 in 2560 (0.37%) are tested as above. A blank with veronal buffer instead of the plasma constitutes the 0% value. Plotting the clotting times in seconds against the plasma concentration in percentage on double logarithmic paper gives a straight line from 100% to 2%, but the 0.5% concentration still gives significantly shorter clotting times than the zero value. This means that the adsorption of factor X by the asbestos is 100%. The authors recommend the 5-40% zone of the dilution curve as the best part, yet that values between

50 and 100% can be assayed with satisfactory accuracy.

Experiments in this work for the assay of factor X were carried out on plasma, both for the dilution curve and the test sample. On some occasions, however, serum was used instead, again both for the calibration curve and the test sample.

19. R.V.V.-cephalin clotting time of plasma

To a clotting tube containing 0.1 ml of plasma is added 0.1 ml of R.V.V.-cephalin reagent (appendix III, 17, B) and left at 37°C. for 30 seconds. As 0.1 ml M/40 CaCl₂ solution is blown into the tube a stop watch is started and the clotting time of the mixture recorded. Various concentrations of R.V.V. in cephalin were used and are mentioned in the appropriate tables.

20. Thromboplastin generation test

(method of Biggs and Douglas, 1953b)

Reagents

1. Control and test adsorbed plasmas: prepared as described in appendix III, 4, A. For use they were diluted 1 in 5 in saline.
2. Control and test sera: (appendix III, 5) They were diluted 1 in 10 with saline and stood at room temperature for about one hour before use.
3. Platelet suspension: appendix III, 15.

4. High-spun plasma: appendix III, 2.
5. M/40 CaCl₂ : appendix I, 10, A.

Procedure

The CaCl₂ solution is kept in the water bath at 37°C. The incubation mixture is pipetted into a clean clotting tube in the water bath in the following order:- 0.3 ml adsorbed plasma diluted 1 in 5, 0.3 ml serum diluted 1 in 10, 0.3 ml platelet suspension, and 0.3 ml M/40 CaCl₂ solution. As the latter is added a stop watch is started and at one minute intervals up to and including 6 minutes, 0.1 ml amounts of the incubation mixture are removed and together with 0.1 ml M/40 CaCl₂ solution are simultaneously added to 0.1 ml amounts of high-spun plasma previously pipetted out in clotting tubes placed in the water bath. The clotting times of the substrate tubes are recorded using extra stop watches.

The incubation mixture sometimes forms a clot which then has to be removed by winding it on a wooden swab stick.

The test is repeated with various alternative combinations of control and test adsorbed plasmas with the control and test sera in the incubation mixture.

Modifications

These were made by substituting some reagents with others as follows:-

- a. Platelet suspension: was sometimes substituted by crude human

cephalin (appendix III, 14), bovine platelets, horse platelets, rabbit platelets, or chicken thrombocyte suspension (appendix III, A, B, and C).

- b. Adsorbed plasma: substituted by purified A.H.G. (appendix III, 11) together with purified factor V (appendix III, 9).
- c. High-spun substrate plasma: substituted by the homologous plasma, namely chicken, bovine, equine, or rabbit high-spun plasmas, to test for substrate reactivity.
- d. Correction of haemophilic plasma: various dilutions of the adsorbed normal or test plasma made in adsorbed haemophilic plasma were tested to detect the ability of the test plasma to correct the deficiency in the haemophilic plasma as compared with that of the normal plasma.
- e. Correction of Christmas serum: various dilutions of normal and test sera in Christmas serum were substituted for the whole serum usually used, to test their ability to correct Christmas serum deficiency.

The above modifications whether applied singly or in combination are mentioned where appropriate.

20, A. Intrinsic thromboplastin dilution curve

(Biggs and Macfarlane, 1957)

An ordinary thromboplastin generation test is set up with

completely normal reagents. When the minimum substrate clotting time has been reached, the tube containing the incubation mixture is placed at the temperature of melting ice, and double-folding dilutions of this mixture are made in saline previously cooled in the ice bath. 0.1 ml amounts of each dilution are then transferred together with 0.1 ml of M/40 CaCl_2 onto 0.1 ml high-spun plasma at 37°C . as performed in an ordinary T.G.T. and the clotting times recorded. The clotting times in seconds are plotted against the intrinsic thromboplastin concentration in percentage on double logarithmic paper, designating the value of 100% to the dilution which clots the substrate in 10 seconds. Using the dilution curve thus obtained (figure 2) all the results of the T.G.T. were converted from clotting times in seconds into per cent values of active thromboplastin. It should be noted that in the construction of the chicken intrinsic thromboplastin dilution curve, since the most potent incubation mixture using homologous chicken reagents was still much weaker than any human mixture, the 100% value was ascribed to the dilution which clotted the substrate in 20 seconds.

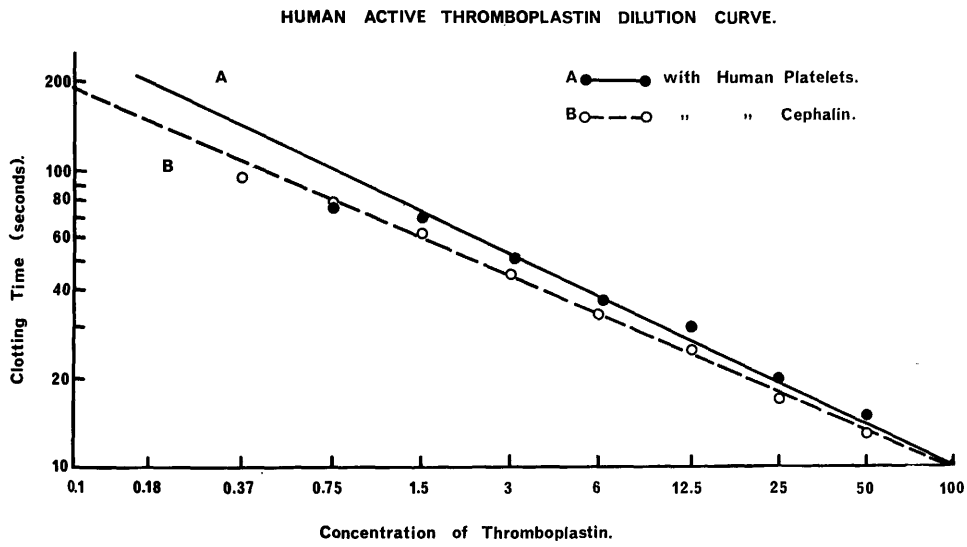


Figure 2. Human intrinsic thromboplastin dilution curve.

21. Measurement of anti-haemophilic globulin

(method of Pitney, 1956)

This method is based on the thromboplastin generation test, estimating the degree of correction of adsorbed haemophilic plasma by the test adsorbed plasma as compared to the control adsorbed plasma assayed in various dilutions to construct a calibration curve.

Reagents

1. Haemophilic plasma: is obtained from a severely haemophilic patient. The venous blood is obtained with a siliconed syringe and is citrated in a siliconed centrifuge tube. The plasma is separated after centrifuging at 3,000 r.p.m. for 10 minutes, then distributed in about 1 ml amounts and stored at -20°C . For use, an aliquot is thawed, tested by Quick's one-stage test which should give a normal result, thus ensuring a normal content of factor V. The plasma is then adsorbed with alumina and the supernatant tested again by Quick's one-stage test which should be more than 60 seconds. The supernatant is then diluted one in five with veronal buffer and kept at 4°C . till use.
2. Control and test plasmas: are collected at the same time, the plasma separated as above, adsorbed with alumina, and dilutions made with veronal buffer. For the control plasma six dilutions are made ranging from 100% to 1% (i.e. 1/20 designated 100%, 1/40,

1/80, 1/200, 1/400, and 1/2000). The test plasma after its adsorption is prepared in three dilutions depending on the probable A.H.G. content. For plasma suspected of having a normal A.H.G. content the dilutions 1/20, 1/40, and 1/80 are suitable; while plasmas with low A.H.G. concentrations are tested at 1/5, 1/10, and 1/20; whereas plasmas with high A.H.G. content are tested at 1/80, 1/200, and 1/400. This is to ensure that the substrate clotting times of such dilutions would fall on the most accurate part of the control dilution curve. The various dilutions are kept at 4°C. till ready for use which should be as soon as possible.

3. Normal serum: normal venous blood is allowed to clot and kept at 37°C. for 24 hours, the serum is then separated and stored at -20°C. until required. On the day before the test 1 ml of the serum is thawed, diluted 1 in 10 with veronal buffer and left at 4°C. overnight.
4. Platelet suspension: appendix III, 15, A.
5. High-spun plasma: appendix III, 2.
6. Veronal buffer : appendix I, 41.
7. M/40 CaCl₂ solution: appendix I, 10, A.

Preparation of the standard dilution curve

Into a clotting tube placed in the water bath are pipetted 0.1 ml volumes of adsorbed haemophilic plasma (diluted 1/5),

adsorbed normal plasma (each dilution at a time), normal serum (diluted 1/10), and platelet suspension. After the mixture has reached the temperature of the water bath, 0.1 ml of previously warmed M/40 CaCl_2 solution is added and a stop watch started. Exactly 4 minutes after the addition of the calcium chloride, 0.1 ml of the incubation mixture together with 0.1 ml M/40 CaCl_2 are transferred to a clotting tube containing 0.1 ml of substrate normal high-spun plasma and its clotting time recorded by another stop watch.

In practice it is possible to set four tubes for the incubation mixtures, each containing a different dilution of the standard plasma and recalcifying them at one minute intervals, thus the subsampling is also carried out at the end of the four minutes again in turn at one minute intervals, using four stop watches to record the clotting times of the substrates. Thus the six dilutions of the normal plasma and the saline blank required for the construction of the standard dilution curve are tested in about 15 minutes.

The thromboplastin times recorded are plotted on ordinary graph paper against the plasma dilution to construct a curve.

Assay of test plasma A.H.G. content

Three suitable dilutions of the adsorbed test plasma are used in place of the normal plasma in the reacting incubation mixture, and the 4 minute thromboplastin times are recorded. The amount

of A.H.G. in each dilution is read off from the dilution curve.

Calculation: A.H.G. concentration in test plasma =

$$\frac{1/20 \text{ (dilution of control designated 100\%)}}{\text{dilution of test plasma}} \times \text{percentage reading.}$$

The mean A.H.G. concentration of the test plasma is obtained by averaging the results obtained for the three dilutions tested.

22. Measurement of Christmas factor

(based on the method of Pitney, 1956)

This method is essentially the same as that for the measurement of A.H.G. except for the substitution of Christmas serum and control and test sera instead of the haemophilic plasma and test and control plasmas, while normal adsorbed plasma is added to the mixture to supply the necessary amounts of A.H.G. and factor V. Thus the method was adapted to measure the degree of correction of Christmas serum by the test serum as compared to the control serum.

Reagents

1. Christmas serum: whole blood is obtained from a patient with a severe deficiency of Christmas factor, and allowed to clot at 37°C. for 4 hours, the serum is then separated by centrifugation and distributed in 1 ml amounts in lusteroid tubes and kept at -20°C. till required. On the day before the test, 1 ml is thawed and diluted 1 in 10 in veronal buffer and kept in the refrigerator overnight.

2. Control and test sera: whole blood is obtained and allowed to clot and stand at 37°C . for 4 hours then the serum is separated. The control serum is diluted in veronal buffer ranging from 1/20 (designated 100%) to 1/1280 (1.5%). The test serum is also diluted from 1/5 to 1/320, sera suspected of having a low Christmas factor activity are tested at three of the low dilutions, while those with high activity are tested at the higher dilutions.
3. Adsorbed normal plasma: prepared as described in appendix III, 4, A, except for being diluted 1 in 5 in veronal buffer instead of in saline.
4. Platelet suspension: appendix III, 15, A.
5. High-spun plasma : appendix III, 2.
6. Veronal buffer : appendix I, 41.
7. M/40 CaCl_2 solution: appendix I, 10, A.

Preparation of the standard dilution curve

Into four clotting tubes placed in the water bath is pipetted 0.1 ml volumes of adsorbed normal plasma (diluted 1/5), Christmas serum (diluted 1/10), control or test serum (various dilutions), and of platelet suspension. As 0.1 ml of M/40 CaCl_2 solution is added to the first tube a stop watch is started and at one minute intervals further 0.1 ml amounts of calcium chloride solution are added consecutively to the other three tubes. At exactly four

minutes after the addition of the calcium chloride solution to each tube, 0.1 ml of each incubation mixture is removed and together with 0.1 ml M/40 CaCl_2 is delivered into a clotting tube containing 0.1 ml high-spun plasma previously distributed and warmed to 37°C . and the clotting time recorded. This procedure is repeated till all the dilutions have been tested.

The thromboplastin times recorded are plotted on ordinary graph paper against the serum dilutions to construct the standard dilution curve.

Assay of test serum Christmas factor content

The same procedure is carried out using various dilutions of the test serum instead of those of the control serum in the incubation mixtures, and the four minute thromboplastin times recorded. The concentration of Christmas factor in each dilution is read off from the dilution curve.

Calculation: Christmas factor concentration in test serum =

$$\frac{1/20 \text{ (dilution of 100\% control)}}{\text{dilution of test serum}} \times \text{percentage reading.}$$

The mean Christmas factor concentration of the test serum is obtained by averaging the results obtained at the various dilutions tested.

23. Demonstration of inhibitors of human intrinsic thromboplastin
in thromboplastin generation mixtures

(Biggs and Macfarlane, 1957)

The presence of an inhibitor of human intrinsic thromboplastin in a plasma or serum sample from various animal species was investigated by testing mixtures of the test sample with normal human reagents, performing a thromboplastin generation test on an aliquot of the mixture, and incubating the other aliquot at 37°C. for two hours at the end of which the mixture is again tested in a T.G.T. Comparison of the substrate clotting times reveals any inhibitory effect that might be present.

Reagents

1. Control and test adsorbed plasmas: prepared as described in appendix III, 4, A, and diluted 1 in 5 in saline.
2. Control and test sera: appendix III, 5, diluted 1 in 10.
3. Cephalin 1/100 : appendix III, 14. This was used instead of the human platelet suspension.
4. High-spun plasma : appendix III, 2.
5. M/40 CaCl₂ solution : appendix I, 10, A.

Procedure

Mixtures are made containing equal parts of human adsorbed plasma (or human serum) and the test adsorbed plasma (or test

serum), and tested in the following incubation mixture immediately after preparation and again after two hours incubation at 37°C.

For a control, saline is substituted for the test preparation.

Incubation mixture: 0.1 ml human ads.pl. (1/5),
0.1 ml test ads.pl. (1/5),
0.2 ml human normal serum (1/10),
0.2 ml cephalin (1/100),
0.2 ml M/40 CaCl₂.

Immediately on recalcification a stop watch is started and at one minute intervals 0.1 ml amounts are transferred together with 0.1 ml amounts of M/40 CaCl₂ solution onto 0.1 ml volumes of the human substrate high-spun plasma previously distributed and warmed to 37°C. and the clotting times recorded.

The incubation mixture for testing the inhibitory activity of serum would be as follows:-

0.2 ml human normal ads.pl. (1/5),
0.1 ml human normal serum (1/10),
0.1 ml test serum (1/10),
0.2 ml cephalin (1/100),
0.2 ml M/40 CaCl₂.

Effect of plasma defibrination: was investigated by heating the test adsorbed plasma for 15' at 56°C., centrifuging and diluting the supernatant 1 in 5 in saline before making the mixtures.

24. Measurement of Hageman factor

I. Method of Ratnoff and Rosenblum, 1958

This method measures the degree of shortening in clotting times of normal platelet-deficient intact plasma on the addition of maximally activated plasma, whether control or test, after recalcification. The degree of shortening in the clotting times is indicative of the Hageman factor content of the plasma added.

Materials

1. Normal platelet-deficient citrated intact plasma: (substrate).

Normal blood is collected with siliconed needles and syringes and citrated 1 : 10 in siliconed graduated centrifuge tubes, then centrifuged at 2,000 r.p.m. for 15 minutes at 4°C. The plasma is removed and high-spun at 27,000 r.p.m. for 10 minutes at 4°C. and the clear supernatant removed and kept in lusteroid tubes at -20°C. till required. It keeps well for up to two months.

2. Test and control plasmas: the blood is collected with siliconed

precautions and oxalated 1 in 10, centrifuged at 2,000 r.p.m.

for 15 minutes and the supernatant plasma adsorbed with washed

BaSO₄ (appendix I, 3, B) using 100 mg/ml, in siliconed lusteroid

tubes. The mixture is stirred thoroughly with a siliconed glass

rod and incubated at room temperature for 10 minutes, then

centrifuged. The supernatant plasma is incubated at 56°C. for 30 minutes and centrifuged again.

Such treated plasmas are rendered deficient in all known coagulation factors except plasma thromboplastin antecedent and Hageman factor. It is incoagulable, and did not alter the recalcification time of normal intact plasma in siliconed tubes. But when the normal plasma preparation is treated with glass, it accelerated the clotting time of the normal intact plasma.

Glass activation

1.5 ml amounts of the control and test plasma preparations are shaken by the hand with 300 mg of glass powder (appendix I, 17) in pyrex tubes for 10 minutes at room temperature, then immediately put in ice-water and used within few minutes of preparation.

Procedure

The test is carried out in duplicate, at room temperature, and in siliconed clotting tubes into each of which is delivered 0.1 ml of normal platelet-deficient citrated intact plasma, 0.1 ml of the test or control plasma preparation before the glass activation, and as 0.1 ml of M/20 CaCl₂ is added a stop watch is started and the clotting time of the mixture recorded. Each tube is tilted once per minute, the end point being a firm clot. The longer clotting time of each pair of duplicate tubes is reported, this is usually within 2 minutes of each other when the clotting time

is 40 minutes or less. The same procedure is carried out at the same time for the test and control plasma preparations after glass activation, the results of which are indicative of the Hageman factor activity as compared to the results of the same preparations before glass activation.

At the time this method was published the authors believed that the shortening of the clotting time was due to the combined activities of Hageman factor and P.T.A. However, later this concept was revised, as it was found that the test is mainly sensitive to P.T.A. activity, (Ratnoff, 1962).

II. By correction of Hageman factor deficient plasma

This method measures the activity of Hageman factor in plasma by its ability to correct the recalcification time of Hageman factor deficient plasma, as compared to the ability of the normal plasma to do so.

Reagents

1. Hageman factor deficient plasma: plasma from a known patient with Hageman factor deficiency was obtained through the kindness of Dr.W.J.Jenkins, The National Blood Transfusion Service, Brentwood, Essex. The plasma was distributed in 3 ml amounts in lusteroid tubes and stored at -20°C . till required.
2. Test and control plasmas: using siliconed glassware throughout,

whole blood was obtained over trisodium citrate in the proportion of 9 to 1, and mixed by inversion while covering the tubes with parafilm covers. The plasma was separated after centrifuging for 15 minutes at 2,000 r.p.m. and kept at 4°C. till used.

3. M/10 CaCl₂ solution : appendix I, 10, A.

4. Cephalin 0.6 gm% (appendix III, 14) diluted 1 in 10 in veronal buffer. It was added to the test mixtures to obviate the effect of the varying concentrations of phospholipids and platelets in the different samples of plasma used.

5. Ballotini glass beads.

Glass activation

Maximal activation of both test and control plasmas was carried out before testing them. This was achieved by contact with glass, shaking by the hand 1 ml of plasma with 1 gm of ballotini glass beads in a bijou bottle for 10 minutes at room temperature, then immediately put in an ice-water bath and tested as soon as possible.

Construction of the standard dilution curve

Immediately after glass activation, 0.1 ml of the normal plasma sample is mixed with 0.4 ml Hageman plasma (this 1 in 5 dilution was designated the 100% value for Hageman factor), then 0.1 ml from this mixture is further diluted in 0.2 ml Hageman plasma previously distributed in siliconed clotting tubes placed in the ice-water bath. This three-fold dilution is repeated three more times, thus

giving a range of dilutions from 1 in 5 (100%) to 1 in 405 (1.2%). Two of these tubes at a time are transferred to a water bath at 37°C. and after few minutes 0.05 ml of cephalin solution together with 0.05 ml of M/10 CaCl₂ solution are added and a stop watch started and the clotting times recorded. The clotting times in seconds are plotted on double logarithmic paper against the calculated percentage of Hageman factor, the 1 in 5 dilution of the control plasma being designated the 100% value.

Assay of test plasma Hageman factor content

This is done in the same way as for the control plasma, using the most suitable three dilutions of the maximally activated plasma depending on a pilot test run. The amount of Hageman factor in each dilution is directly read from the standard dilution curve.

Calculation: concentration of Hageman factor in test plasma =
$$\frac{1/5 \text{ (dilution of control designated 100\%)}}{\text{dilution of test sample}} \times \text{percentage reading}$$

The mean Hageman factor concentration of the test plasma is obtained by averaging the results obtained for the various dilutions tested.

25. Measurement of the esteratic activity of thrombins

(method of Sherry and Troll, 1954)

This method demonstrates the esteratic activity of thrombin, utilizing TAME (p-Toluene sulphonyl-L-arginine methyl ester) as

substrate. Such activity is followed by the liberation of carboxyl groups which are split at the ester link, the activity can thus be measured by titrating the liberated carboxyl groups.

Reagents

1. TAME substrate, 0.2 molar solution: appendix I, 37.
2. Tris buffer, 0.5 molar, pH 9 : appendix I, 39.
3. Formaldehyde, 37%, pH 8 : appendix I, 15.
4. Phenol red indicator, 0.01% : appendix I, 24.
5. Sodium hydroxide, N/20 : appendix I, 34, B.
6. Thrombin solution under test.

Method

To 0.2 ml TAME solution is added 0.2 ml thrombin solution containing about 1-10 units (or other test solution), then 0.6 ml of Tris buffer is added and the mixture shaken and incubated for 30 minutes exactly at 37°C., after which time 1 ml of 37% solution of formaldehyde is added to stop further digestion of TAME.

A blank is prepared as above except for adding the formaldehyde solution at zero time before the addition of the thrombin solution.

Titration

To each of the above tubes, after completion, is added 0.2 ml phenol red indicator, and the solution is titrated against 0.05 N standard sodium hydroxide using a syringe type microburette (appendix II, 9).

A unit of enzymatic activity (TAMe unit) is defined as that amount of thrombin which will release 1 μM of acid from TAMe in 10 minutes under the conditions of the test. This unit has been so selected as to be approximately comparable in size to the N.I.H. clotting unit.

To calculate the arbitrarily defined units, the alkali consumed by the blank is subtracted from the amount consumed by the test, and converted to micromoles (μM) of acid liberated, then divided by 3 to give $\mu\text{M}/10$ minutes, i.e.

$$\begin{aligned} \text{TAMe units in 0.2 ml test solution} &= \frac{(\text{Test} - \text{Blank}) \times 50,000}{1,000 \times 3} \\ &= \frac{(\text{T} - \text{B}) \times 50}{3} \end{aligned}$$

APPENDIX V

RESULTS OF EXPERIMENTS

CONCENTRATION OF COAGULATION FACTORS

IN THE CONTROL HUMAN PLASMA USED AS STANDARD

The plasma used as reference control for all the quantitative assays was from the same individual (the author) throughout as to maintain a conceivably constant concentration of the various blood coagulation factors compared to which the concentration of animal coagulation factors were expressed in terms of percentages.

The control plasma itself was assayed against human plasma pooled from ten normal individuals to ascertain the exact levels of its coagulation factors as shown in table i. The methods used for these assays were as follows:-

Factor V : correction of factor V deficient plasma (appendix IV, 14).

Factor VII : correction of phenindione plasma (appendix IV, 15, A).

Factor VIII: correction of haemophilic plasma by the method of Pitney, 1956, (appendix IV, 21).

Factor IX : correction of Christmas serum (appendix IV, 22).

Factor X : correction of Seitz-filtered bovine plasma by the method of Bachmann et al, 1958, (appendix IV, 18).

For qualitative tests, pooled animal test samples were compared with control blood collected from three normal human subjects, the plasmas being pooled after centrifugation

Table i. The concentration of coagulation factors
in the control human plasma

Plasma dilution	F V	F VII	F VIII	F IX	F X
	Clotting times (seconds)				
P O O L E D H U M A N P L A S M A					
1/10	20	23	—	—	24½
1/20	26	31	10	10	32½
1/40	34	33	12	13	38½
1/80	40	37	15	17	43
1/160	49½	39	21	22	61½
1/320	53	40	49	40	73
1/640	55	41	65	60	83½
1/1280	58	44	80	72	96½
C O N T R O L P L A S M A					
1/10	20	24	—	—	25
1/20	25½	31	8½	9	32
1/40	33	33	10½	11	39
1/80	—	—	14	13	—
1/160	—	—	18	—	—
Concentration	105 %	90 %	151 %	200 %	91 %

Table ii. Thrombin-fibrinogen dilution curves

The reference thrombin-fibrinogen dilution curve (figure 1) was drawn from the results obtained for the first experiment. Later batches of thrombin or fibrinogen preparations were tested against stock standardised fibrinogen or thrombin solutions and only reagents giving results conforming to the original curve were released for use in later experiments. In experiments 2 and 4 the solution under test was fibrinogen, while in experiments 3 and 5 thrombin was the solution under test. These experiments are cited as examples of the several batches that were similarly tested as described in appendix IV, 7.

	Concentration of thrombin (nominal units/ml)															
	50	40	30	25	20	15	12½	10	8	6¼	5	4	3	3.1	2	1.55
	Clotting times of fibrinogen solutions (seconds)															
Expt.1	4	5½	6	--	8	11	--	14.4	19	--	34½	35.2	40	--	64	--
Expt.2	5			7			12			21					36	75
Expt.3	4½			7			13			21					38	74
Expt.4	4½			7			13			22					37½	69
Expt.5	4½			7½			12			21½					39	76

- 100 -

APPENDIX V, A

RESULTS OF EXPERIMENTS
ON FOWL BLOOD

RESULTS OF EXPERIMENTS
ON FOWL BLOOD

All the following data were obtained from experiments carried out on blood samples obtained from chicken of the Light Sussex strain, using pooled plasma or serum samples from at least three animals for each experiment to obviate as much as possible any individual variations from affecting the final levels of the various coagulation factors.

Blood samples were obtained fresh on the day of the test concerned as described in appendix II, 1, ii.

Table 1. Chicken plasma fibrinogen estimation

(micro-Kjeldahl method of Cullen and van Slyke, 1920, appendix IV,2,I)

Experiment 1

This will be described in detail as an example for the calculation.

Blank titration (B) = 14 mls.

Test titration (T) = 12.1 mls.

Standard titration (S) = 9.5 mls.

$$\text{Conversion factor} = \frac{10 \times 0.28 \times 6.25}{A} = 1.25$$

Calculation for standard:

$$(14 - 9.5) \times 1.25 = 5.62 \text{ mg protein/ 5 mls standard solution.}$$

Since this should have been 6.25 mg (equivalent to 1 mg N/ 5 mls)

$$\text{we have to correct for a recovery factor which equals } \frac{6.25}{5.62} = 1.1$$

Calculation for test:

$$\begin{aligned} \text{mg fibrinogen/ ml citrated plasma} &= (B - T) \times 1.25 \\ &= (14 - 12.1) \times 1.25 \\ &= 2.375 \end{aligned}$$

$$\text{mg fibrinogen/100 mls plasma} = 237.5$$

$$\text{Correction for recovery: } 237.5 \times 1.1 = 261.25$$

Correction for plasma dilution by the citrate: in this example

the hematocrit values for the individual blood samples were estimated and the supernatant plasma volumes read off accurately, giving an average of 8 mls plasma from each 10 mls citrated blood.

Since the plasma contains virtually all the citrate added, i.e. 1 ml, this 8 mls actually contains 7 mls of absolute plasma. Therefore the result should be multiplied by the factor $\frac{8}{7}$ as follows:-

$$\text{Plasma fibrinogen} = 261.25 \times \frac{8}{7} = 298.5 \text{ mg/100 mls absolute plasma.}$$

Table 1 (ctd.). Chicken plasma fibrinogen content

	mg/100 mls citrated plasma	mg/100 mls absolute plasma
Experiment 1	261.25	298.5
Experiment 2	200.70	250.8
Experiment 3	222.59	261.8
Experiment 4	209.10	246.0
Average	223.41	264.0

Table 2. Chicken plasma fibrinogen estimation

(Stirland's turbidimetric method, 1956, appendix IV, 2, III)

	mgs fibrinogen/100 mls plasma
Expt. 1	130
Expt. 2	100
Expt. 3	25.7
Expt. 4	100

Plasma used in experiment 3 was also estimated by the micro-kjeldahl method which gave a fibrinogen concentration of 172.3 mg per 100 mls of plasma. In view of this gross discrepancy, Stirland's method was found unsuitable for the estimation of chicken plasma fibrinogen which may have different precipitation characteristics.

Table 3. Thrombin-fibrinogen reaction of the plasma

(method described in appendix IV, 8 and 9)

Substrate Plasma	Thrombin topical (P.D.& Co.) in units/ml					
	50	25	12.5	6.25	3.1	1.55
Mixture	Clotting times in seconds (at 37°C.)					
0.2 ml Chicken pl.	90	180 +	180 +	180 +	180 +	180 +
0.2 ml Saline	115	180 +	180 +	180 +	180 +	180 +
0.2 ml Thrombin	125	180 +	180 +	180 +	180 +	180 +
0.2 ml Human pl.	5	6	7½	13	22	43
0.2 ml Saline	6	6	10	16	28	58
0.2 ml Thrombin	6	6	9	15	24	50
0.2 ml Chicken pl.	40	38	42	55	90	150
0.1 ml Saline	42	41	44	60	75	120
0.1 ml M/40 CaCl ₂	41	42	43	58	80	135
0.2 ml Thrombin						
0.2 ml Human pl.	3	4	5	8	16½	31
0.1 ml Saline	3	3½	5½	13	18	34
0.1 ml M/40 CaCl ₂	3	4	6½	12	18	35
0.2 ml Thrombin						
0.2 ml Chicken pl.	11	13	15	25	64	132
0.1 ml Pyrocatechol	13	15	18	30	58	97
0.1 ml M/40 CaCl ₂	11	13½	16	29	60	130
0.2 ml Thrombin						
0.2 ml Human pl.	2	3	4	6	11	19
0.1 ml Pyrocatechol	2	3	4	9	12	20
0.1 ml M/40 CaCl ₂	2	3	4	8	12	20
0.2 ml Thrombin						
0.2 ml Chicken pl.	49	50	52	67	86	143
0.1 ml Toluidine blue	47	48	50	65	87	139
0.1 ml M/40 CaCl ₂	46	49½	51	64½	86	141
0.2 ml Thrombin						
0.2 ml Human pl.	3	3	5	9	16	32
0.1 ml Tol. blue	3	3½	5	10	16½	33
0.1 ml M/40 CaCl ₂	3	3	5	10	16	32
0.2 ml Thrombin						
	Clotting times in seconds (at 41°C.)					
0.2 ml Chicken pl.						
0.1 ml Saline	45	41	44	60	85	137
0.1 ml M/40 CaCl ₂						
0.2 ml Thrombin						
Human control pl.	3	4	5	10	16	32

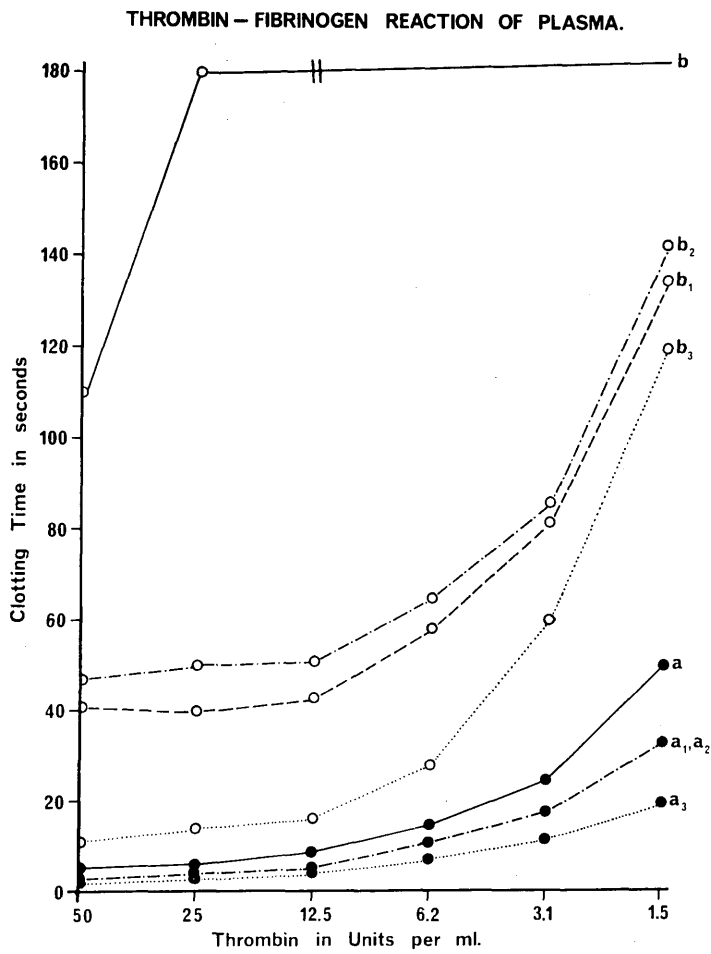


Figure 3. Thrombin-fibrinogen reaction of chicken plasma.

- Curve a : human plasma + saline.
- Curve a₁ : human plasma + CaCl₂ + saline.
- Curve a₂ : human plasma + CaCl₂ + toluidine blue.
- Curve a₃ : human plasma + CaCl₂ + pyrocatechol.
- Curve b : chicken plasma + saline.
- Curve b₁ : chicken plasma + CaCl₂ + saline.
- Curve b₂ : chicken plasma + CaCl₂ + toluidine blue.
- Curve b₃ : chicken plasma + CaCl₂ + pyrocatechol.

Table 4. Antithrombin assay in chicken plasma

(modified method of Astrup and Darling, 1942d, appendix IV, 11, B)

Experiment 1

Mixture			A	B	C	D
Saline	ml.	0.3	0.2	0.15	0.1	0.0
Plasma (dil.1/5)	ml.	0.0	0.1	0.15	0.2	0.3
Thrombin(30 U/ml)	ml.	1	1	1	1	1
Clotting times of fibrinogen in seconds*	Ck.	14	15	16	17	20
	Hn.	14	15	16	18	20
T_o/T_n	Ck.	1	0.93	0.87	0.82	0.70
	Hn.	1	0.93	0.87	0.77	0.70
Corrected T_o/T_n	Ck.	1	0.91	0.87	0.82	0.72
	Hn.	1	0.90	0.85	0.80	0.70
n values in ml.		0	0.02	0.03	0.04	0.06

* Mean of duplicate estimations.

Calculation: is described in detail as an example for later experiments.

The concentration of antithrombin in chicken plasma in mixture

A according to the equation that antithrombin concentration equals

$$\frac{a}{n} (1 - T_o/T_n) \text{ would therefore be } \frac{30}{0.02} (1 - 0.91) = 135 \text{ units.}$$

Similarly mixture B was found to contain 130 units, while mixtures C and D contained 135 and 140 units respectively. The average of the four mixtures was 135 units.

The antithrombin concentration in human mixture A was 150 units, while mixtures B, C, and D all had values of 150 units, with an average of 150 units.

Therefore, chicken plasma antithrombin = $\frac{135 \times 100}{150}$ which equals 90% of human plasma antithrombin content.

This experiment is illustrated in figure 4 from which the corrected T_o/T_n values were obtained.

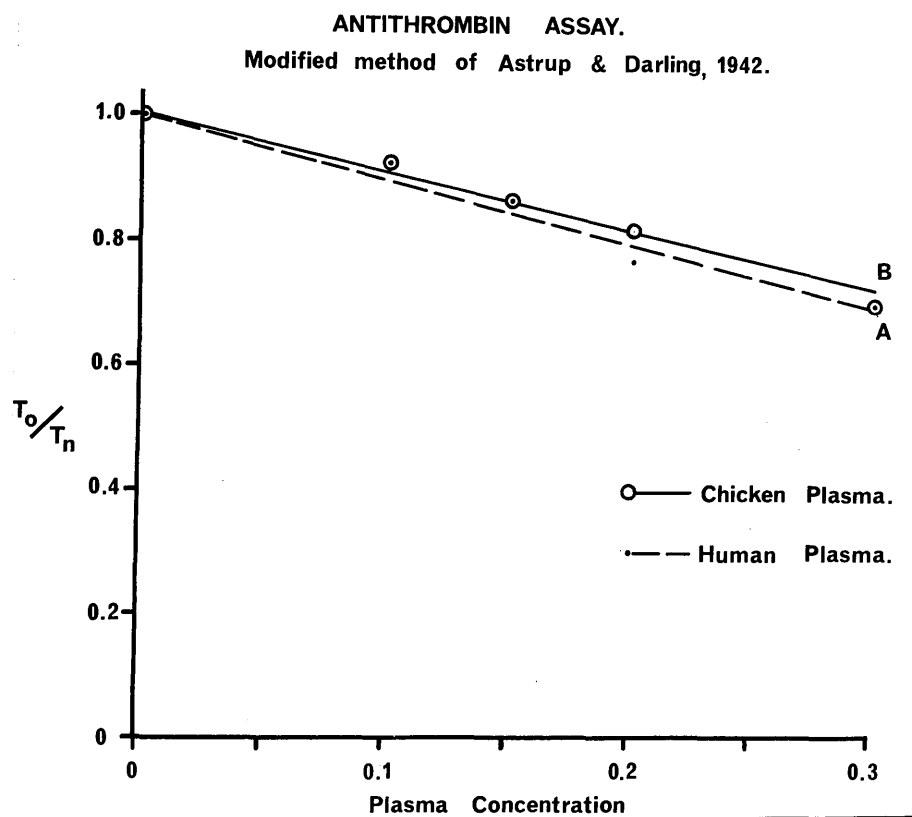


Figure 4. Antithrombin assay in chicken plasma.

Curve A : values obtained with human plasma, experiment 1, table 4.

Curve B : values obtained with chicken plasma, experiment 1, table 4.

Table 5. Antithrombin assay in chicken plasma

Experiment 2

		A	B	C	D	
Clotting times of fibrinogen	Ck.	13	15	18	20	23
	Hn.	13	14½	17	19	22
Computed conc. of antithrombin	Ck.	240	240	240	235	235
	Hn.	225	220	217	215	215

Experiment 3

		A	B	C	D	
Clotting times of fibrinogen	Ck.	13	14	16	19	24
	Hn.	13	14	15	18½	23
Computed conc. of antithrombin	Ck.	225	230	232	230	230
	Hn.	195	200	195	200	200

Table 6. Average antithrombin concentration of chicken plasmas

Experiment	Chicken plasma antithrombin concentration	
	in units	in per cent of human antithrombin
1	135	90 %
2	238	108 %
3	229	116 %
Average	200.6	104.6 %

Table 7. Species specificity of the thrombin-fibrinogen reaction

(method described in appendix IV, 10)

Substrate	Thrombin solution		
	Human	Chicken	Bovine (50 U/ml)
	Clotting times (seconds)*		
0.4 ml human fibrinogen 0.1 ml saline	3½	8	3½
0.4 ml chicken fibrinogen 0.1 ml saline	21	26	27
0.4 ml human fibrinogen 0.1 ml pyrocatechol 1%	4	6	3
0.4 ml chicken fibrinogen 0.1 ml pyrocatechol 1%	15	13	12

* Average of two estimations.

Table 8. Thrombin clotting times of chicken plasma

0.1 ml amounts of thrombin solution (P.D.& Co.) containing 50 units/ml were added to 0.4 ml of the plasma mixture and the clotting times recorded. The plasma was heated at 56°C. for 15 minutes to defibrinate it, then it was spun to get rid of the precipitate, and the supernatant tested as to its inhibitory effect when added to normal untreated human plasma.

Hum.Pl. + Ck.Pl.	Hum.Pl. + Heated Ck.Pl.	Heated Ck.Pl.	Hum.Pl.	Ck.Pl.
37"	19"	180 +	4"	77"

Table 9. Quick's one-stage prothrombin time

(method described in appendix IV, 6)

Test plasma	Human brain	Chicken brain
	Clotting times (seconds)	
Chicken plasma	217	16
	435	14
	146	14
Human plasma	15	132
	13	172
	14	138
Human pl. + chicken pl.	24	29
	22	28
	23	24
Human pl. + saline	20	205
	19	160
	19	150
Chicken pl. + saline	355	22
	318	24
	162	15

Table 11. Factor V assay

In view of the strong species specificity of chicken factor V and brain thromboplastin, dilution curves of normal plasma in the homologous plasmas rendered deficient in factor V were compared with each other, each being clotted with the homologous brain extract.

	Factor V concentration (per cent)							
	100	50	25	12.5	6.2	3.1	1.5	0
	Clotting times (seconds)							
Human experiment 1	25½	28½	33	38	42½	45	49	50
Human experiment 2	22	30	37	42	50	55	62	70
Human experiment 3	21	26	30	37	50	54	62	86
Chicken experiment 1	27	31	34	37	39	40	41	42
Chicken experiment 2	29	36	45	53	62	70	80	87
Chicken experiment 3	27	32	36½	40	46	52	57½	61

The 1 in 10 dilution of the normal fresh plasma in the stored factor V deficient plasma was designated the 100% value for factor V concentration.

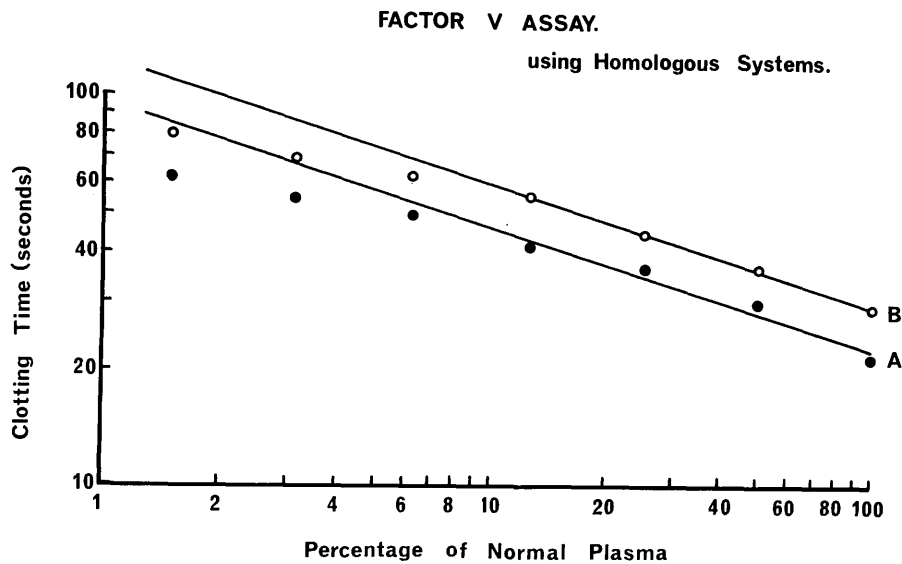


Figure 5. Factor V assay.

Curve A

Human factor V dilution curve, constructed by clotting the normal human plasma dilutions in stored human plasma by the addition of human brain thromboplastin and recalcification with M/40 CaCl_2 . It illustrates experiment 2 from table 11.

Curve B

Chicken factor V dilution curve, constructed by clotting the chicken normal plasma dilutions made in stored chicken plasma by the addition of chicken brain thromboplastin and recalcification with M/40 CaCl_2 . It illustrates experiment 2 from table 11.

Table 12. Factors VII and X - brain thromboplastin species specificity

(method described in appendix IV, 17)

Incubation mixture	Substrate	Incubation time (minutes)					
		1	2	3	4	5	6
		Clotting Times (seconds)					
Hum. brain 1/4 Hum. serum 1/5 CaCl ₂ M/40	Fresh hum.pl.	22	18	18	19	19½	18
	Phenindione pl.	18	18	15½	17	18	17
Hum. brain 1/4 Ck. serum 1/5 CaCl ₂ M/40	Fresh hum.pl.	45	35	35	38	38	38
	Phenindione pl.	65	63	59	59	58	60
Hum. brain 1/4 Saline CaCl ₂ M/40	Fresh hum.pl.	36	37	36	36	38½	38½
	Phenindione pl.	66	63	60	64	64	63
Ck. brain 1/2 Ck. serum 1/5 CaCl ₂ M/40	Fresh Ck.pl.	12½	12	12½	13	14½	14
	Fresh Hum.pl.	34	38	46	50	51	57
	Phenindione pl.	67	63	62	60	100	100
Ck. brain 1/2 Hum. serum 1/5 CaCl ₂ M/40	Fresh Ck.pl.	24	22½	23	22	23	23½
	Fresh hum.pl.	168	162	162	180	180	190
	Phenindione pl.	180	191	-	194	196	201
Ck. brain 1/2 Saline CaCl ₂ M/40	Fresh Ck.pl.	23	22	22½	22	22	22
	Fresh hum.pl.	190	180	163	188	190	190
	Phenindione pl.	240	210	260	332	264	335

Table 13. Stuart-Prower factor assay

(method of Bachmann, Duckert, and Koller, 1958; appendix IV, 18)

Plasma dilution	Concentration of factor X	Clotting times (seconds)		
		Expt. 1	Expt. 2	Expt. 3
		Hum.Plasma	Hum.Plasma	Hum.Serum
1/10	100 %	20	19½	22
1/20	50 %	24½	25	25
1/40	25 %	30	32	31
1/80	12 %	38	36	36
1/160	6 %	46	45	44
1/320	3 %	55	51	50
1/640	1.5%	63	58	65
1/1280	0.75%	80	70	77
1/2560	0.36%	—	80½	83
Buffer	0.0 %	141	91	100
		Ck.Plasma	Ck.Plasma	Ck.Serum
1/10		73	72	92
1/20		81	83	—
1/2		—	—	99
Calculated factor X conc.		1.2 %	0.62 %	0.1 %

Table 14. R.V.V.-cephalin clotting time of plasma

(method described in appendix IV, 19)

Concentration of R.V.V.-cephalin	Plasma	Plasma dilution in veronal buffer				
		1	1/2	1/4	1/8	1/16
		Clotting time (sec.)*				
1/40,000 - 0.06 gm %	Human	8	9	13	20	40
	Chicken	17	18	23	35	54
1/80,000 - 0.06 gm %	Human	8	10	13	20	35
	Chicken	17	18	22	35	53
1/200,000- 0.06 gm %	Human	9	12			
	Chicken	20	21½			

* Average of three estimations.

Table 15. Measurement of prothrombin

Prothrombin was estimated by the two-stage area method of Biggs and Douglas, 1953a (appendix IV, 12, C), using the brain thromboplastin of the same species as the plasma under test, and subsampling onto human fibrinogen solution.

Incubation mixture	Expt.	Incubation time (minutes)						Number of squares under curve*
		$\frac{1}{2}$	$\frac{3}{4}$	1	2	3	4	
		Clotting times (seconds)						
Hum. pl. with Hum. brain	1	12	13	14	29	86	180+	
	2	12 $\frac{1}{2}$	13 $\frac{1}{2}$	15	31	90	180+	
	3	--	--	12	21	43	133	
Ck. pl. with Ck. brain	1	32	73	100	180+	180+	180+	
	2	30	40	93	180+	180+	--	
	3	32	60	81	180+	180+	--	
		Thrombin units / ml.						
Human	1	13 $\frac{1}{2}$	12	11	4.4	1.2	0.0	508
	2	12 $\frac{1}{2}$	11.2	10.4	4.3	1.2	0.0	480
	3	--	--	13.5	7	2.9	0.7	592
Chicken	1	4.1	1.5	1.0	0.0	0.0		63
	2	4.5	3.3	1.1	0.0	0.0		75
	3	4.1	1.9	1.38	0.0	0.0		73

* Each square = 1/100 th square inch.

The average area for human plasma = 526.6 squares.

The average area for chicken plasma = 70.3 squares.

Thus, chicken plasma prothrombin = $\frac{70.3 \times 100}{526.6}$
 = 13.3 % of human prothrombin.

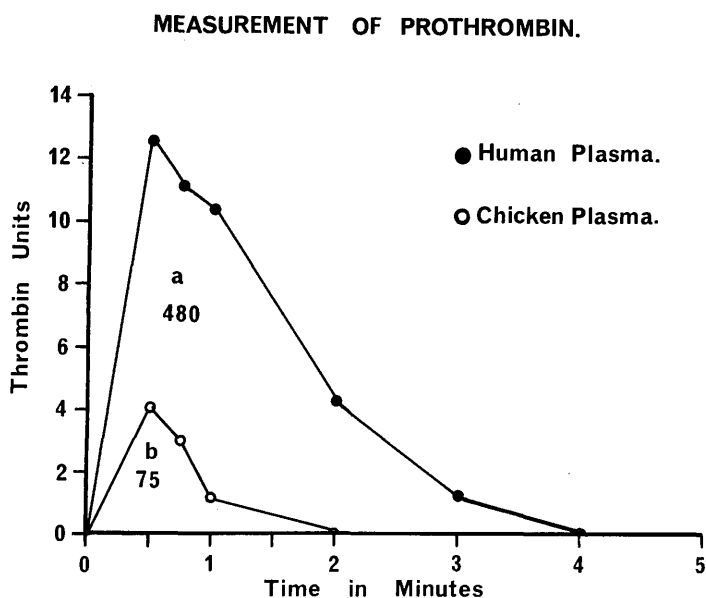


Figure 6. Measurement of prothrombin.

(the two-stage area method of Biggs & Douglas, 1953a)

Curve a

Human plasma tested with human brain, (table 15, experiment 2).

Curve b

Chicken plasma tested with chicken brain, (table 15, experiment 2).

Subsampling in both cases was onto human fibrinogen, and the clotting times converted into thrombin units from the thrombin-fibrinogen dilution curve illustrated in figure 1.

Table 16. Measurement of prothrombin

Prothrombin was estimated by the globulin fraction method of Douglas and Biggs, 1953, (appendix IV, 12, B), using human brain thromboplastin only for both plasmas.

	Experiment 1		Experiment 2	
	Human	Chicken	Human	Chicken
Clotting times of substrate (seconds)	12½	41	13½	46
Thrombin units/ml.	13	3.1	11.5	2.7
Prothrombin conc.	100 %	23.8 %	100 %	23.4 %

Table 17. Thrombocyte count

(method described in appendix IV, 1, B)

Experiment	Total count of W.B.Cs./c.mm.	Percentage of thrombocytes	Calculated No. of thrombocytes
1	38,500	50 %	19,250
2	40,500	52 %	21,060
3	45,000	45 %	20,250
4	38,000	55 %	20,900
5	28,000	60 %	16,800

The average thrombocyte count = 19,650 /c.mm. whole blood.

Table 18. Chicken whole blood clotting time

(method of Lee and White, 1913; appendix IV, 3)

	Clotting times*	
	in ordinary glass	in siliconed glass
Hen 1	11' 12"	13' 23"
Hen 2	32'	41' 30"
Hen 3	45'	51' 20"
Hen 4	16' 30"	18'
Hen 5	17'	17' 20"
Average	24' 20"	28' 18"

* Average of two tubes' readings.

Table 19. Recalcification time of plasma

(method: appendix IV, 5)

Clotting times (seconds)	
Human plasma	Chicken plasma
165	1157
160	480
160	512

Table 20. Hageman factor and plasma thromboplastin antecedent activity

(method of Ratnoff and Rosenblum, 1958; appendix IV, 24, I)

Test plasma preparation	Clotting time in minutes of substrate plasma (normal citrated platelet-deficient Intact plasma)	
	Human	Chicken
Human, intact	64	60
	43 (66)*	120+ (120+)
	90	120+
Chicken, intact	120+	120+
	120+ (120+)	120+ (120+)
	120+	120+
Human, glass activated	13	50
	15 (13)	38 (38)
	10	27
Chicken, glass activated	120+	120+
	120+ (120+)	120+ (120+)
	120+	120+

* Values between brackets are the average of the three experiments.

Table 21. Thromboplastin generation test

(method of Biggs and Douglas, 1953b; appendix IV, 20)

Human platelets and M/40 CaCl₂ solutions were constant (0.2 ml each).

Substrate: human high-spun plasma.

No.	Incubation mixture (ml)	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
1	0.2 Chicken plasma 0.2 Human serum	1	63	59	48	44	36	31
		2	24	14	15	15	15	15
		3	96	82	73	62	50	29
		Avg.	61	52	45	40	34	25
2	0.2 Chicken plasma 0.2 Chicken serum	1	157	70	49	28	25	25
		2	62	35	25	28	37	45
		3	150	140	127	90	84	53
		Avg.	123	82	67	49	49	41
3	0.2 Chicken plasma 0.2 Saline	1	100	91	81	78	95	96
		2	132	115	105	92	83	63
		3	107	112	120	145	152	180+
		Avg.	113	106	102	105	110	113
4	0.2 Human plasma 0.2 Human serum	1	30	11	11	11	11	11
		2	60	11	9	9	9	9
		3	73	27	10	9½	10	10
		Avg.	54	16	10	10	10	10
5	0.2 Human plasma 0.2 Chicken serum	1	70	72	130	77	117	100
		2	88	105	90	120	111	102
		3	122	108	104	100	110	120
		Avg.	93	95	108	99	113	107
6	0.2 Human plasma 0.2 Saline	1	98	94	101	99	130	105
		2	93	98	92	104	104	99
		3	180	178	127	144	132	120
		Avg.	124	123	107	116	122	108
7	0.2 Saline 0.2 Chicken serum	1	180+	118	180+	180+	180+	180+
		2	97	133	100	145	132	120
		3	180+	180	163	170	175	92
		Avg.	152	144	147	165	162	131
8	0.2 Saline 0.2 Human serum	1	80	71	60	52	38	32
		2	79	64	50	41	33	26
		3	78	70	58	50	45	37
		Avg.	79	68	56	48	38	32
9	0.2 Haemophilic pl. 0.2 Human serum	1	85	64	53	40	32	32
		2	78	62	45	33	33	34
		3	90	75	62	52	37	37
		Avg.	84	67	53	42	34	34

Table 21, continued

No.	Incubation mixture (ml)	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
10	0.2 Human plasma 0.2 X-mas serum	1	91	79	64	45	34	32
		2	73	55	45	39	32	32
		3	172	160	135	104	68	45
		Avg.	112	98	81	63	45	36
11	0.2 Haemophilic pl. 0.1 Human ads.pl. 0.2 Human serum	1	60	21	11	11	11	11
		2	55	15	9	9	9	9
		3	68	30	11	9	10	10
		Avg.	61	22	10	10	10	10
12	0.15 Haemophilic pl. 0.05 Human.ads.pl. 0.2 Human serum	1	72	40	12½	12½	13	13
		2	--	--	--	--	--	--
		3	70	39	11	12	12	12
		Avg.	71	39½	11½	12¼	12½	12½
13	0.175 Haemoph.pl. 0.025 Hum.ads.pl. 0.2 Hum.serum	1	76	47	16	15	14	14
		2	--	--	--	--	--	--
		3	71	44	15	14	13½	14
		Avg.	73	45	15½	14½	13½	14
14	0.19 Haemoph.pl. 0.01 Hum.ads.pl. 0.2 Hum.serum	1	90	49	18	17	17	16
		2	--	--	--	--	--	--
		3	70	47	19	16½	16½	16
		Avg.	80	48	18½	16¾	16¾	16
15	0.1 Haemophilic pl. 0.1 Chicken plasma 0.2 Human serum	1	60	42	30	30	21	21
		2	46	24	19	18	17	15½
		3	78	62	55	43	28	24
		Avg.	62	43	37	30	22	20
16	0.1 Haemophilic pl. 0.1 Saline 0.2 Human serum	1	65	56	42	31	30	29
		2	67	50	38	26	28	29
		3	63	62	46	37	31	32
		Avg.	65	56	42	31	30	30

The results in every fourth line for each mixture are the average of the above three experiments. The first experiment for all mixtures are directly comparable with each other as they were obtained with the same platelet preparation and the same set of reagents. Likewise, all second and third experiments are comparable with each other respectively being obtained with the same set of reagents for each experiment, though different from those of other experiments. However, all the results are approximately of the same magnitude of activity or lack of activity, sufficiently enough to justify taking their average as a general guide for the comparison between the activity of the various mixtures. These averages were converted into percentage active thromboplastin in the following table.

Table 22. Thromboplastin generation test

Compilation of data in table 21 in terms of percentage active human thromboplastin as read from the thromboplastin dilution curve A in figure 2 (page 80) which was constructed with human platelets.

No.	Incubation mixture (ml)		Incubation time (minutes)					
			1	2	3	4	5	6
			Percentage active thromboplastin					
1	0.2	Chicken plasma	2.2	3	4.2	5.4	7.5	15
	0.2	Human serum						
2	0.2	Chicken plasma	0.4	1.2	1.8	3.5	3.5	5.2
	0.2	Chicken serum						
3	0.2	Chicken plasma	0.5	0.6	0.7	0.6	0.6	0.5
	0.2	Saline						
4	0.2	Human plasma	3	40	100	100	100	100
	0.2	Human serum						
5	0.2	Human plasma	0.9	0.8	0.6	0.7	0.6	0.6
	0.2	Chicken serum						
6	0.2	Human plasma	0.4	0.4	0.6	0.5	0.5	0.6
	0.2	Saline						
7	0.2	Saline	0.3	0.3	0.3	0.2	0.2	0.4
	0.2	Chicken serum						
8	0.2	Saline	1.3	1.7	2.5	3.7	6	8.5
	0.2	Human serum						
9	0.2	Haemophilic plasma	1.1	1.7	3	5.2	7.5	7.5
	0.2	Human serum						
10	0.2	Human plasma	0.5	0.8	1.2	2	4.2	6.8
	0.2	X-mas serum						
11	0.1	Haemophilic plasma	2.2	19	100	100	100	100
	0.1	Human plasma						
	0.2	Human serum						
12	0.15	Haemophilic plasma	1.6	5.6	78	68	65	65
	0.05	Human plasma						
	0.2	Human serum						
13	0.175	Haemophilic plasma	1.5	4.2	42	47	54	50
	0.025	Human plasma						
	0.2	Human serum						
14	0.19	Haemophilic plasma	1.2	3.7	28	35	35	38
	0.01	Human plasma						
	0.2	Human serum						
15	0.1	Haemophilic plasma	2.1	4.6	6.4	10	19	24
	0.1	Chicken plasma						
	0.2	Human serum						
16	0.1	Haemophilic plasma	1.9	2.6	4.9	9.2	10	10
	0.1	Saline						
	0.2	Human serum						

Table 23. Thromboplastin generation test

(method of Biggs and Douglas, 1953b; appendix IV, 20)

Human cephalin (appendix III, 14) and M/40 CaCl₂ solutions (0.2 ml each) were constant. Substrate: human high-spun plasma.

The fifth line for each mixture represents the percentage of active thromboplastin as read from the dilution curve B in figure 2, which was constructed using human cephalin, plasma, and serum.

No.	Incubation mixture (ml)	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
17	0.2 Chicken plasma 0.2 Human serum	1	15	15	15	15	15	15
		2	14	12	12	14	13½	15
		3	51	23	20	19	23	21
		Avg.	27	17	16	16	17	17
		%	9.5	29	33	33	29	29 %
18	0.2 Chicken plasma 0.2 Chicken serum	1	86	28	24	24	25	27
		2	45	20	23	33	39	55
		3	45	31	29	30	32	31
		Avg.	59	26	25	29	32	38
		%	1.5	10	11.5	8	6.4	4.2%
19	0.2 Chicken plasma 0.2 Saline	1	180+	180+	97	92	93	95
		2	88	85	72	50	46	42
		3	93	100	90	120	87	130
		Avg.	120	122	86	87	75	89
		%	0.2	0.2	0.5	0.5	0.8	0.5%
20	0.2 Human plasma 0.2 Human serum	1	45	11	9	9	10	9
		2	50	11	8½	8½	9	9
		3	75	35	10	10	10	9
		Avg.	57	19	9	9	9	9
		%	1.6	22	130	130	130	130
21	0.2 Human plasma 0.2 Chicken serum	1	95	91	95	88	110	90
		2	110	93	95	88	120	96
		3	97	104	100	120	110	110
		Avg.	101	96	97	99	113	99
		%	0.4	0.4	0.4	0.4	0.3	0.4%
22	0.2 Human plasma 0.2 Saline	1	99	99	121	98	130	134
		2	109	93	76	78	120	110
		3	101	98	102	107	124	112
		Avg.	103	99	100	94	124	119
		%	0.4	0.4	0.4	0.4	0.2	0.2%

Table 23, continued

No.	Incubation mixture (ml)	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
23	0.2 Saline 0.2 Chicken serum	1	93	91	105	95	85	80
		2	104	110	104	173	180+	180+
		3	113	110	180+	104	129	173
		Avg.	103	103	129	124	131	141
		%	0.4	0.4	0.2	0.2	0.2	0.1 %
24	0.2 Saline 0.2 Human serum	1	96	97	85	73	78	93
		2	65	65	68	64	62	60
		3	81	72	71	63	70	68
		Avg.	81	78	75	67	70	74
		%	0.7	0.7	0.8	1	0.9	0.8 %
25	0.2 Haemophilic pl. 0.2 Human plasma	1	86	75	73	71	69	63
		2	69	69	62	63	57	54
		3	72	73	71	67	63	60
		Avg.	76	72	69	67	63	59
		%	0.8	0.9	1	1	1.2	1.4 %
26	0.2 Human plasma 0.2 X-mas serum	1	103	66	30	28	30	34
		2	127	97	87	90	82	68
		3	134	133	101	115	84	108
		Avg.	121	99	73	78	65	70
		%	0.3	0.4	0.9	0.7	1.1	0.9 %
27	0.1 Haemophilic pl. 0.1 Human plasma 0.2 Human serum	1	70	25	10	9	9	9
		2	68	19	9	9	8½	9
		3	67	30	11	10	10	10
		Avg.	68	25	10	9.3	9.1	9.3
		%	1	11	100	120	125	120 %
28	0.15 Haemophilic pl. 0.05 Human plasma 0.2 Human serum	1	68	30	11	9	9	9
		2	55	18	9	9	9	9
		3	72	45	13	10	10	10
		Avg.	65	31	11	9.3	9.3	9.3
		%	1.1	7	80	120	120	120 %
29	0.175 Haemophilic pl. 0.025 Human plasma 0.2 Human serum	1	70	48	19	12	12	12
		2	67	55	21	10	10	10
		3	67	56	26	14	12	12
		Avg.	68	53	22	12	11.3	11.3
		%	1	1.9	17	66	75	75
30	0.19 Haemophilic pl. 0.01 Human plasma 0.2 Human serum	1	80	66	25	20	16	15
		2	70	59	21	16	15	14
		3	76	58	20	15	15	15
		Avg.	75	61	22	17	15.3	14.6
		%	0.8	1.4	17	29	36	40 %
31	0.1 Haemophilic pl. 0.1 Chicken plasma 0.2 Human serum	1	68	30	16	15½	15	15
		2	25	15	15	15	14	14½
		3	70	41	23	17	17	17
		Avg.	54	29	18	16	15.3	15.5
		%	1.8	8	25	33	36	35
32	0.1 Haemophilic pl. 0.1 Saline 0.2 Human serum	1	100	98	104	90	92	90
		2	87	110	134	127	133	90
		3	92	85	82	80	75	70
		Avg.	93	98	107	99	100	83
		%	0.4	0.4	0.3	0.4	0.4	0.6 %

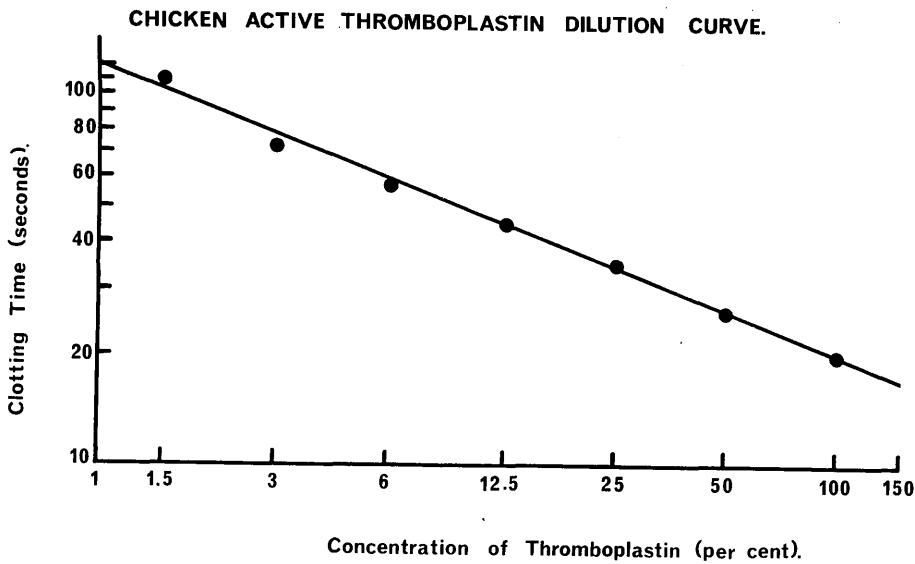


Figure 7. Chicken active thromboplastin dilution curve.

The same method as for the construction of the human dilution curve was used as described in appendix IV, 20, A, except for the substitution of chicken reagents instead of the human in the incubation mixture as follows:-

Incubation mixture: 0.2 ml chicken adsorbed plasma, diluted 1/5,
0.2 ml human serum, diluted 1/10,
0.2 ml chicken thrombocyte suspension,
0.2 ml M/40 CaCl_2 solution.

The shortest thromboplastin time which was 20 seconds was designated the 100 per cent value.

Table 24. Modified thromboplastin generation test

(method of Biggs and Douglas, 1953b; appendix IV, 20)

Chicken thrombocyte suspension (appendix III, 15, B) and the M/40 CaCl₂ solution were constant (0.2 ml each) in all mixtures.

Substrate: human high-spun plasma. Percentage thromboplastin was read off from the chicken active thromboplastin dilution curve illustrated in figure 7.

No.	Incubation mixture (ml)	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
33	0.2 Chicken plasma 0.2 Human serum	1	53	25	25	24	22½	24
		2	66	26	21	19	21	19
		3	72	30	21	20	19	18
		Avg.	64	27	22	21	21	20
			5.4	45	80	90	90	100 %
34	0.2 chicken plasma 0.2 chicken serum	1	49	24	25	32	38	42
		2	124	98	82	45	30	25
		3	125	75	30	25	22	22
		Avg.	99	66	46	34	30	30
			1.8	5	12	30	35	35 %
35	0.2 Chicken plasma 0.2 saline	1	75	70	50	45	42	40
		2	102	106	93	95	115	97
		3	105	101	125	95	97	90
		Avg.	94	92	89	78	85	76
			2	2.2	2.4	3.2	2.6	3.5 %
36	0.2 Human plasma 0.2 Human serum	1	76	53	45	39	39	37½
		2	75	67	55	35	26	20
		3	120	90	73	62	41	22
		Avg.	90	70	58	45	35	27
			2.3	4.3	6.8	13	24	45 %
37	0.2 Human plasma 0.2 Chicken serum	1	122	82	150	130	85	140
		2	104	104	102	90	97	97
		3	98	92	99	92	97	140
		Avg.	108	93	110	104	93	126
			1.5	2.1	1.3	1.6	2.1	1 %

Table 24, continued

No.	Incubation mixture	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
38	0.2 Human plasma 0.2 Saline	1	77	65	63	63	66	64
		2	95	90	100	130	105	110
		3	110	110	112	115	122	120
		Avg.	94	88	92	102	101	98
		%	2	2.5	2.2	1.7	1.7	1.9 %
39	0.2 Saline 0.2 Chicken serum	1	120	180+	144	145	180+	180+
		2	102	98	110	134	116	109
		3	85	90	89	84	90	92
		Avg.	102	123	114	121	129	127
		%	1.7	1.1	1.3	1.1	0.9	1 %
40	0.2 Saline 0.2 Human serum	1	105	134	87	180+	180+	180+
		2	85	82	89	89	85	76
		3	85	99	92	89	79	100
		Avg.	92	105	89	119	114	118
		%	2.2	1.6	2.4	1.1	1.3	1.2 %
41	0.2 Haemophilic pl. 0.2 Human serum	1	93	92	85	85	80	82
		2	85	78	88	84	85	74
		3	90	90	100	102	100	98
		Avg.	89	87	91	90	88	84
		%	2.4	2.5	2.2	2.3	2.5	2.7 %
42	0.2 Human plasma 0.2 X-mas serum	1	160	162	150	155	130	102
		2	115	130	132	160	162	105
		3	180	180	120	90	157	110
		Avg.	152	157	134	135	150	106
		%	less than one per cent					1.5 %
43	0.1 Haemophilic pl. 0.1 Human plasma 0.2 Human serum	1	90	85	66	64	43	24
		2	80	78	64	58	40	25
		3	85	80	68	62	45	26
		Avg.	85	81	66	61	43	25
		%	2.6	3	5	6	14	56 %
44	0.1 Haemophilic pl. 0.1 Chicken plasma 0.2 Human serum	1	102	83	45	29	27	27
		2	88	80	43	26	26½	26
		3	96	79	40	26	26	25
		Avg.	95	81	43	27	26	26
		%	2	3	14	45	50	50 %
45	0.15 Haemophilic pl. 0.05 Chicken plasma 0.2 Human serum	1	110	92	80	72	49	38
		2	--	--	--	--	--	--
		3	107	90	84	70	50	40
		Avg.	109	91	82	71	49	39
		%	1.4	2.2	2.9	4.1	10	18 %

Table 24, continued

No.	Incubation mixture (ml)	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
46	0.2 chicken plasma 0.1 X-mas serum 0.1 Human serum	1	90	38	29	20	20	20
		2	--	--	--	--	--	--
		3	90	38	30	20	20	20
		Avg.	90	38	29½	20	20	20
		%	2.3	19	35	100	100	100 %
47	0.2 Chicken plasma 0.15 X-mas serum 0.05 Human serum	1	96	43	20	20	20	20
		2	--	--	--	--	--	--
		3	97	42	20	19	19	19
		Avg.	96½	42½	20	19½	19½	19½
		%	1.9	15	100	105	105	105 %
48	0.2 Chicken plasma 0.175 X-mas serum 0.025 Human serum	1	105	61	21	21	21	21
		2	--	--	--	--	--	--
		3	100	62	20	19	19	19
		Avg.	102½	61½	20½	20	20	20
		%	1.7	5.9	94	100	100	100 %
49	0.2 Chicken plasma 0.19 X-mas serum 0.01 Human serum	1	95	92	40	21	21	21
		2	--	--	--	--	--	--
		3	93	98	44	19	19	19
		Avg.	94	95	42	20	20	20
		%	2	2	15	100	100	100 %
50	0.2 Chicken plasma 0.1 X-mas serum 0.1 Chicken serum	1	101	98	66	35	26	26
		2	--	--	--	--	--	--
		3	100	98	67	35	26	25
		Avg.	100½	98	66½	35	26	25½
		%	1.8	1.9	4.8	24	50	56 %
51	0.2 Chicken plasma 0.15 X-mas serum 0.05 Chicken serum	1	100	101	95	60	40	31
		2	--	--	--	--	--	--
		3	92	150	105	55	36	29
		Avg.	96	125	100	57	38	30
		%	1.9	1	1.8	7	19	35 %
52	0.2 Chicken plasma 0.2 X-mas serum	1	105	110	111	110	112	110
		2	--	--	--	--	--	--
		3	102	104	115	115	117	115
		Avg.	103½	107	113	112½	114½	112½
		%	1.7	1.5	1.3	1.3	1.3	1.3%

Table 25. Chicken substrate reactivity in T.G.T.

Incubation mixture: 0.2 ml adsorbed plasma, diluted 1/5, as shown,
 0.2 ml serum, diluted 1/10, as shown,
 0.2 ml human platelets,
 0.2 ml M/40 CaCl₂ solution.

Substrate: chicken high-spun plasma.

No.	Incubation mixture	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
53	Chicken ads.pl. Human serum	1	46	40	31	26	20	19
		3	64	42	27	24	24	24
		Avg.	55	41	29	25	22	21½
		%	2.7	5.1	11	15	19	20 %
54	Chicken ads.pl. Chicken serum	1	155	110	63	41	36	30
		3	170	140	115	105	65	55
		Avg.	162	125	89	73	50	42
		%	0.2	0.4	1	1.5	3.4	4.9%
55	Human ads.pl. Human serum	1	60	27	23	24	26	27
		3	62	25	25	30	30	32
		Avg.	61	26	24	27	28	29½
		%	2.2	13	16	12	11	10 %
56	Human ads.pl. Chicken serum	1	180+	180+	170	150	145	157
		3	180+	180+	180+	180+	180+	180+
		Avg.	180+	180+	180+	180+	180+	180+
		%	0	0	0	0	0	0 %
57	Haemophilic ads.pl. Human serum	1	65	62	63	51	46	49
		3	60	62	62	75	30	30
		Avg.	62½	62	62½	63	38	40
		%	2	2	2	2	6	5 %
58	Human ads.pl. Christmas serum	1	180+	180+	160	123	140	120
		3	180+	180+	180+	180+	160	140
		Avg.	180+	180+	180+	180+	150	130
		%	0	0	0	0	0.3	0.4%

Experiments 1 and 3 have been performed on the same day and with the same preparations of plasma, serum, and platelets as was used for experiments 1 and 3 in table 21. Therefore, mixtures number 53, 54, 55, 56, 57, and 58 are directly comparable with mixtures No. 1, 2, 4, 5, 9, and 10 respectively, as regards the effect of different substrate plasmas. Experiment 2 was not continued due to the exhaustion of the particular platelet preparation used in that experiment.

Table 26. Chicken substrate reactivity in T.G.T.

Incubation mixture: 0.2 ml. adsorbed plasma, diluted 1/5, as shown,
 0.2 ml. serum, diluted 1/10, as shown,
 0.2 ml. cephalin 1/100,
 0.2 ml. M/40 CaCl₂ solution.

Substrate: chicken high-spun plasma.

No.	Incubation mixture	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
59	Chicken ads.pl. Human serum	1	16	20	23	25	24	24
		2	37	33	25	22	21	22
		3	32	17	18	19	20	20
		Avg.	28	23	22	22	22	22
		%	8.7	14	15½	15½	15½	15½
60	Chicken ads.pl. Chicken serum	1	40	39	39	45	52	55
		2	137	80	95	120	142	163
		3	98	40	48	50	65	64
		Avg.	92	53	61	72	86	94
		%	0.5	1.9	1.3	0.9	0.6	0.5%
61	Human ads.pl. Human serum	1	62	21	19	22	20½	22
		2	45	20	18	18	19	20
		3	44	39	20	18	19	19
		Avg.	50	27	19	19	19½	20
		%	2.2	9.5	22	22	21	19.5%
62	Human ads.pl. Chicken serum	1	180+	180+	180+	180+	180+	180+
		2	180+	180+	180+	180+	180+	180+
		3	150	150	150	155	160	155
		Avg.	180+	180+	180+	180+	180+	180+
		%	0	0	0	0	0	0%
63	Haemophilic ads.pl. Human serum	1	69	70	75	77	78	76
		2	68	71	69	71	70	64
		3	50	50	48	51	52	55
		Avg.	62	64	64	66	66	65
		%	1.3	1.2	1.2	1.1	1.1	1.1%
64	Human ads.pl. Christmas serum	1	180+	180+	180+	180+	180+	180+
		2	180+	180+	180+	180+	180+	180+
		3	180+	180+	180+	180+	180+	180+
		Avg.	180+	180+	180+	180+	180+	180+
		%	0	0	0	0	0	0%

Experiments 1, 2, and 3 were performed with the same reagents used for experiments 1, 2, and 3 in table 23, and on the same day. Thus, mixtures 59, 60, 61, 62, 63, and 64 are directly comparable with mixtures 17, 18, 20, 21, 25, and 26 respectively, as regards substrate reactivity, as well as with each other.

Table 27. Chicken substrate reactivity in T.G.T.

Incubation mixture: 0.2 ml adsorbed plasma, diluted 1/5, as specified,
 0.2 ml serum, diluted 1/10, as specified,
 0.2 ml chicken thrombocyte suspension,
 0.2 ml M/40 CaCl₂ solution.

Substrate: chicken high-spun plasma.

No.	Incubation mixture	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
65	Chicken ads.pl. Human serum	1	45	32	28	29	32	31
		2	67	26	23	24	21	25
		3	50	46	45	40	35	31
		Avg.	54	35	32	31	29	29
		%	8	24	30	33	38½	38½ %
66	Chicken ads.pl. Chicken serum	1	103	37	41	55	80	130
		2	180+	180+	180+	140	80	130
		3	180+	180+	180+	177	105	96
		Avg.	154	132	133	126	88	97
		%	0	0	0	1	2.4	1.9%
67	Human ads.pl. Human serum	1	124	180+	175	180+	180+	163
		2	146	125	130	113	87	74
		3	99	97	92	65	60	60
		Avg.	123	134	132	119	109	99
		%	1.1	0	0	1.2	1.4	1.8%
68	Human ads.pl. Chicken serum	1	more than 180 in all tubes					
		2	more than 180 in all tubes					
		3	more than 180 in all tubes					
		Avg.	more than 180 in all tubes					
		%	0	0	0	0	0	0 %
69	Haemophilic ads.pl. Human serum	1	--	--	--	--	--	--
		2	97	97	94	99	104	98
		3	106	120	124	122	117	124
		Avg.	101	108	109	110	110	111
		%	1.7	1.5	1.4	1.4	1.4	1.4%
70	Human ads.pl. Christmas serum	1	--	--	--	--	--	--
		2	more than 180 in all tubes					
		3	more than 180 in all tubes					
		Avg.	more than 180 in all tubes					
		%	0	0	0	0	0	0 %

Experiments 1, 2,&3 were performed using the same reagents used for the same numbered experiments in table 24 and on the same day. Thus, mixtures 65, 66, 67, 68, 69, and 70 are directly comparable with mixtures 33, 34, 36, 37, 41, and 42 respectively.

Percentage active thromboplastin is expressed in terms of chicken active thromboplastin as read from figure 7.

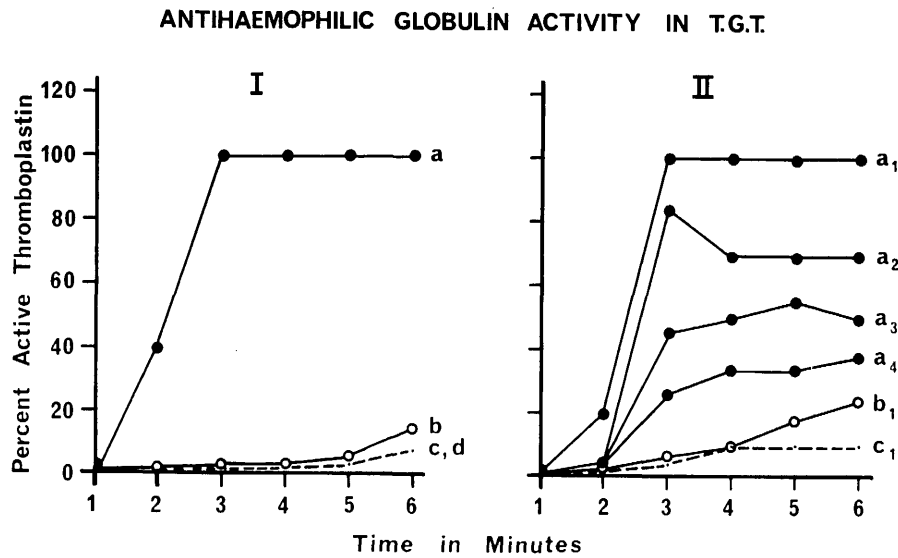


Figure 8. Antihæmophilic globulin activity of chicken adsorbed plasma.

Incubation mixture: 0.2 adsorbed plasma 1/5, variable,
 0.2 human serum 1/10,
 (ml.) 0.2 human platelets,
 0.2 M/40 CaCl₂ solution.
 Substrate: human high-spun plasma.

Figure I

- Curve a : Human ads.pl. 1/5 (Mixture No. 4, tables 21 and 22)
- Curve b : Chicken ads.pl. 1/5 (Mixture No. 1, tables 21 and 22)
- Curve c : Saline (Mixture No. 8, tables 21 and 22)
- Curve d : Haemophilic pl. 1/5 (Mixture No. 9, tables 21 and 22)

Figure II

- Curve a₁ : Hum.ads.pl. 1/2 in hæmophilic ads.pl.(Mixture 11, tables 21 & 22)
- Curve a₂ : Hum.ads.pl. 1/4 in hæmophilic ads.pl.(Mixture 12, tables 21 & 22)
- Curve a₃ : Hum.ads.pl. 1/8 in hæmophilic ads.pl.(Mixture 13, tables 21 & 22)
- Curve a₄ : Hum.ads.pl. 1/20in hæmophilic ads.pl.(Mixture 14, tables 21 & 22)
- Curve b₁ : Ck.ads.pl. 1/2 in hæmophilic ads.pl.(Mixture 15, tables 21 & 22)
- Curve c₁ : Saline 1/2 in hæmophilic ads.pl.(Mixture 16, tables 21 & 22)

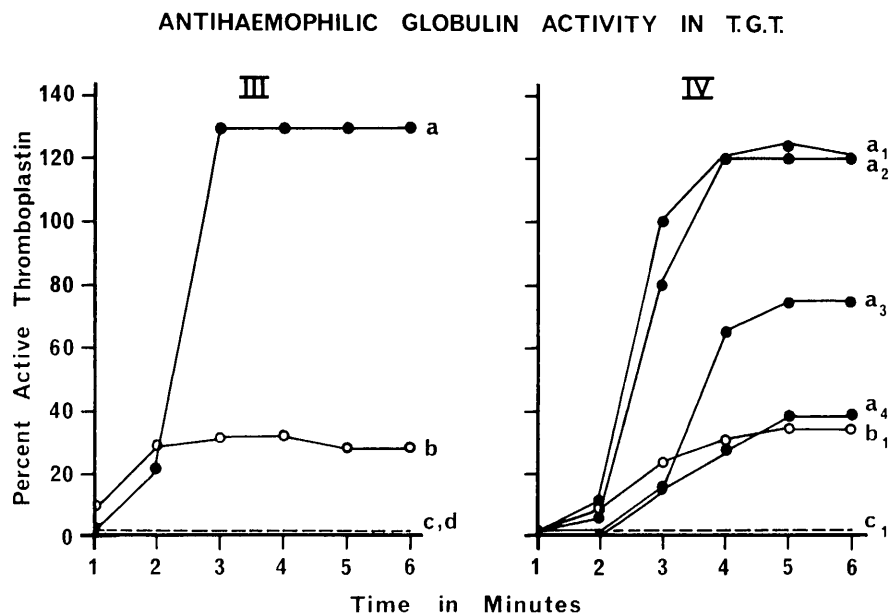


Figure 9. Antihæmophilic globulin activity of chicken adsorbed plasma.

Incubation mixture: 0.2 ml adsorbed plasma, variable,
 0.2 ml human serum 1/10,
 0.2 ml human cephalin 1/100,
 0.2 ml M/40 CaCl₂ solution.

Substrate: human high-spun plasma.

Figure III

- Curve a : Hum.ads.pl. 1/5 (Mixture 20, table 23).
- Curve b : Ck.ads.pl. 1/5 (Mixture 17, table 23).
- Curve c : Saline (Mixture 24, table 23).
- Curve d : Haemoph.ads.pl.1/5 (Mixture 25, table 23).

Figure IV

- Curve a₁ : Hum.ads.pl. 1/2 in hæmophilic ads.pl.(Mixture 27, Table 23).
- Curve a₂ : Hum.ads.pl. 1/4 in hæmophilic ads.pl.(Mixture 28, table 23).
- Curve a₃ : Hum.ads.pl. 1/8 in hæmophilic ads.pl.(Mixture 29, table 23).
- Curve a₄ ; Hum.ads.pl. 1/20 in hæmophilic ads.pl.(Mixture 30, table 23).
- Curve b₁ : Ck.ads.pl. 1/2 in hæmophilic ads.pl.(Mixture 31, table 23).
- Curve c₁ : Hæmophilic ads.pl. diluted 1/10 with saline(Mixt.32, table 23).

CHICKEN A.H.G. ACTIVITY IN MODIFIED T.G.T.

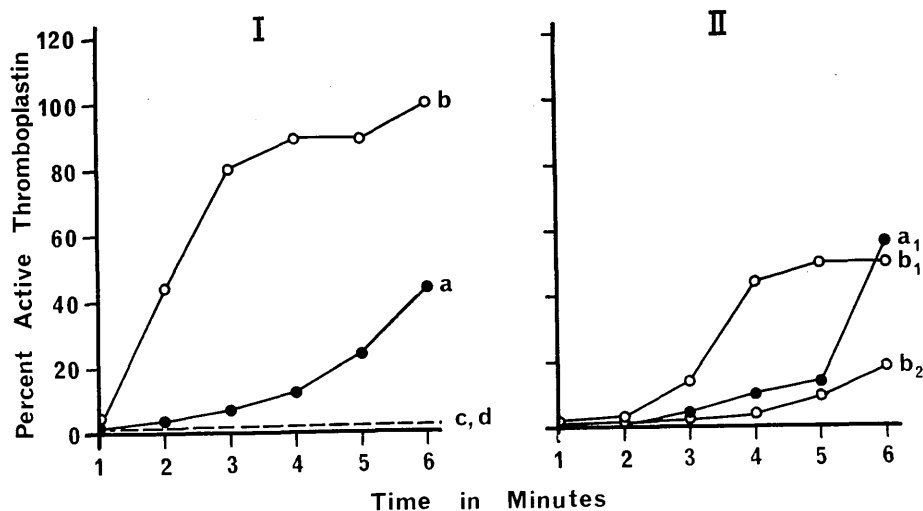


Figure 10. Antihaemophilic globulin activity of chicken adsorbed plasma.

Incubation mixture: 0.2 ml adsorbed plasma, 1/5, variable,
 0.2 ml human serum, 1/10,
 0.2 ml chicken thrombocyte suspension,
 0.2 ml M/40 CaCl₂ solution.

Substrate: human high-spun plasma.

Figure I :

Curve a : Hum.ads.pl., (Mixture 36, table 24)

Curve b : Ck.ads.pl., (Mixture 33, table 24)

Curve c : Saline, (Mixture 40, table 24)

Curve d : Haemophilic ads.pl. (Mixture 41, table 24)

Figure II

Curve a₁ : Hum.ads.pl. 1/2 in haemophilic ads.pl.(Mixture 43, table 24)

Curve b₁ : Ck.ads.pl. 1/2 in haemophilic ads.pl.(Mixture 44, table 24)

Curve b₂ : Ck.ads.pl. 1/4 in haemophilic ads.pl.(Mixture 45, table 24).

Table 28. Assay of chicken plasma A.H.G. activity

(method of Pitney, 1956; appendix IV, 21)

Plasma dilution	A.H.G. concentration	Clotting times (seconds)		
		Expt. 1	Expt. 2	Expt. 3
		Hum. pl.	Hum. pl.	Hum. pl.
1/20	100 %	12	10	15
1/40	50 %	15	14	20
1/80	25 %	16	20	27
1/200	10 %	19	35	40
1/400	5 %	25	40	48
1/2000	1 %	26	45	63
Buffer	0 %	31	55	66
		Ck. pl.	Ck. pl.	Ck. pl.
1/5		23	40	82
1/10		23½	43	66
1/20		27	—	69
1/40		27½	46	68
1/80		29	50	—
Calculated	A.H.G. conc.	1.5 %	1.5 %	0 %

Such nearly nil activity of chicken adsorbed plasma in correcting haemophilic adsorbed plasma thromboplastin time is attributed to the specificity of chicken A.H.G. and factor V against the human platelets which were used as the thromboplastic agent in this assay system, as demonstrated in tables 29 and 30, and figure 11 to follow. Substituting chicken thrombocytes for the human platelets would be of no benefit, since they are also species specific as regards human A.H.G., thus there are no means of directly comparing chicken and human A.H.G. activities in one system.

Table 29. Chicken A.H.G. specificity in modified T.G.T.

Incubation mixture:-

- 0.2 ml purified A.H.G. (appendix III, 11), variable,
- 0.2 ml purified factor V, (appendix III, 9), variable,
- 0.2 ml human serum, diluted 1/10,
- 0.2 ml human cephalin 1/100 (appendix III, 14),
- 0.2 ml M/40 CaCl₂ solution.

Substrate : human high-spun plasma.

Mixture No.	Variable	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
71	Human A.H.G.	1	76	40	16	12	12	11
		2	65	32	15	12	12	11
	Human factor V	Avg.	70	36	15½	12	12	11
	%	1	4.8	36	65	65	80	%
71 b	Chicken A.H.G.	1	139	120	121	117	116	121
		2	113	112	112	113	117	123
	Human factor V	Avg.	126	116	117	115	116½	122
	%	0.2	0.3	0.3	0.3	0.3	0.3	0.2%
71 c	Chicken A.H.G.	1	100	83	89	82	82	80
		2	96	78	92	79	78	76
	Chicken factor V	Avg.	98	80	90	80	80	78
	%	0.4	0.7	0.5	0.7	0.7	0.7	0.7%

Percentage thromboplastin was expressed in terms of human active thromboplastin as read from the dilution curve B in figure 2, which was constructed with human cephalin.

Table 30. Chicken factor V specificity in modified T.G.T.

Incubation mixture:-

- 0.2 ml purified human A.H.G., (appendix III, 11),
- 0.2 ml purified factor V, variable, (appendix III, 9),
- 0.2 ml human serum, diluted 1/10,
- 0.2 ml human cephalin 1/100 (appendix III, 14),
- 0.2 ml M/40 CaCl₂ solution.

Substrate : human high-spun plasma.

Mixture No.	Species of factor V	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
71	Human	1	76	40	16	12	12	11
		2	65	32	15	12	12	11
		Avg.	70	36	15½	12	12	11
		%	1	4.8	36	65	65	80 %
72	Chicken	1	80	66	56	49	41	24
		2	66	60	54	46	35	22
		Avg.	73	63	55	47½	38	23
		%	0.8	1.2	1.7	2.5	4.2	14 %
73	Saline	1	90	81	65	58	47	31
		2	88	82	65	60	48	30
		Avg.	89	81½	65	59	47½	30½
		%	0.5	0.6	1.1	1.5	2.5	7 %

Percentage thromboplastin was expressed in terms of human active thromboplastin as read from the dilution curve B in figure 2, which was constructed with human cephalin.

HUMAN BLOOD THROMBOPLASTIN - FACTOR V SPECIFICITY.
(modified T.G.T.)

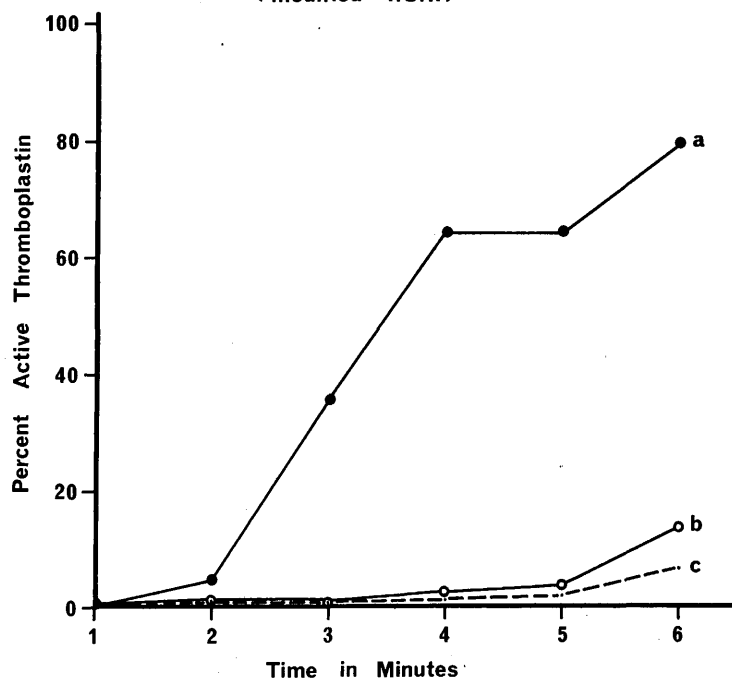


Figure 11. Chicken factor V specificity in modified T.G.T.

Incubation mixture: 0.2 ml purified human A.H.G.,
0.2 ml purified factor V, variable,
0.2 ml human serum 1/10,
0.2 ml human cephalin 1/100,
0.2 ml M/40 CaCl₂ solution.

Substrate: human high-spun plasma.

Curve a : Human factor V preparation, (Mixture 71, table 30).

Curve b : Chicken factor V preparation, (Mixture 72, table 30).

Curve c : Saline, (Mixture 73, table 30).

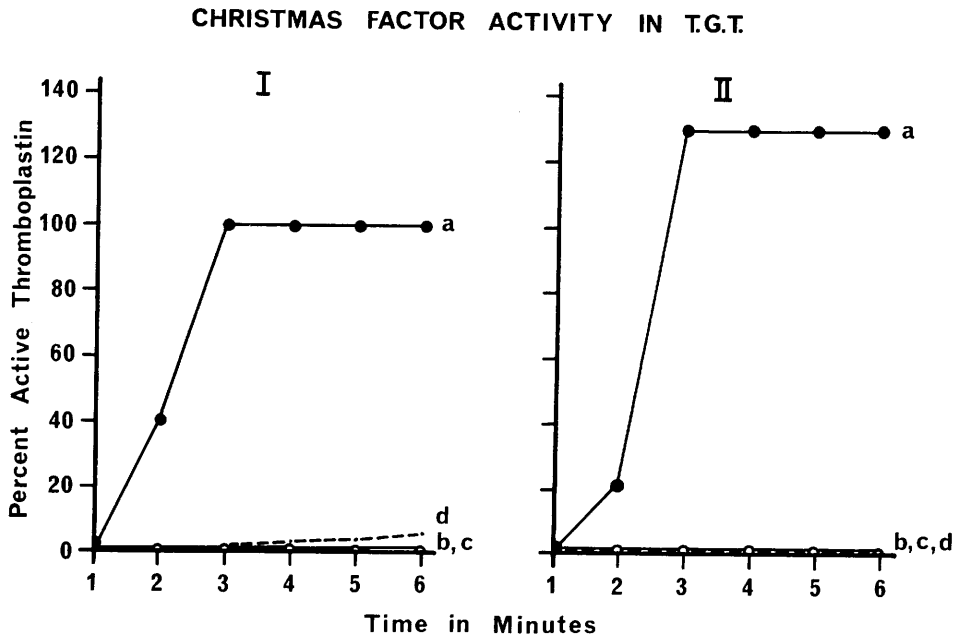


Figure 12. Christmas factor activity of chicken serum in T.G.T.

Incubation mixture : 0.2 ml human ads.pl. 1/5,
 0.2 ml serum, variable,
 0.2 ml human platelets, or cephalin 1/100,
 0.2 ml M/40 CaCl₂ solution.

Substrate : human high-spun plasma.

<u>Figure I</u>	<u>Figure II</u>
(with platelets)	(with cephalin)

Curve a : Human serum, 1/10	Mixture 4	Mixture 20
Curve b : Chicken ser. 1/10	Mixture 5	Mixture 21
Curve c : Saline	Mixture 6	Mixture 22
Curve d : Christmas serum 1/10..	Mixture 10	Mixture 26

CHICKEN X-MAS FACTOR ACTIVITY IN MODIFIED T.G.T.

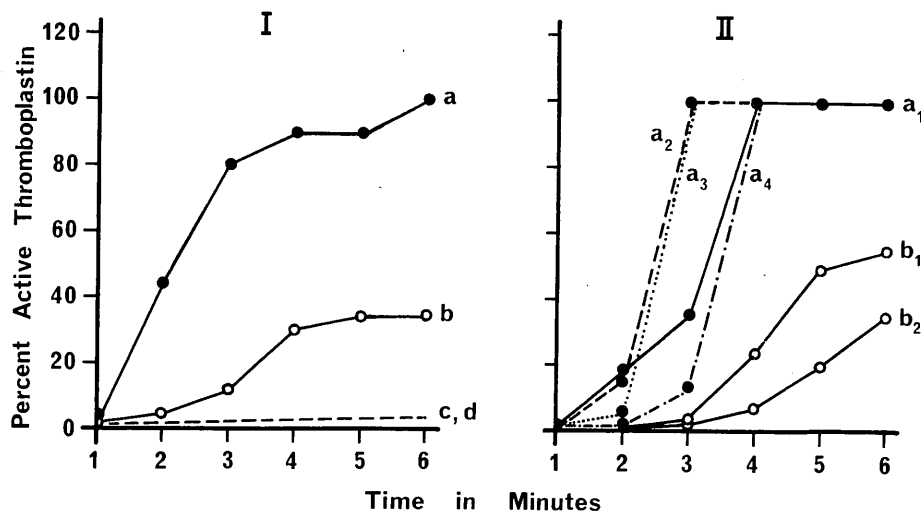


Figure 13. Christmas factor activity of chicken serum in modified T.G.T.

Incubation mixture : 0.2 ml chicken ads.pl., 1/5,
 0.2 ml serum, 1/10, variable,
 0.2 ml chicken thrombocyte suspension,
 0.2 ml M/40 CaCl₂ solution.

Substrate : human high-spun plasma.

Figure 13, I

- Curve a : human serum (Mixture 33, table 24)
- Curve b : chicken serum (Mixture 34, table 24)
- Curve c : saline (Mixture 35, table 24)
- Curve d : Christmas serum (Mixture 52, table 24)

Figure 13, II

- Curve a₁ : human serum 1/2 in X-mas serum (Mixture 46, table 24)
- Curve a₂ : human serum 1/4 in X-mas serum (Mixture 47, table 24)
- Curve a₃ : human serum 1/8 in X-mas serum (Mixture 48, table 24)
- Curve a₄ : human serum 1/20 in X-mas serum (Mixture 49, table 24)
- Curve b₁ : chicken ser.1/2 in X-mas serum (Mixture 50, table 24)
- Curve b₂ : chicken ser.1/4 in X-mas serum (Mixture 51, table 24)

Table 31. Christmas factor assay in chicken serum

(based on the method of Pitney, 1956, as described in appendix IV, 22)

Serum dilution	Concentration of X-mas factor	Clotting times (seconds)		
		Expt. 1	Expt. 2	Expt. 3
		HUMAN	CONTROL	SERUM
1/20	100 %	18	10	10
1/40	50 %	35	10	11
1/80	25 %	62	11	12½
1/200	10 %	120	12	15
1/400	5 %	113	14	23
1/2000	1 %	121	21	45
Buffer	0 %	155	55	85
		CHICKEN	TEST	SERUM
1/5		162	88	120
1/10		171	90	132
1/20		118	133	129
Calculated X-mas factor conc.		0 %	0 %	0 %

Incubation mixture:-

- 0.1 ml adsorbed normal human plasma, diluted 1/5,
- 0.1 ml Christmas disease serum, diluted 1/10,
- 0.1 ml human platelets,
- 0.1 ml control or test serum, various dilutions,
- 0.1 ml M/40 CaCl₂ solution.

Subsampling performed at exactly 4 minutes, onto human H.S.plasma.

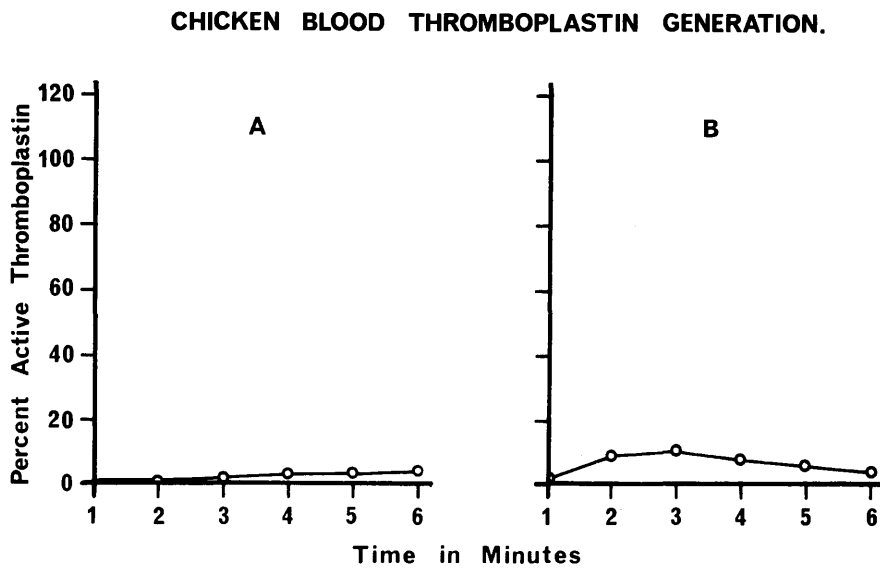


Figure 14. Chicken blood thromboplastin generation.

Incubation mixture: 0.2 ml chicken adsorbed plasma, diluted 1/5,
0.2 ml chicken serum, diluted 1/10,
0.2 ml Variable,
0.2 ml M/40 CaCl₂ solution.

Substrate: human high-spun plasma.

Curve A

With human platelets, (Mixture 2, tables 21 and 22).

Curve B

With human cephalin 1/100, (Mixture 18, table 23)

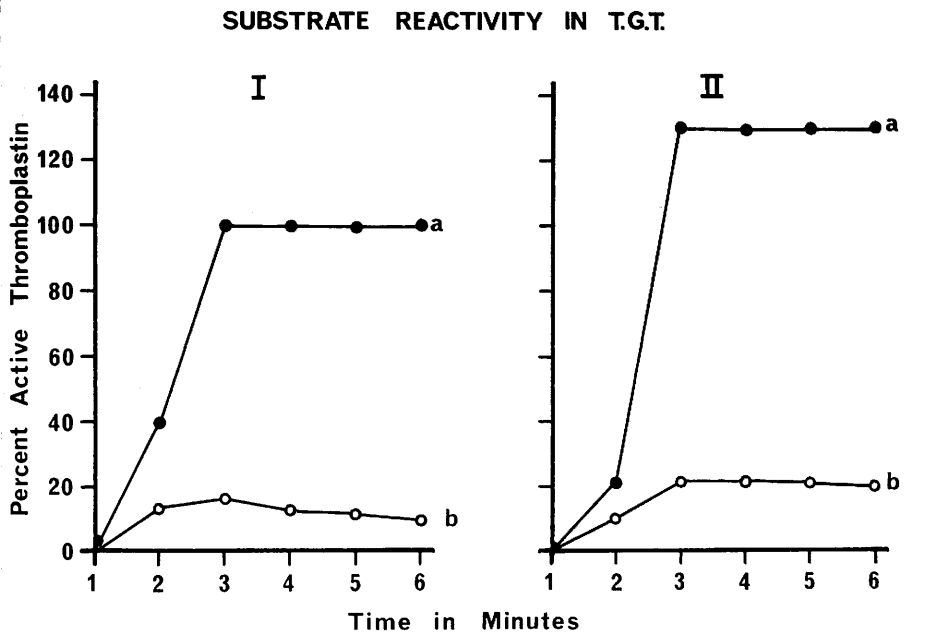


Figure 15. Chicken plasma substrate reactivity in T.G.T.

Incubation mixture: 0.2 ml human ads.pl., diluted 1/5,
0.2 ml human serum, diluted 1/10,
0.2 ml human platelets (figure I),
or human cephalin (figure II),
0.2 ml M/40 CaCl₂ solution.

Substrate : variable.

Curves a : using human H.S.plasma as substrate, (Mixtures 4 and 20)

Curves b : using chicken H.S.pl. as substrate, (Mixtures 55 and 61)

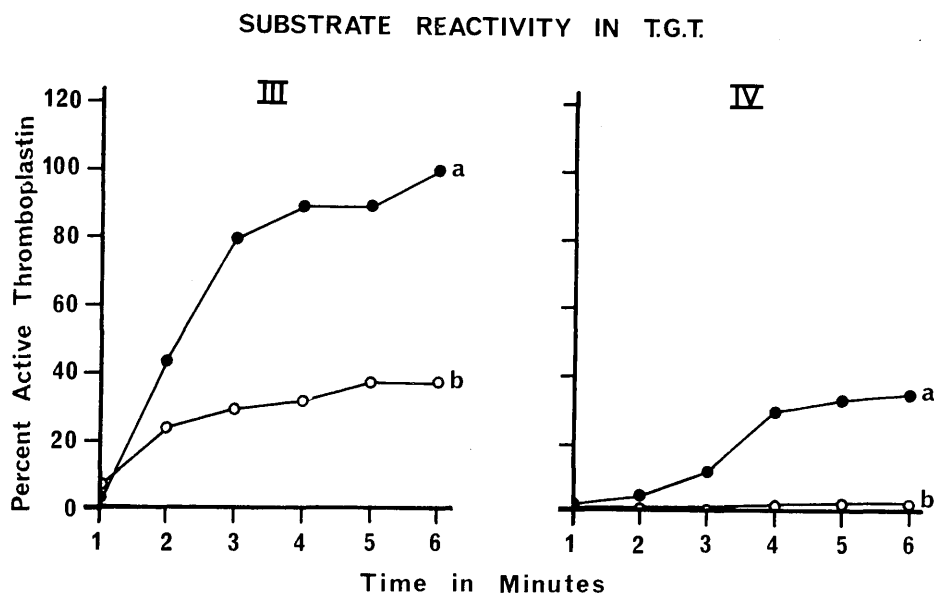


Figure 16. Chicken plasma substrate reactivity in T.G.T.

Figure III

Incubation mixture: 0.2 ml chicken ads.pl., diluted 1/5,
 0.2 ml human serum, diluted 1/10,
 0.2 ml chicken thrombocyte suspension,
 0.2 ml M/40 CaCl₂ solution.

Curve a : subsampling over human H.S.plasma, (Mixture 33, table 24)

Curve b : subsampling over chicken H.S.pl., (Mixture 65, table 27)

Figure IV

Incubation mixture: 0.2 ml chicken ads.pl., diluted 1/5,
 0.2 ml chicken serum, diluted 1/10,
 0.2 ml chicken thrombocyte suspension,
 0.2 ml M/40 CaCl₂ solution.

Curve a : subsampling over human H.S.plasma, (Mixture 34, table 24)

Curve b : subsampling over chicken H.S.pl., (Mixture 66, table 27)

This curve represents a completely homologous chicken thromboplastin generation mixture and substrate, which it is noted has virtually no activity.

Table 32. Demonstration of inhibitor against human intrinsic thromboplastin in chicken adsorbed plasma in T.G.T.

(method described in appendix IV, 23)

Incubation mixture: 0.1 ml human normal adsorbed plasma, diluted 1/5,
 0.1 ml variable,
 0.2 ml human serum, diluted 1/10,
 0.2 ml human cephalin 1/100,
 0.2 ml M/40 CaCl₂ solution.

Substrate : human high-spun plasma.

A- Before incubation

No.	Plasma mixture (ml)	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
74	0.1 Hum.ads.pl.	1	65	24	14	11	10	10
		2	60	21	13	10	10	10
	Avg.	62½	22½	13½	10½	10	10	
	%	1.3	16	50	90	100	100 %	
75	0.1 Hum.ads.pl.	1	68	25	16	11	11	10
		2	62	20	14	10	10	10½
	Avg.	65	22½	15	10½	10½	10¼	
	%	1.2	16	39	90	90	94 %	
76	0.2 Hum.ads.pl.	1	48	16	10	9	9	9
		2	49	15	10	9½	9	9½
	Avg.	48½	15½	10	9¼	9	9¼	
	%	2.3	36	100	120	130	120 %	

Percentage figures for the human active thromboplastin were read from the thromboplastin dilution curve B in figure 2.

Table 32, continued

B- After incubation at 37°C. for 2 hours

No.	Plasma mixture (ml)	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
77	0.1 Hum.ads.pl.	1	72	26	15	12	10	10
		2	65	25	14½	12	11	11
	0.1 Saline	Avg.	68½	25½	14¾	12	10½	10½
		%	1	11	42	66	90	90 %
78	0.1 Hum.ads.pl.	1	99	59	31	25	18	16
		2	110	60	30	24	18½	17
	0.1 Ck.ads.pl.	Avg.	104½	59½	30½	24½	18¼	16½
		%	0.4	1.4	7	12	24	31 %
79	0.2 hum.ads.pl.	1	50	17	11	10½	10	10½
		2	53	15½	11	10	10	10
		Avg.	51½	16¼	11	10¼	10	10¼
		%	2	32	80	95	100	95 %
80	0.1 Hum.ads.pl.	1	76	30	17	13½	11	11
		2	70	23	16	12	11½	11
	0.1 Heated Ck.ads.pl.	Avg.	73	26½	16½	12¾	11¼	11
		%	0.9	10	31	58	78	80 %

Percentage values for the active human thromboplastin were read from the thromboplastin dilution curve B in figure 2.

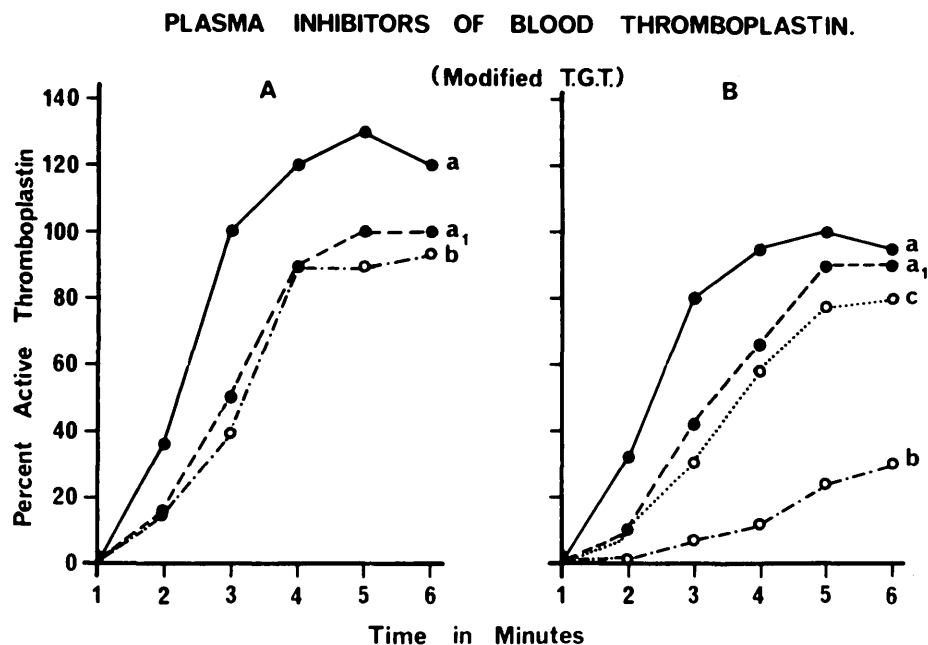


Figure 17. Chicken plasma inhibitors of human intrinsic thromboplastin.

Incubation mixture: 0.2 ml plasma mixture, diluted 1/5,
 0.2 ml human serum, diluted 1/10,
 0.2 ml human cephalin 1/100,
 0.2 ml M/40 CaCl₂ solution.

Substrate : normal human high-spun plasma.

Figure A : Before incubation of the plasma mixture.

Curve a : Hum.ads.pl. alone (Mixture 76, table 32)

Curve a₁ : Hum.ads.pl. + saline (Mixture 74, table 32)

Curve b : Hum.ads.pl. + Ck.ads.pl. (Mixture 75, table 32)

Figure B : After 2 hours incubation at 37°C.

Curve a : Hum.ads.pl. alone (Mixture 79, table 32, B)

Curve a₁ : Hum.ads.pl. + saline (Mixture 77, table 32, B)

Curve b : Hum.ads.pl. + Ck.ads.pl. (Mixture 78, table 32, B)

Curve c : Hum.ads.pl. + Heated Ck.ads.pl. (Mixture 80, table 32, B)

Table 33. Investigation for inhibitors against human intrinsic thromboplastin in chicken serum in the T.G.T.

(method described in appendix IV, 23)

Incubation mixture: 0.2 ml human adsorbed plasma, diluted 1/5,
 0.2 ml serum mixture, diluted 1/10,
 0.2 ml human cephalin 1/100,
 0.2 ml M/40 CaCl₂ solution.

Substrate : human normal high-spun plasma.

A- Before incubation

No.	Serum mixture (ml)	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
81	0.2 Human serum	1	60	20	10	9	10	9
		2	56	18	10	9	8	9
		Avg.	58	19	10	9	9	9
		%	1.5	22	100	130	130	130 %
82	0.1 Human serum 0.1 Saline	1	69	24	11½	10	10½	10½
		2	67	20	10½	10	9½	9½
		Avg.	68	22	11	10	10	10
		%	1	15	80	100	100	100 %
83	0.1 Human serum 0.1 Chicken serum	1	67	24	12	11	11	10
		2	66	22	11	10	10	10
		Avg.	66½	23	11½	10½	10½	10
		%	1.1	14	74	90	90	100 %

Percentage values of human active thromboplastin were read from the thromboplastin dilution curve B, figure 2.

Table 33, continued

B- After incubation at 37°C. for 2 hours

No.	Serum mixture (ml)	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
84	0.1 Human serum	1	66	20	10½	10	10	10
		2	67	20	10	10	10	10
	Avg.	66½	20	10¼	10	10	10	
	%	1.1	19.5	100	100	100	100 %	
85	0.1 Human serum	1	73	22	12	11	10	11
		2	67	20	10	10	10	10
	Avg.	70	21	11	10½	10	10½	
	%	0.9	17	80	90	100	90 %	

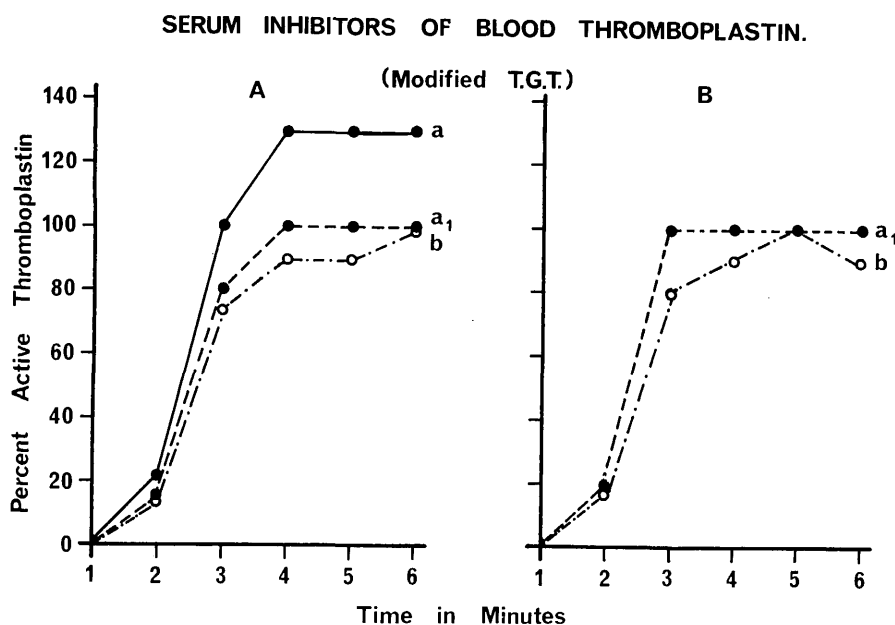


Figure 18. Chicken serum inhibitors of human intrinsic thromboplastin.

Incubation mixture: 0.2 ml human adsorbed plasma, diluted 1/5,
 0.2 ml serum mixture, diluted 1/10,
 0.2 ml human cephalin 1/100,
 0.2 ml M/40 CaCl₂ solution.

Substrate : human normal high-spun plasma.

Figure A Before incubation of the serum mixture

Curve a : human serum alone, (Mixture 81, table 33 A)
 Curve a₁ : human serum + saline (Mixture 82, table 33 A)
 Curve b : human serum + chicken serum (Mixture 83, table 33 A)

Figure B After incubation at 37°C. for 2 hours

Curve a₁ : human serum + saline (Mixture 84, table 33 B)
 Curve b : human serum + chicken serum (Mixture 85, table 33 B)

Table 34. Clot retraction

(method of Macfarlane, 1938; appendix IV, 4)

	Percentage retraction in 1 hour*
Experiment 1	15 %
Experiment 2	25 %
Experiment 3	22 %
Experiment 4	17 %
Experiment 5	20 %
Average	19.8 %

* Mean of duplicate estimations.

Table 35. Prothrombin consumption index

(method of Biggs and Macfarlane, 1957; appendix IV, 13, A)

	Expt. 1	Expt. 2	Expt. 3
Chicken pl. time (sec)	33	30	32
Chicken ser.time (sec)	360+	360+	360+
P.C.I.	9.1 %	4.1 %	8.8 %

Average P.C.I. of chicken blood = 7.3 %

- 155 -

APPENDIX V, B

RESULTS OF EXPERIMENTS
ON BOVINE BLOOD

RESULTS OF EXPERIMENTS
ON BOVINE BLOOD

All the following results were obtained from experiments carried out on blood samples obtained from domestic cattle of several breeds at the slaughter house, using pooled plasma or serum samples from at least three animals for each experiment to obviate as much as possible any individual variations from affecting the final levels of the various coagulation factors.

Blood samples were obtained fresh on the day of the test concerned as described in appendix II, 1, vi.

Table 36. Measurement of fibrinogen in bovine plasma

	Turbidimetric method of Stirland, 1956 (appendix IV, 2, III)	Micro-Kjeldahl method of Cullen & van Slyke, 1920 (appendix IV, 2, I)
	mgms fibrinogen /100 mls absolute plasma	
Expt. 1	750	744
Expt. 2	620	661.1
Expt. 3	720	692
Average	696.6	699

The average of both methods = 697.8 mg fibrinogen/100 mls plasma.

Table 37. Thrombin-fibrinogen reaction of plasma

(method described in appendix IV, 8 and 9)

Clotting mixture (ml)	Expt.	Thrombin topical (P.D.& Co.) in units / ml.					
		50	25	12.5	6.25	3.1	1.55
		Clotting times (seconds)					
O.1 Bovine pl. O.1 Saline O.1 Thrombin	1	5	7	15	33	68	90
	2	6	7	10	15½	29	58
	3	6	8	11	16	30	58
	Avg.	5.4	7.3	12	21.5	42	69
O.1 Human pl. O.1 Saline O.1 Thrombin	1	4	5	10	20	32	65
	2	5	7	9	13	26	52
	3	5	6	10	14	27	44
	Avg.	4.6	6	9.6	15.6	28.3	53.6
O.1 Bovine pl. O.1 Pyrocatechol O.1 Thrombin	1	2½	3½	5	8	16	24
	2	3	3½	5	11	13½	25
	3	2½	3½	4½	8	14½	24
	Avg.	2.6	3.5	4.8	9	14.6	24.3
O.1 Human pl. O.1 Pyrocatechol O.1 Thrombin	1	2½	3½	5	8	14	22
	2	3	3	4	9	13	19
	3	3	3½	5	8½	16	23
	Avg.	2.8	3.3	4.6	8.5	14.3	21.3

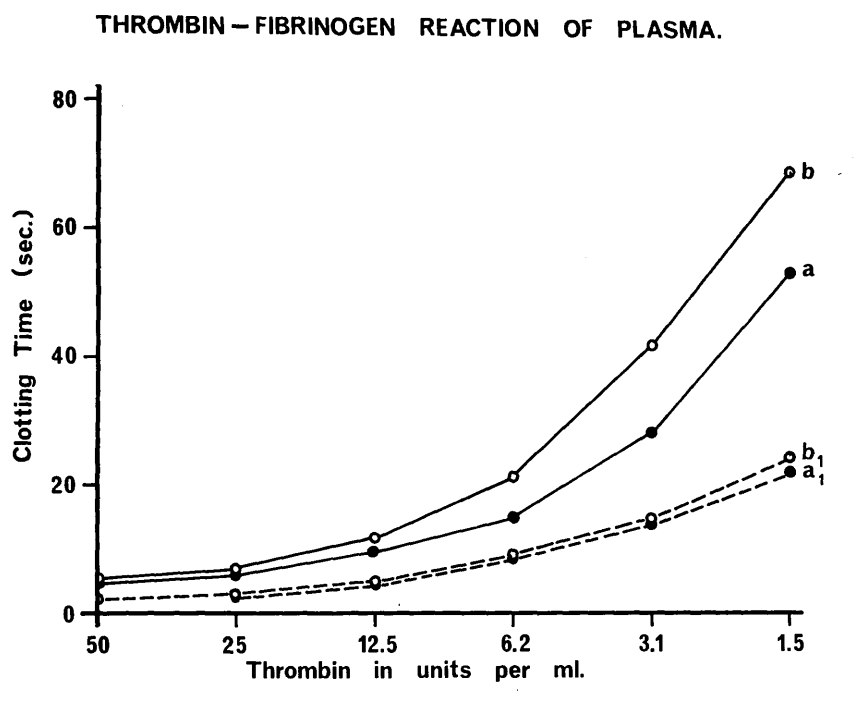


Figure 19. Thrombin-fibrinogen reaction of bovine plasma.

Curve a : Human plasma + saline.

Curve b : Bovine plasma + saline.

Curve a₁ : Human plasma + pyrocatechol 1%.

Curve b₁ : Bovine plasma + pyrocatechol 1%.

Table 38. Antithrombin assay in bovine plasma

(modified method of Astrup and Darling, 1942d, appendix IV, 11, B)

Mixture			A	B	C	D	
Saline	ml.		0.3	0.2	0.15	0.1	0.0
Plasma (dil. 1/5)	ml.		0.0	0.1	0.15	0.2	0.3
Thrombin(30 U/ml)	ml.		1	1	1	1	1
		Expt.					
Clotting time of fibrinogen (seconds*)	Human	1	10	12	14½	16	22
		2	18	21	25	30	38
	Bovine	1	10	13	15	15½	24
		2	18	20	23	28	35
T _o / T _n	Human	1	1	0.83	0.71	0.62	0.45
		2	1	0.86	0.72	0.60	0.47
	Bovine	1	1	0.77	0.66	0.64	0.41
		2	1	0.90	0.78	0.64	0.51
Corrected T _o / T _n	Human	1	1	0.81	0.72	0.63	0.45
		2	1	0.83	0.74	0.65	0.47
	Bovine	1	1	0.80	0.70	0.60	0.40
		2	1	0.84	0.76	0.68	0.53
Calculated units of Antithrombin per ml plasma	Human	1		270	260	240	265
		2		212	216	219	220
	Bovine	1		255	290	285	290
		2		200	200	200	204

* Mean of duplicate estimations.

	Expt. 1	Expt. 2
Average value for human plasma =	258	216.7 U./ml.
Average value for bovine plasma =	280	201 U./ml.

Bovine plasma antithrombin content
in percentage of human antithrombin = 108.5% 93 %

Therefore, bovine plasma antithrombin content equals 100.75 % of human
plasma antithrombin content.

ANTITHROMBIN ASSAY.

Modified method of Astrup & Darling, 1942.

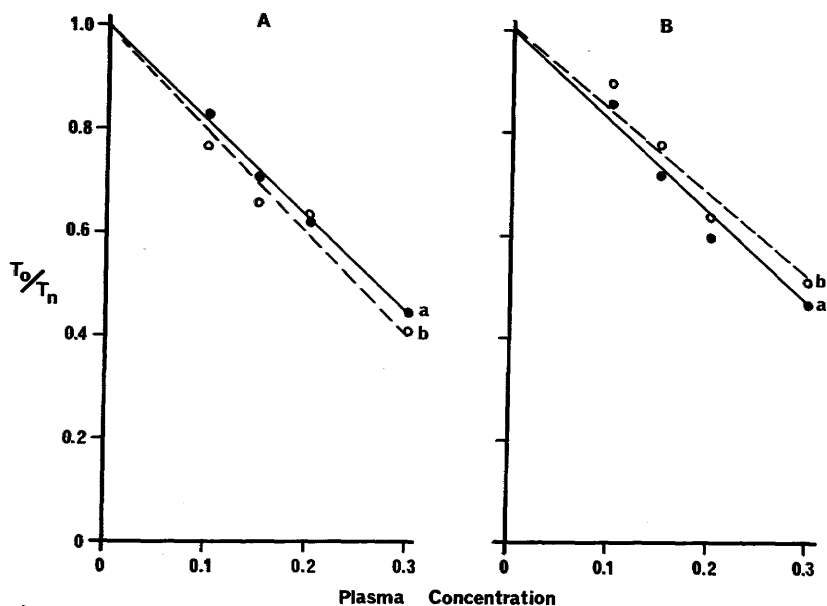


Figure 20. Antithrombin assay in bovine plasma.

Figure A

Curve a : human plasma (experiment 1, table 38)

Curve b : bovine plasma (experiment 1, table 38)

Figure B

Curve a : human plasma (experiment 2, table 38)

Curve b : bovine plasma (experiment 2, table 38)

Table 39. Antithrombin assay in bovine plasma

(method of Douglas and Biggs, 1953; appendix IV, 11, A)

Incubation mixture	Expt.	Incubation time (minutes)						
		$\frac{1}{2}$	$\frac{3}{4}$	1	2	3	4	5
		Clotting times (seconds)						
0.5 ml Hum.pl. 0.5 ml Thrombin (50 units/ml)	1	22	28	35	53	128	180+	180+
	2	--	--	16	37	90	180+	180+
	3	26	29	42	80	170	180+	180+
	Avg.	24	28 $\frac{1}{2}$	31	57	129	180+	180+
0.5 ml Bov.pl. 0.5 ml Thrombin (50 units/ml)	1	22	30	42	80	180+	180+	
	2	--	--	20	61	180+	180+	
	3	24	35	45	100	180+	180+	
	Avg.	23	32 $\frac{1}{2}$	36	80	180+	180+	
Human plasma	Avg.	Units of residual thrombin						
		5.8	4.8	4.3	2.1	0.8	0	
Bovine plasma	Avg.	6.2	4	3.6	1.4	0.5	0	

The incubation time at which thrombin reaches zero activity is read from curves a and b in figure 21 and is as follows:-

Value for human plasma = 3

Value for bovine plasma = 2.3

$$\begin{aligned} \text{Therefore, bovine plasma antithrombin} &= \frac{3 \times 100}{2.3} \\ &= 130.4\% \text{ of human plasma antithrombin.} \end{aligned}$$

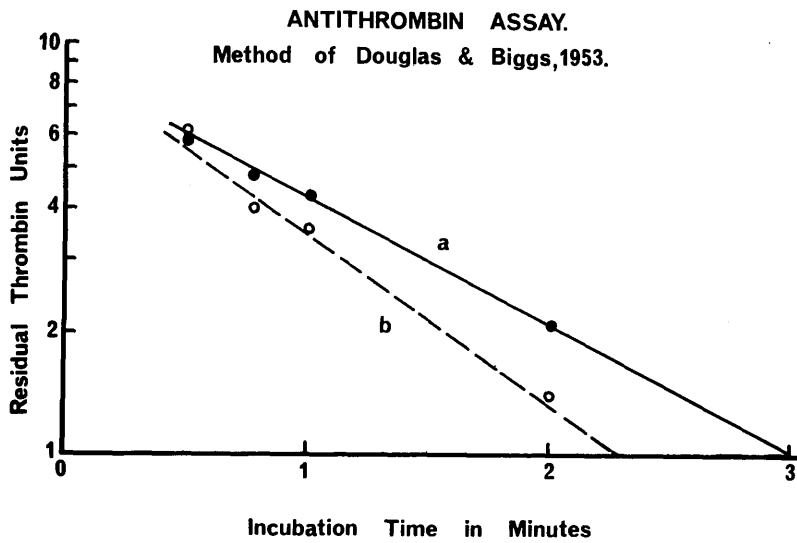


Figure 21. Antithrombin assay in bovine plasma.

Curve a : human plasma , average values (Table 39)

Curve b : bovine plasma, average values (Table 39)

Table 40. Quick's one-stage prothrombin time test

Test plasma	Human brain	Bovine brain
	Clotting times (seconds)	
Bovine plasma	43	18
	21 (31)	13 (14.8)
	29	13½
Human plasma	16½	37
	15 (15.8)	34 (36.3)
	16	38
Bov.pl. + Hum.pl.	18	22
	16 (16.3)	18 (19)
	15	17
Bov.pl. + Saline	37	17
	27 (32.6)	15 (16.3)
	34	17
Hum.pl. + Saline	20	44
	16½(18.8)	44 (45.3)
	20	48

The figure between brackets is the mean of each group of three experiments carried out with different batches of brain thromboplastin and different plasma pools.

Table 41. Factor V - brain thromboplastin species specificity

(method described in appendix IV, 16)

Incubation mixture: 0.3 ml Brain extract, diluted 1/4, variable,
 0.3 ml Purified factor V, (appendix III, 9), variable,
 0.3 ml human serum, diluted 1/10,
 0.3 ml M/40 CaCl₂ solution.

Substrate: Human normal plasma.

Variables	Expt.	Incubation time (minutes)						
		$\frac{1}{2}$	1	2	3	4	5	6
		Clotting times (seconds)						
Human brain	1	13	11	10	9	10	10	10
Human factor V	2	15	9	7	7	8	8	9
Human brain	1	10	7	6	5 $\frac{1}{2}$	5 $\frac{1}{2}$	6	6
Bovine factor V	2	10	5	5	5	5	5	5 $\frac{1}{2}$
Human brain	1	13	13	12 $\frac{1}{2}$	13	13	12 $\frac{1}{2}$	13
Saline	2	15	14	13	13	13	13 $\frac{1}{2}$	14
Bovine brain	1	16	13	12	11	11	12	12
Human factor V	2	20	15	9 $\frac{1}{2}$	8	10	9	10
Bovine brain	1	16	11	8	6	7	8	8 $\frac{1}{2}$
Bovine factor V	2	15	6 $\frac{1}{2}$	5 $\frac{1}{2}$	5 $\frac{1}{2}$	5 $\frac{1}{2}$	6 $\frac{1}{2}$	6
Bovine brain	1	20	17	16	14	14	15	15
Saline	2	17	16	15	14	15	16	17

Table 42. Assay of bovine plasma factor V activity

By correction of human stored factor V-deficient plasma, as described in appendix IV, 14. Human brain thromboplastin was used for all mixtures.

Plasma dilution	Factor V percentage	Clotting times (seconds)		
		Expt. 1	Expt. 2	Expt. 3
		Human control plasma		
1/10	100 %	21	30	32½
1/20	50 %	26	35	37
1/40	25 %	30	45	42
1/80	12.5 %	37	50	44
1/160	6.2 %	50	55	46
1/320	3.1 %	54	59	52
1/640	1.5 %	62	64	51
	0 %	86	66	51
		Bovine test plasma		
1/10		19½	23	26½
1/20		20	29½	29
1/40		25	33	34
1/80		30	40	40
1/160		34	47	44
Calculated factor V conc.		244 %	272 %	288 %

The average of the three experiments for bovine plasma factor V concentration equals 268 % of human plasma factor V activity.

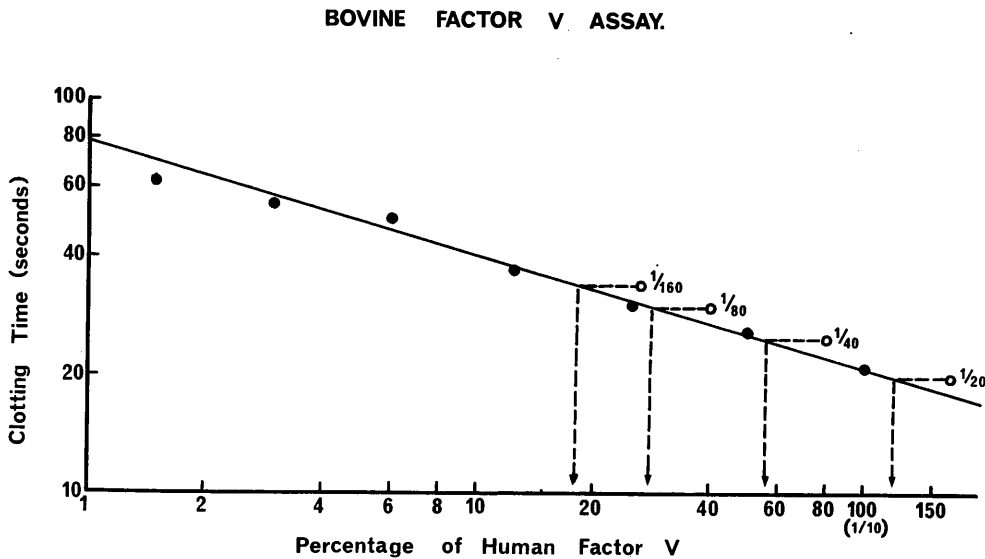


Figure 22. Bovine plasma factor V assay.

The dilution curve shown above is for human normal plasma diluted in human stored plasma, and clotted with human brain in the presence of calcium chloride solution. It represents the results obtained in experiment 1, table 42.

The dotted lines show how the clotting times obtained with every dilution of the test bovine plasma were converted into percentage values of factor V activity which is then multiplied by the dilution factor to arrive at the final bovine plasma factor V concentration in percentage of human plasma factor V activity.

Table 43. Factors VII and X - brain thromboplastin species specificity

(method described in appendix IV, 17)

Incubation mixture:-

0.3 ml brain extract, diluted 1/4, variable,

0.3 ml purified factors VII and X, variable, (appendix III, 10),

0.3 ml M/40 CaCl₂ solution.

Variables	Substrate	Incubation time (minutes)						
		$\frac{1}{2}$	1	2	3	4	5	6
		Clotting times (seconds)						
Human brain	Fresh hum.pl.	11	10	9	9	9	9	9
Hum. VII & X	Phenindione pl.	15	13	13	12 $\frac{1}{2}$	13	13	13
Human brain	Fresh hum.pl.	20	16	11 $\frac{1}{2}$	10	10	10	9
Bov. VII & X	Phenindione pl.	27	20	15	12	10	10	10
Human brain	Fresh hum.pl.	29	26 $\frac{1}{2}$	26	27	26	26	26
Saline	Phenindione pl.	50	55	60	58	60	60	60
Bovine brain	Fresh hum.pl.	20	17	12	11	10	10	10
Hum. VII & X	Phenindione pl.	32	25	18	19	17	18	17
Bovine brain	Fresh hum.pl.	8	7	7	7	8	7	7
Bov. VII & X	Phenindione pl.	9	9	8	8	8	8	8
Bovine brain	Fresh hum.pl.	47	47	49	52	48	49	50
Saline	Phenindione pl.	73	115	90	115	105	108	110

Table 44. Bovine plasma factor VII assay

Method A : by correction of human phenindione plasma, using human brain extract, (method of Biggs and Macfarlane, 1957, appendix IV, 15, A).

Method B : by correction of Seitz-filtered bovine plasma using either human or bovine brain extracts, (method of Koller et al, 1951, appendix IV, 15, B).

Plasma dilution	Factor VII percentage	Clotting times (seconds)			
		Method A		Method B	
		Expt. 1	Expt. 2	Human brain	Bovine brain
		Hum. pl.	Hum. pl.	Hum.pl.	Bov.pl.
1/10	100 %	28	28	40	48
1/20	50 %	33	34	57	65
1/40	25 %	37	36½	97	84
1/80	12.5 %	42	42	167	117
1/160	6.2 %	45	45	265	169
1/320	3.1 %	48	49	556	215
1/640	1.5 %	50	52	930	272
	0 %	60	62	—	321
		Bov.pl.	Bov.pl.	Bov.pl.	Hum.pl.
1/10		38	40	142	82
1/20		43	45	254	123
1/40		46½	47	—	175
Calculated F VII conc.		21 %	18 %	13.7 %	23 %

It should be noted that in the first two experiments using human brain, the bovine factor VII gave a value of 18-21 % of human factor VII activity. While in method B, with human brain, the human factor was still much higher than the bovine factor VII, but with bovine brain it was 23 % of the bovine factor VII.

It should also be mentioned that method B is sensitive to factor X in the test plasma, since the Seitz-filtered bovine plasma is virtually free from both factors VII and X.

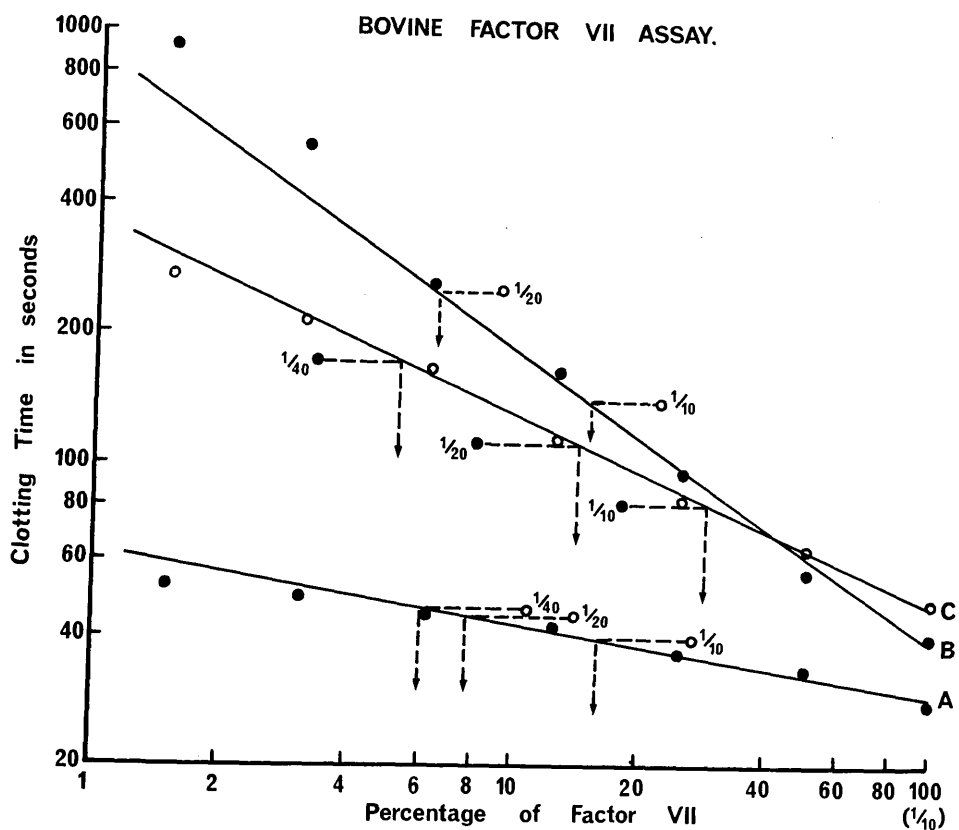


Figure 23. Bovine plasma factor VII assay.

(Human plasma : black dots. Bovine plasma : white dots.)

- Curve A : human plasma dilution curve in phenindione human plasma, using human brain, (table 44, method A, experiment 2).
The bovine plasma factor VII reads 18 % of the human.
- Curve B : human plasma dilution curve in bovine Seitz-filtered plasma, using human brain, (table 44, method B, human brain).
The bovine plasma factor VII reads 13.7 % of the human.
- Curve C : bovine plasma dilution curve in bovine Seitz-filtered plasma, using bovine brain, (table 44, method B, bovine brain).
The human plasma factor VII reads 23 % of the bovine.

The dotted lines show how the clotting times obtained with each dilution of plasma were converted into percentage values of factor VII, which when multiplied by the dilution factor gave the final factor VII percentages.

Table 45. Bovine plasma and serum factor X assay

(method of Bachmann, Duckert, and Koller, 1958; appendix IV, 18)

Plasma or serum dilution	Concentration of factor X	Expt.1	Expt.2	Expt.3	Expt.4	Expt.5
		Clotting times (seconds)				
		Human plasma			Human serum	
1/10	100 %	21	22½	19½	22	20
1/20	50 %	24	25	23½	25	23½
1/40	25 %	29	32	31½	31	32
1/80	12.5 %	36½	37	39	36	38
1/160	6.2 %	42	48	42	44	46
1/320	3.1 %	51	51½	53	50	53
1/640	1.5 %	60	61	62	65	62
1/1280	0.75%	75	74	73	77	72½
1/2560	0.36%	82½	83	84	83	—
	0 %	94½	93	94	100	100
		Bovine plasma			Bovine serum	
1/10		20	22	21	22½	23½
1/20		24	25	24½	26	26
1/40		31	30½	31½	32	30
1/80		36	36	38	—	37
Calculated factor X conc.		101.5%	109 %	94 %	89.5%	92.2%

Average factor X concentration in bovine plasma = 101.5 % of human plasma.

Average factor X concentration in bovine serum = 90.6 % of human serum.

Table 46. Bovine plasma prothrombin assay

(by the one-stage method of Koller et al, 1951; appendix IV, 12, A)

Incubation mixture: 0.1 ml test plasma diluted in veronal buffer,
 0.05 ml adsorbed bovine plasma,
 0.05 ml oxalated human serum (prothrombin-free),
 0.1 ml brain extract, as shown,
 0.1 ml M/40 CaCl₂ solution.

Plasma dilution	Prothrombin concentration	Human brain	Bovine brain	
		Expt. 1	Expt. 2	Expt. 3
		Clotting times (seconds)		
		Hum.pl.	Bovine plasma	
1/10	100 %	28	15	20
1/20	50 %	40	21	24
1/40	25 %	64	27½	34
1/80	12.5%	73	33	38
1/160	6.2%	79	46	41
1/320	3.1%	87	51	44
1/640	1.5%	103	63	55
	0 %	200	—	—
		Bov.pl.	Human plasma	
1/10		40	21	22
1/20		58	28	31
1/40		—	32	40
Prothrombin conc.		45 %	48 %	57 %

Bovine plasma prothrombin estimated with human brain (expt. 1) had a concentration of 45% of the human control plasma prothrombin. In experiments 2 and 3, the use of bovine brain gave shorter clotting times with bovine plasma, which therefore was considered the control for the construction of a dilution curve, from which the human plasma prothrombin was calculated to be 48 - 57% of the bovine plasma prothrombin activity.

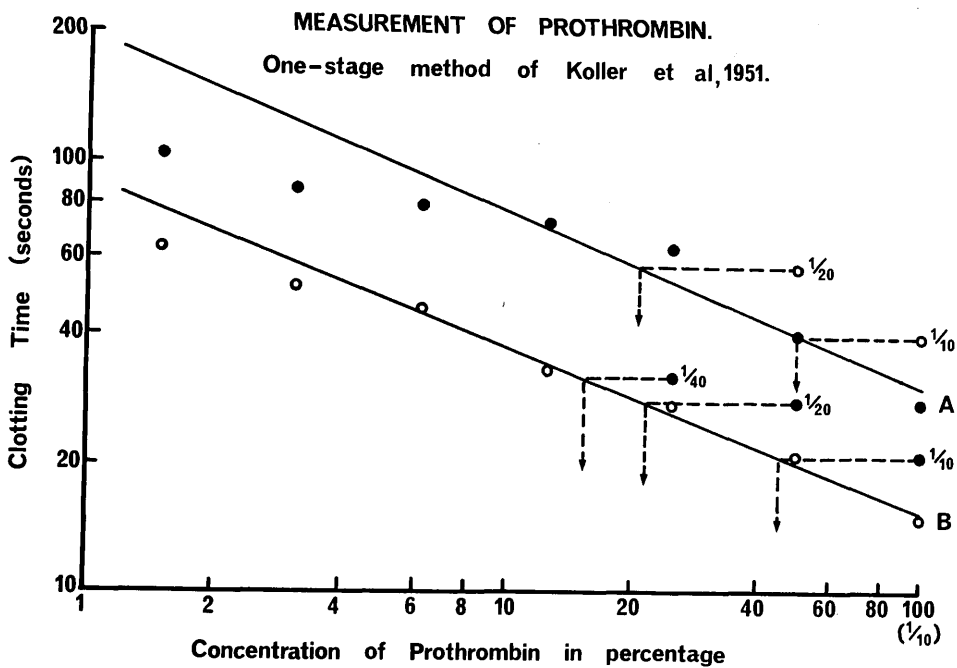


Figure 24. Bovine plasma prothrombin assay.

(Human plasma : black dots. Bovine plasma : white dots)

Curve A : human plasma dilution curve, using human brain extract, illustrating experiment 1, table 46.

Curve B : bovine plasma dilution curve, using bovine brain extract, representing experiment 2, table 46.

Table 47. Bovine plasma prothrombin assay

(Two-stage area method of Biggs and Douglas, 1953a; appendix IV, 12, C)

Incubation mixture	Expt.	Incubation time (minutes)								Weight of area under curve & its percentage	
		$\frac{1}{4}$	$\frac{1}{2}$	$\frac{3}{4}$	1	2	3	4	5		
		Clotting times (seconds)									
Human plasma with human brain	1	14	--	15	22	55	180+	180+			
	2	--	--	13	20	61	180+	180+			
	3	14	--	21	28	82	180+	180+			
Bovine plasma with bovine brain	4	--	15	16	25	42	150	180+			
	5	--	13	14	16	25	69	180+			
	6	--	--	10	8	14	35	94	180-		
Bovine plasma with human brain	7	--	--	17	13	12 $\frac{1}{2}$	36	91	155		
	8	--	40	--	20	25	66	180+	180-		
		Thrombin units / ml.								Weight	%
	1	11	--	10	6.4	2.3	0.5	0			
	2	--	--	12	7.3	1.6	0.3	0			
	3	11	--	7	4.8	1.3	0.2	0			
	Avg.	11	--	9.6	6.1	1.7	0.3	0	175	100	
	4	--	10	9.6	5.6	3	0.7	0	178	101.7	
	5	--	12	11	9.5	5.6	1.6	0.3	284	162.2	
	6	--	--	16.5	22	11	3.7	1.1	524	299.4	
	7	--	--	8.6	12	13	3.6	1.2	408	233.1	
	8	--	3	--	7.3	5.6	1.7	0.3	203	116	

To compare the effect of using brain extracts of different species the bovine plasmas used in experiments 5 and 8 were from the same pool, and similarly plasmas used in experiments 6 and 7 were the same.

The human control plasma was from the same subject.

With bovine brain, bovine plasma prothrombin was 162% of human plasma prothrombin (experiment 5), while on using human brain its prothrombin was 116% of human plasma prothrombin (experiment 8).

Similarly, another plasma pool gave a result of 299% of human prothrombin when estimated with bovine brain (experiment 6), and of 233% of human prothrombin when estimated with human brain (experiment 7).

A third bovine plasma pool had 101% of human plasma prothrombin even on using the homologous bovine brain extract (experiment 4).

MEASUREMENT OF PROTHROMBIN.

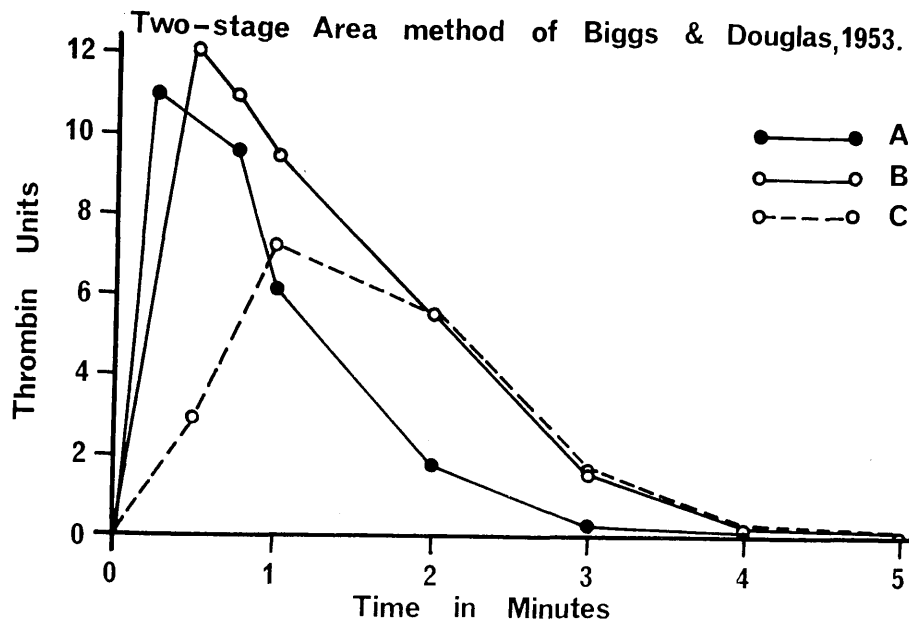


Figure 25. Bovine plasma prothrombin assay.

Curve A : obtained for human plasma using human brain extract.

It represents the average of the experiments 1, 2, and 3, from table 47. Weight of cut-out area under curve equals 175 mg which was designated the 100 % value of prothrombin.

Curve B : represents experiment 5 (table 47) on bovine plasma with bovine brain. Weight of cut-out area under curve equals 284 mg which was equivalent to 162.2% of human prothrombin.

Curve C : represents experiment 8 (table 47) on the same plasma pool using human brain. Weight of enclosed area equals 203 mg which was equivalent to 116 % of human plasma prothrombin.

It is noted that the use of the homologous brain extract yields a higher prothrombin activity.

Table 48. Bovine plasma prothrombin assay

(Globulin fraction method of Douglas and Biggs, 1953; appendix IV, 12, B)

Both globulin fractions were tested with human brain extract.

	Experiment 1		Experiment 2	
	Hum.pl.	Bov.pl.	Hum.pl.	Bov.pl.
Clotting time of substrate (seconds)	25½	26	21	23
Thrombin units / ml.	5.2	5	6.6	5.9
Prothrombin concentration	100 %	96 %	100 %	89.3 %

Table 49. Bovine prothrombin consumption index

A. Method of Biggs and Macfarlane, 1957, (appendix IV, 13, A) using bovine brain extract.

	Incubation time (seconds)			
	45	90	45	90
	Expt. 1		Expt. 2	
Bovine plasma time (sec.)	16	16	15½	15½
Bovine serum time (sec.)	40	44	32	51
Prothrombin consumption index	40 %	36 %	48 %	30 %
Average P.C.I.	38 %		39 %	

B. Globulin fraction method of Douglas and Biggs, 1953, (appendix IV, 13, B), using human brain extract.

	Bovine plasma	Bovine serum
30 minute clotting time (sec.)	26	120
Thrombin units per ml.	5	0.78
Prothrombin consumption index	15.6 %	

Table 50. R.V.V.-cephalin clotting time of plasma

(method described in appendix IV, 19)

Concentration of R.V.V. - cephalin R.	Plasma	Clotting times (seconds)*		
		Expt. 1	Expt. 2	Expt. 3
1/40,000 - 0.06 gm %	Human	7	8½	8
	Bovine	10	10	12
1/200,000- 0.06 gm %	Human	8½	9	9
	Bovine	12	13	14

* Average of duplicate estimations.

Table 51. Bovine blood platelets' count

(method described in appendix IV, 1, A)

	Total count / c.mm. whole blood*
Experiment 1	331,000
Experiment 2	261,000
Experiment 3	324,400
Experiment 4	278,000
Experiment 5	387,700
Experiment 6	292,000
Average	312,350

* Mean of duplicate estimations.

Table 52. Bovine whole blood clotting time

(method of Lee and White, 1913; appendix IV, 3)

	Clotting times*	
	Ordinary glass	Siliconed glass
Experiment 1	7' 20"	13' 30"
Experiment 2	6' 29"	12' 10"
Experiment 3	6' 15"	14' 5"
Average	6' 41"	13' 15"

* Mean of two tubes' readings.

Table 53. Recalcification time of plasma

(method described in appendix IV, 5)

	Clotting times	
	Human plasma	Bovine plasma
Experiment 1	106"	164"
Experiment 2	129"	151"
Experiment 3	120"	122"

Table 54. Hageman factor and plasma thromboplastin antecedent activity in bovine plasma

(method of Ratnoff and Rosenblum, 1958; appendix IV, 24, I)

Test plasma preparation	Clotting time (minutes) of substrate normal citrated platelet-deficient Intact plasma		
	Experiment 1	Experiment 2	Experiment 3
Human untreated plasma	145	125	120+
Bovine untreated plasma	160	120+	120+
Human activated plasma	14	16	13
Bovine activated plasma	15	15	14

Table 55. Thromboplastin generation test

(method of Biggs and Douglas, 1953b; appendix IV, 20)

Incubation mixture: 0.2 ml adsorbed plasma, variable, diluted 1/5,
 0.2 ml serum, variable, diluted 1/10,
 0.2 ml human platelets,
 0.2 ml M/40 CaCl₂ solution.

Substrate : human H.S.plasma.

No.	Incubation mixture (ml)	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
86	0.2 Hum.ads.pl. 0.2 Hum.serum	1	41	9	9	9	9½	9½
		2	62	11	9½	9	8½	9
		3	70	24	10	10	10	11
		Avg.	58	14.6	9½	9.3	9.3	9.8
		%	2.6	46	110	117	117	103 %
87	0.2 Bov.ads.pl. 0.2 Bov.serum	1	12	10	10	10	9½	11
		2	10	10	10	10	10	9½
		3	9	11	10	10	11	11
		Avg.	10.3	10.3	10	10	10.2	10.4
		%	94	94	100	100	96	90 %
88	0.2 Bov.ads.pl. 0.2 Hum.serum	1	8	9	9	8½	9½	9
		2	10	10	9	9	9	9
		3	22	12	11	12	11	10½
		Avg.	13.3	10.3	9.6	9.8	9.8	9.5
		%	56	94	110	103	103	110 %
89	0.2 Hum.ads.pl. 0.2 Bov.serum	1	15	9½	10½	10	10½	10½
		2	11	9	10	10	10	9
		3	15	11	10	10½	10	11
		Avg.	13.6	9.8	10.2	10.2	10.2	10.2
		%	54	103	96	96	96	96
90	0.15 Haemophilic pl. 0.05 Hum.ads.pl. 0.2 Hum.serum	1	25	9½	9½	10	10	10
		2	31	11	10	9½	9½	9½
		3	91	32	13	12½	11	11
		Avg.	49	17½	10.8	10.5	10.2	10.2
		%	3.5	31	88	90	96	96 %

Table 55, continued

No.	Incubation mixture (ml)	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
91	0.175 Haemophilic pl. 0.025 Hum.ads.pl. 0.2 Hum.serum	1	30	16	15	15	13	12½
		2	35	17	17	16	16	15
		3	58	20	17	15	14	15
		Avg.	41	17.6	16.3	15.3	14.3	14.2
		%	5	31	36	41	48	49 %
92	0.19 Haemophilic pl. 0.01 Hum.ads.pl. 0.2 Hum.serum	1	88	71½	29	18	17	17
		2	90	73½	30	17	17	17½
		3	92	77	31	19	18	18
		Avg.	90	74	30	18	17½	17½
		%	1	1.5	9.8	29	28	28 %
93	0.15 Haemophilic pl. 0.05 Bovine ads.pl. 0.2 Hum.serum	1	12½	8½	8½	9	9	9
		2	15	9	9	9½	9	9
		3	45	12	12	11	10½	11
		Avg.	24	9.8	9.8	9.8	9.5	9.6
		%	15	103	103	103	110	110 %
94	0.175 Haemophilic pl. 0.025 Bovine ads.pl. 0.2 Hum.serum	1	25	10	9½	9½	9	9
		2	36	11½	10½	10½	10	10
		3	47	13	11½	11	11	11
		Avg.	36	11½	10½	10.3	10	10
		%	6.8	76	90	95	100	100 %
95	0.19 Haemophilic pl. 0.01 Bovine ads.pl. 0.2 Hum.serum	1	37	10	10	10	9	9
		2	52	13½	11	11	10½	10½
		3	68	17	12	12	12	12
		Avg.	51½	13½	11	11	10.8	10.8
		%	3	54	84	84	90	90 %
96	0.19 Haemophilic pl. 0.01 Bov.ads.pl. 1/10 0.2 Hum.serum	1	58	17	16	12½	12½	12
		2	—	—	—	—	—	—
		3	86	22	16	14	13	13
		Avg.	72	19½	16	13¼	12.7	12.5
		%	1.5	25	38	56	64	65 %
97	0.19 Haemophilic pl. 0.01 Bov.ads.pl. 1/20	1	72	28	20	17	16	16
		2	—	—	—	—	—	—
		3	88	30	28	25	18	18
		Avg.	80	29	24	21	17	17
		%	1.3	10.5	16	21	33	33 %

Table 55, continued

No.	Incubation mixture (ml)	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
98	0.20 Human ads.pl. 0.15 X-mas serum 0.05 Human serum	1	39	19	11	10	10	9
		2	50	19	10	10	10	10
		3	100	30	12	11	10	10
		Avg.	63	23	11	10.3	10	9.6
		%	2	17	84	94	100	110 %
99	0.20 Human ads.pl. 0.175 X-mas serum 0.025 Human serum	1	88	25	13	11	11	11
		2	90	27	13	12	12	11
		3	105	28	14	13	12	12
		Avg.	94.3	26.6	13.3	12	11.6	11.3
		%	0.9	12.5	56	70	74	80 %
100	0.20 Human ads.pl. 0.19 X-mas serum 0.01 Human serum	1	101	82	30	16	14	14
		2	110	91	30	16	15	15
		3	126	97	33	16	15	14
		Avg.	112	90	31	16	14.6	14.3
		%	0.6	1	9	37.5	46	48 %
101	0.20 Human ads.pl. 0.10 X-mas serum 0.10 Bovine serum	1	12½	11	11	11½	11	12
		2	12	12	12	11½	11	11
		3	13	12½	12	11½	12	12½
		Avg.	12½	11.8	11.6	11.5	11.3	11.8
		%	54	74	76	76	80	74 %
102	0.20 Human ads.pl. 0.15 X-mas serum 0.05 Bovine serum	1	40	16	15½	14	14½	14½
		2	32	17	15	15	15	15
		3	23	15½	15	15	15	15
		Avg.	31½	16.2	15.2	14.6	14.8	14.8
		%	8.8	36	41	46	45	45 %
103	0.20 Human ads.pl. 0.175 X-mas serum 0.025 Bovine serum	1	42	19	17	17½	16½	16
		2	38	21	19	19	18	18
		3	34	20	19	19	18½	17½
		Avg.	38	20	18.3	18.4	17.6	17.2
		%	6	23	28	28	31	32 %

Experiments 1, 2, and 3 each was made with a different set of reagents. Thromboplastin percentages were read from the human active thromboplastin dilution curve constructed with human platelets, (figure 2, curve A).

Table 56. Thromboplastin generation test, modified

(method of Biggs and Douglas, 1953b; appendix IV, 20)

Incubation mixture: 0.2 ml adsorbed plasma, variable, diluted 1/5,
 0.2 ml serum, variable, diluted 1/10,
 0.2 ml bovine platelets, (appendix III, 15, C)
 0.2 ml M/40 CaCl₂ solution.

Substrate : human H.S.plasma.

No.	Incubation mixture (ml)	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
104	0.2 Human ads.pl. 0.2 Human serum	1	40	22	12	10	10	10
		2	82	28	12	12	10½	10½
		3	68	15	10	11	11	11
		Avg.	63	23	11.3	11	10.5	10.5
		%	2.1	17	80	84	92	92 %
105	0.2 Bovine ads.pl. 0.2 Bovine serum	1	20	12	12	12	13	12½
		2	12	11	11	11	12½	12½
		3	20	11½	11½	12	13	13
		Avg.	17.3	11.5	11.5	11.7	12.7	12.7
		%	32	76	76	74	62	62 %
106	0.2 Bovine ads.pl. 0.2 Human serum	1	18½	13	12½	10	9	9
		2	13½	10	10	9½	9½	9½
		3	15	11	11	11½	11	11
		Avg.	15.3	11.3	11.1	10.3	9.8	9.8
		%	40	80	82	94	103	103 %
107	0.2 Human ads.pl. 0.2 Bovine serum	1	20	12	12	13	13	13½
		2	16	11	11	11½	12	11
		3	20	12	12	13½	13	13
		Avg.	18.6	11.7	11.7	12.7	12.7	12.5
		%	27	74	74	62	62	64 %

In view of the similar activities of human and bovine platelet suspensions, the percentage values of active thromboplastin were read from the human dilution curve A, figure 2.

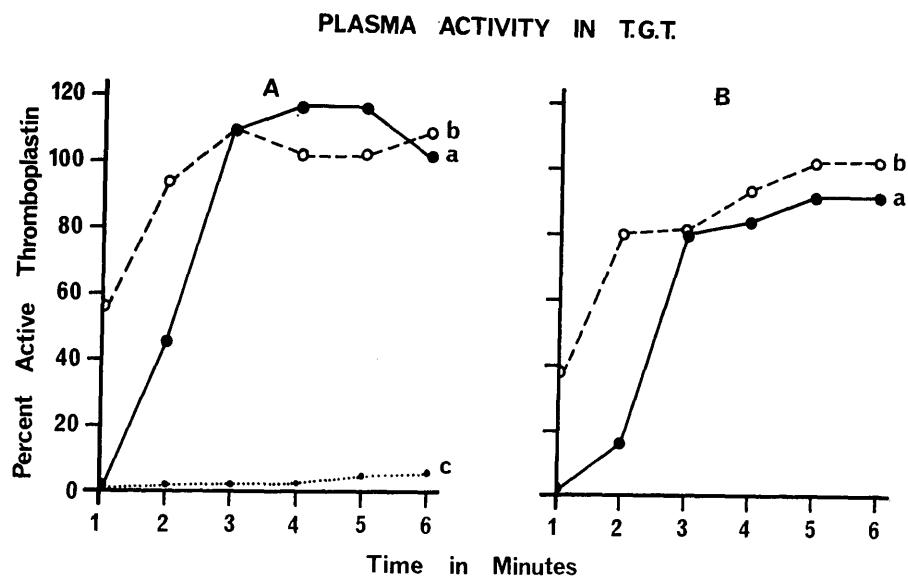


Figure 26. Bovine plasma activity in the T.G.T.

Figure A : using human platelets

Curve a : human ads.pl. + human serum (Mixture 86, table 55)

Curve b : bovine ads.pl. + human serum (Mixture 88, table 55)

Figure B : using bovine platelets

Curve a : human ads.pl. + human serum (Mixture 104, table 56)

Curve b : bovine ads.pl. + human serum (Mixture 106, table 56)

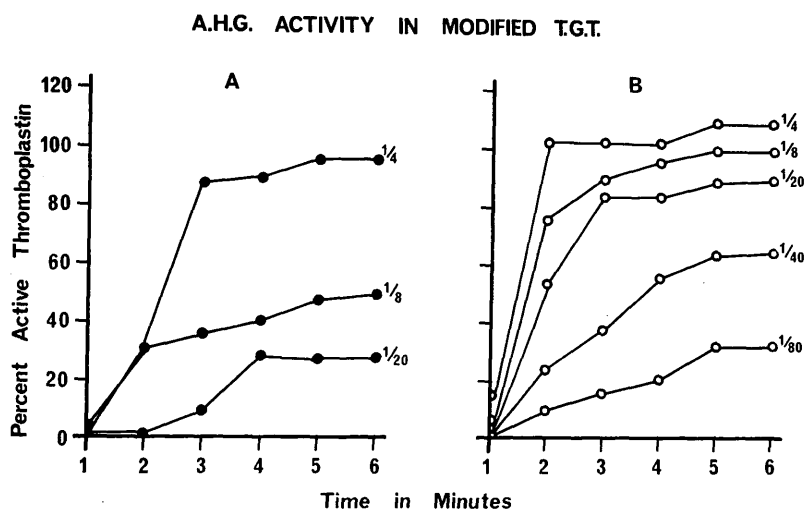


Figure 27. Bovine plasma A.H.G. activity in T.G.T.

Figure A

Human adsorbed plasma diluted 1/4, 1/8, and 1/20 in haemophilic adsorbed plasma, (Mixtures 90, 91, and 92 respectively, table 55).

Figure B

Bovine adsorbed plasma diluted 1/4, 1/8, 1/20, 1/40, and 1/80 in haemophilic adsorbed plasma, (Mixtures 93, 94, 95, 96, and 97, table 55).

Table 57. A.H.G. assay in bovine plasma

(method of Pitney, 1956; appendix IV, 21)

Plasma dilution	A.H.G. concentration	Clotting times (seconds)					
		Expt.1	Expt.2	Expt.3	Expt.4	Expt.5	Expt.6
		Human control plasma					
1/20	100 %	16½	19	18	20	15	20
1/40	50 %	22½	25	23	25	23	27
1/80	25 %	35	30	28	32	26	34
1/200	10 %	52	40	43	38	35	42
1/400	5 %	59	45	47	57	38	65
1/2000	1 %	60	55	53	80	42	74
	0 %	78	60	56	82	38	80
		Bovine test plasma					
1/80		14	--	--	--	--	--
1/100		16	14	18	18	14	17
1/200		26	17	22	23	16	20
1/400		29	24	28	30	21	28
1/800		42	29	34	35	29	36
Calculated A.H.G. conc.		633 %	1107 %	590 %	673 %	973 %	920 %

The average A.H.G. concentration in the six bovine plasma pools tested is 816 % of the human control plasma A.H.G. content.

Statistical analysis of the data obtained from the above table gave a mean value of A.H.G. of 781.05 % of the human, with a standard error (S_m) of 63.3, and a coefficient of variation (fractional S) of 34.85 %. The corrected coefficients of variation amongst the values obtained for the various dilutions tested of each plasma pool were 29.54 %, 11.68 %, 23.18 %, 4.55 %, 21.8 %, and 8.7 % respectively.

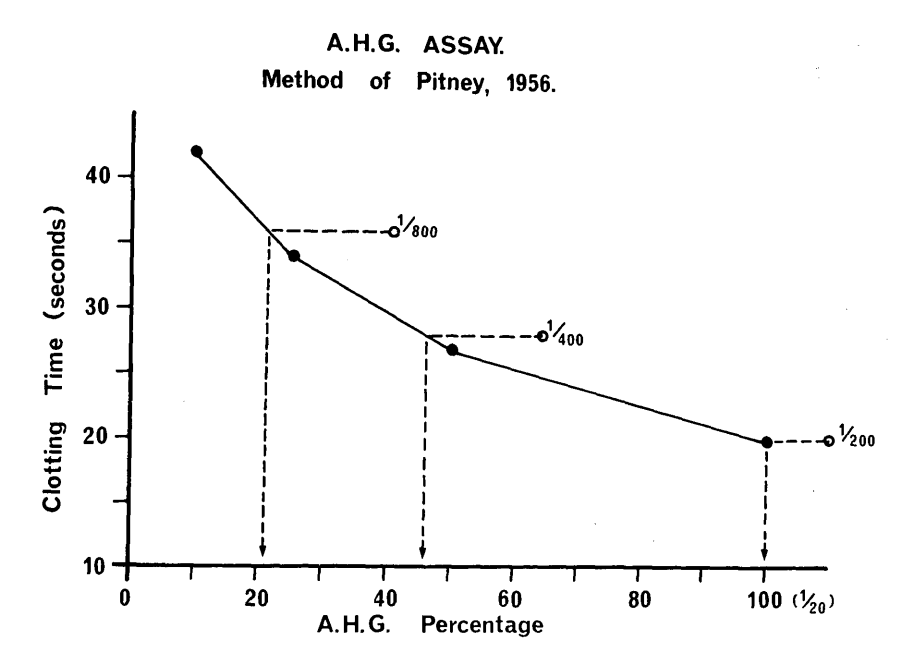


Figure 28. A.H.G. assay in bovine plasma.

A diagrammatic presentation of experiment 6, table 57, showing the curve obtained with various dilutions of normal human adsorbed plasma in haemophilic adsorbed plasma, when plotting the percentage of A.H.G. in each mixture (dilution 1/20 designated 100%) against substrate clotting times.

Calculation is made as follows:-

Dilution 1/200 reads 100% thus bov.pl. contains $\frac{100 \times 200}{20} = 1000$ % A.H.G.
 Dilution 1/400 reads 46% thus bov.pl. contains $\frac{46 \times 200}{20} = 920$ % A.H.G.
 Dilution 1/800 reads 21% thus bov.pl. contains $\frac{21 \times 200}{20} = 840$ % A.H.G.

Average of the three dilutions = 920 %

Standard of deviation (S) = 80.41

Coefficient of variation = 8.7 %

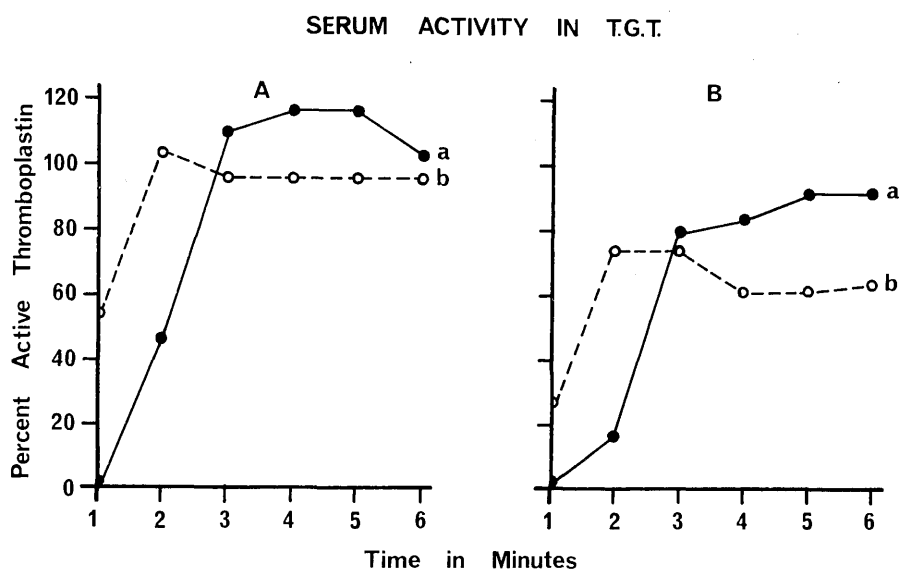


Figure 29. Activity of bovine serum in T.G.T.

Figure A : using human platelets

Curve a : human ads.pl. + human serum (Mixture 86, table 55)

Curve b : human ads.pl. + bovine serum (Mixture 89, table 55)

Figure B : using bovine platelets

Curve a : human ads.pl. + human serum (Mixture 104, table 56)

Curve b : human ads.pl. + bovine serum (Mixture 107, table 56)

It is noted that the use of bovine serum causes very rapid generation of active thromboplastin which is apparent in the first minute reading.

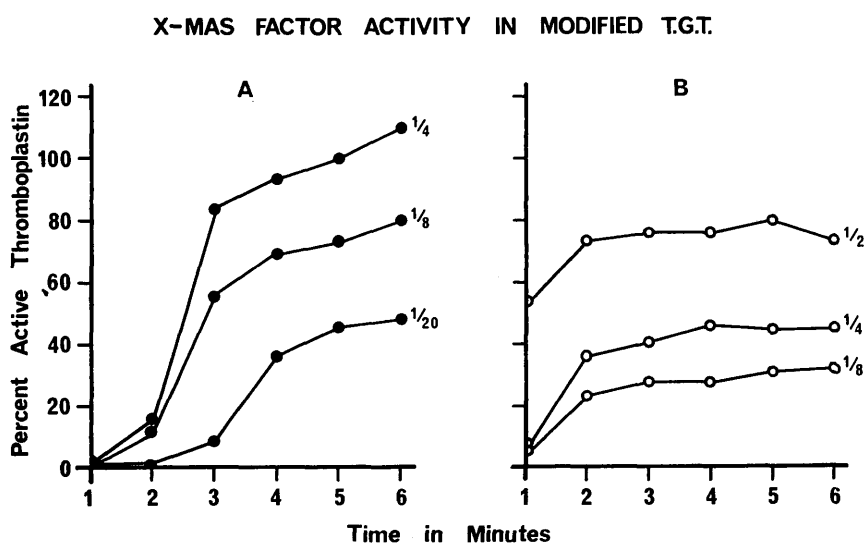


Figure 30. Bovine serum Christmas factor activity in the T.G.T.

Figure A

Correction of Christmas serum activity by human normal serum diluted 1/4, 1/8, and 1/20 in X-mas serum, (Mixtures 98, 99, and 100 respectively, table 55).

Figure B

Correction of the Christmas serum by bovine serum diluted in it in the proportion of 1/2, 1/4, and 1/8, (Mixtures 101, 102, and 103 respectively, table 55).

Table 58. Christmas factor assay in bovine serum

(based on method of Pitney, 1956; appendix IV, 22)

Serum dilution	X-mas factor concentration	Clotting times (seconds)					
		Expt.1	Expt.2	Expt.3	Expt.4	Expt.5	Expt.6
		Human control serum					
1/20	100 %	9	10	10	14	11½	11
1/40	50 %	10	11	11	16	12½	12½
1/80	25 %	12	12½	12	21	14	13½
1/160	12.5%	—	—	14	27½	16½	16
1/200	10 %	15	15	—	—	—	—
1/320	6.2%	—	—	19	49	20	17½
1/400	5 %	18	23	—	—	—	—
1/640	3.1%	—	—	29	63	27	25
1/1280	1.6%	—	—	40	72	42	35
1/2000	1 %	31	45	—	—	—	—
	0 %	80	85	85	81	64	56
		Bovine test serum					
1/5		—	12	—	—	13	—
1/10		—	—	11	18	15	14
1/20		13	13	12	23	18	16
1/40		15	14½	13	25	20	17½
1/80		18	18	14	27	—	19½
Calculated X-mas factor		20 %	22.5%	33.5%	25 %	10.5%	13.3%

The average Christmas factor concentration for the six bovine serum pools is 20.8 % of the human control serum Christmas factor content.

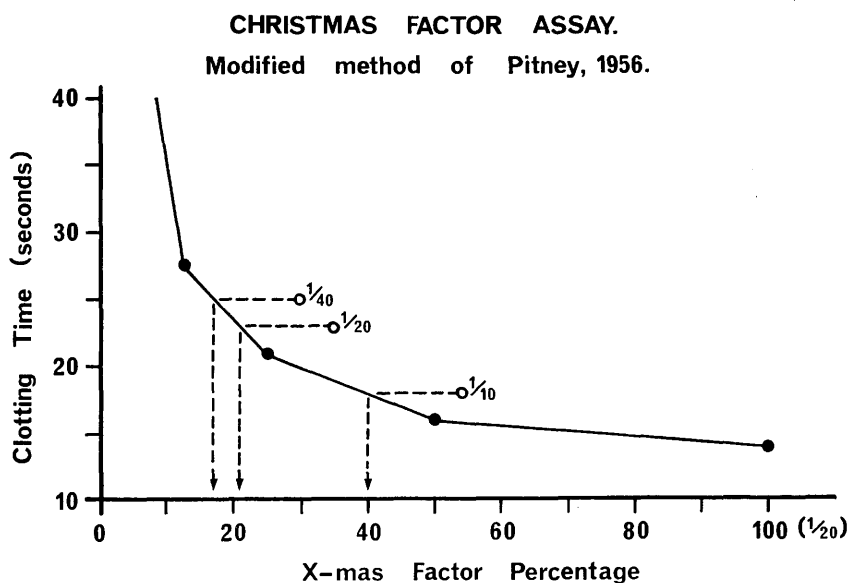


Figure 31. Christmas factor assay in bovine serum.

A schematic illustration of experiment 4, table 58, showing the curve obtained with various dilutions of normal human serum in Christmas disease serum, when plotting the percentages of Christmas factor in the mixtures (dilution 1/20 designated 100 %) against the substrate clotting times.

Dilution 1/10 reads 40 % thus bov.serum contains $\frac{40 \times 10}{20} = 20$ % X-mas F.
 Dilution 1/20 reads 21 % thus bov.serum contains $\frac{21 \times 20}{20} = 21$ % X-mas F.
 Dilution 1/40 reads 17 % thus bov.serum contains $\frac{17 \times 40}{20} = 34$ % X-mas F.

The average Christmas factor concentration of this particular bovine serum pool therefore contains 25 % of human serum Christmas factor activity.

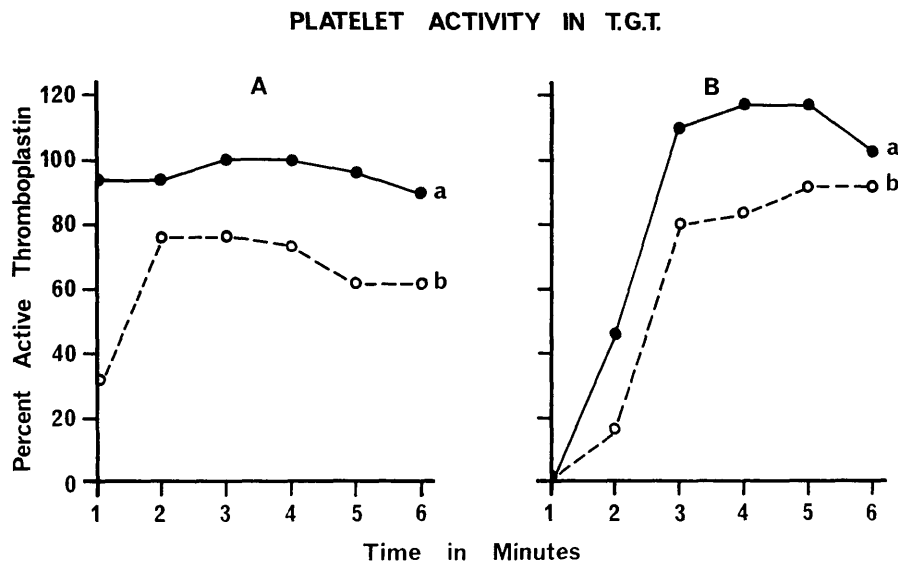


Figure 32. Thromboplastic activity of bovine platelets in T.G.T.

Figure A : Bovine ads.pl. and bovine serum are constants.

Curve a : using human platelets, (Mixture 87, table 55)

Curve b : using bovine platelets, (Mixture 105, table 56)

Figure B : Human ads.pl. and human serum are constants.

Curve a : using human platelets, (Mixture 86, table 55)

Curve b : using bovine platelets, (Mixture 104, table 56).

It is noted that the bovine platelet preparations used are weaker than the human preparations which were similarly made.

Table 59. Bovine plasma substrate reactivity in the T.G.T.

Incubation mixture : 0.2 ml adsorbed plasma, diluted 1/5,
 0.2 ml serum, diluted 1/10,
 0.2 ml human platelets,
 0.2 ml M/40 CaCl₂ solution.

Substrate : bovine high-spun plasma.

No.	Incubation mixture	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
108	Human plasma Human serum	1	25	15	17	19	19	18
		2	39	13	15	14½	15	16
		Avg.	32	14	16	16¾	16½	17
		%	8.6	50	37	34	35	33 %
109	Bovine plasma Bovine serum	1	25	15	16	17	17½	17½
		2	17	16½	15	15	16	16
		Avg.	21	15¾	15½	16	16¼	16¾
		%	21	39	40	37	34	34 %
110	Bovine plasma Human serum	1	14	15	15	16	16	16
		2	13½	15	15	14½	15	15
		Avg.	13¾	15	15	15¼	15½	15½
		%	54	43	43	41	40	40 %
111	Human plasma Bovine serum	1	37	20	20	25	24	25
		2	21	20	18½	18½	20	21
		Avg.	29	20	19¼	21¼	22	23
		%	10.5	23	25	20	19	17 %

Percentage active thromboplastin values were read from the human thromboplastin dilution curve A, figure 2.

Incubation mixtures 108, 109, 110, and 111 were from the same reagents used in making mixtures 86, 87, 88, and 89, table 55, and are therefore directly comparable with them as regards the effect of using bovine substrate plasma instead of the human substrate used in the latter mixtures.

Table 60. Bovine plasma substrate reactivity in the T.G.T.

Incubation mixture: 0.2 ml adsorbed plasma, diluted 1/5,
 0.2 ml serum, diluted 1/10,
 0.2 ml bovine platelets,
 0.2 ml M/40 CaCl₂ solution.

Substrate : bovine high-spun plasma.

No.	Incubation mixture	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
112	Human ads.pl. Human serum	1	61	30	22	20	21	20
		2	63	33	21	20	22	20½
		3	60	29	21	21	23	23
		Avg.	61	30.7	21.3	20.3	22	21
			2.7	9.4	20	21.5	19	21 %
113	Bovine ads.pl. Bovine serum	1	27	19	19	17	18½	18½
		2	20	17	17	19	18½	18½
		3	35	18	17½	17½	18	19
		Avg.	27.3	18	17.8	17.8	18.3	18.6
			12	29	30	30	28	27 %
114	Bovine ads.pl. Human serum	1	30	18	17	17	17	17
		2	25	17	17½	18	19	19
		3	30	17½	17½	18½	18½	21
		Avg.	28.3	17.5	17.3	17.8	18.1	19
			11	31	32	30	29	26 %
115	Human ads.pl. Bovine serum	1	33	26	25	25	25	27
		2	27	24	24	24	27	26
		3	42	25	25	29	29	33
		Avg.	34	25	24.6	26	27	28.6
			7.5	14.5	15	13.5	12.5	11 %

Mixtures 112, 113, 114, and 115 were tested on the same day as, and from the same reagents used for mixtures 104, 105, 106, and 107, table 56, respectively. They are directly comparable with each other as regards the effect of using bovine substrate plasma instead of the human.

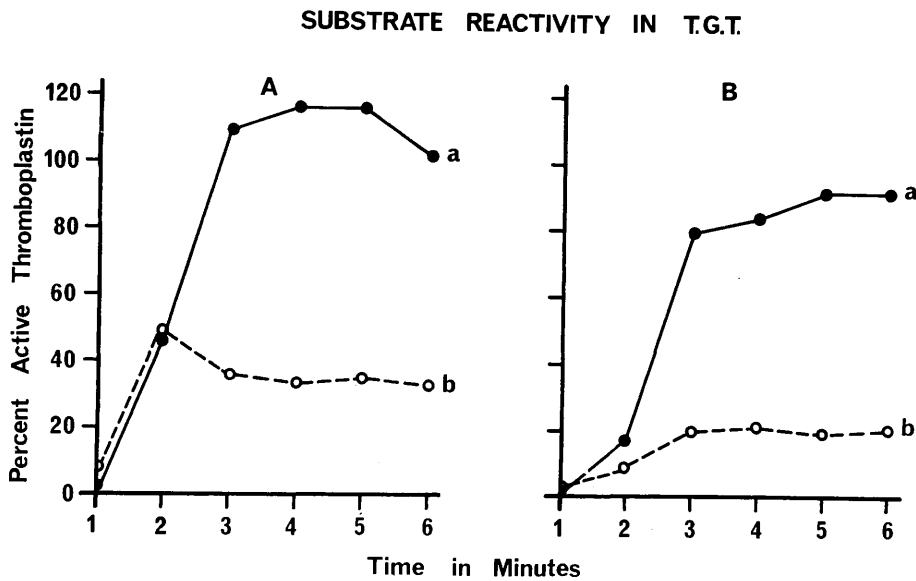


Figure 33. Bovine plasma substrate reactivity in the T.G.T.

Figure A (Human ads.pl., human serum, and human platelets are constant)

Curve a : on human high-spun plasma substrate (Mixture 86, table 55)

Curve b : on bovine high-spun plasma substrate (Mixture 108, table 59)

Figure B (Human ads.pl., human serum, and bovine platelets are constant)

Curve a : on human high-spun plasma substrate (Mixture 104, table 56)

Curve b : on bovine high-spun plasma substrate (Mixture 112, table 60)

It should be noted that the use of bovine high-spun plasma as substrate in the T.G.T. does not reflect the true level of active thromboplastin generated due to the delay in its reactivity as compared with the human high-spun plasma.

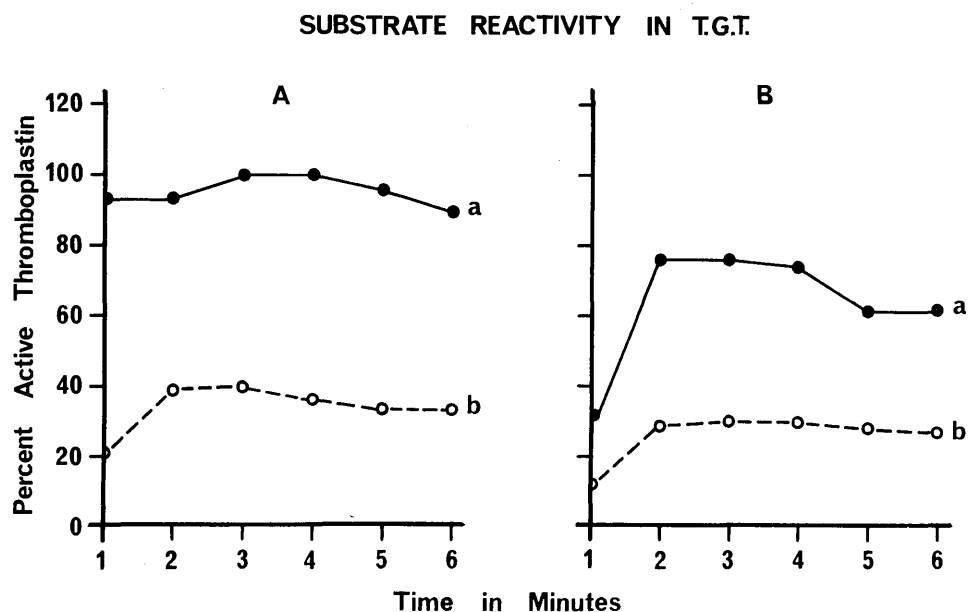


Figure 34. Bovine plasma substrate reactivity in the T.G.T.

Figure A (Bovine ads.pl., bovine serum, and human platelets are constant)

Curve a : on human H.S.plasma substrate (Mixture 87, table 55)

Curve b : on bovine H.S.plasma substrate (Mixture 109, table 59)

Figure B (Bovine ads.pl., bovine serum, and bovine platelets are constant)

Curve a : on human H.S.plasma substrate (Mixture 105, table 56)

Curve b : on bovine H.S.plasma substrate (Mixture 113, table 60)

It is noted that even when using bovine ads.pl. and serum, the bovine substrate plasma exhibited the same delayed reactivity to the active thromboplastin formed, compared to the human substrate plasma.

Attention is specially drawn to curve b in figure B which represents a completely homologous bovine thromboplastin generation system, and shows very weak thromboplastin generation as compared to a normal human system.

APPENDIX V, C

RESULTS OF EXPERIMENTS
ON HORSE BLOOD

RESULTS OF EXPERIMENTS
ON HORSE BLOOD

All the following data were obtained from experiments carried out on blood samples obtained from horses, mainly English thoroughbred racing horses and Clydesdale horses, using pooled plasma or serum samples from at least three animals for each experiment to obviate as much as possible any individual variations from affecting the final levels of the various coagulation factors.

Blood samples were obtained fresh on the day of the test concerned as described in appendix II, 1, iv.

Table 61. Fibrinogen estimation in horse plasma

(modified Greenberg method, appendix IV, 2, II)

	mg fibrinogen per 100 mls absolute plasma
Experiment 1	325
Experiment 2	433.8
Experiment 3	567.2
Experiment 4	420
Average	436.5

Table 62. Thrombin-fibrinogen reaction of plasma

(method described in appendix IV, 8 and 9)

Clotting mixture (ml)	Expt.	Thrombin topical (P.D.& Co.) in units / ml					
		50	25	12.5	6.25	3.1	1.55
		Clotting times (seconds)					
0.2 Horse pl. 0.2 Saline 0.2 Thrombin	1	16	19	25	34	66	171
	2	13	18½	21	30	55	152
	3	11	19½	23	38	64	163
	Avg.	13.3	19	23	34	61.6	162
0.2 Human pl. 0.2 Saline 0.2 Thrombin	1	5½	7	10	16	26	51
	2	5	6½	9	15	27	48
	3	5	6½	10	16	25	48
	Avg.	5.1	6.6	9.6	15.6	26	49
0.2 Horse pl. 0.1 Saline 0.1 Pyrocatechol 0.2 Thrombin	1	5	7	8½	13	22	37½
	2	5	6	8	13	22	35
	3	5	6	8	12½	22	32
	Avg.	5	6.3	8.1	12.8	22	34.8
0.2 human pl. 0.1 Saline 0.1 Pyrocatechol 0.2 Thrombin	1	3	4½	5½	9	14	26
	2	3	4½	5½	9	15	26
	3	3	3½	5½	9	14	22
	Avg.	3	4.1	5.5	9	14.3	24.6
0.2 Horse pl. 0.1 Pyrocatechol 0.1 M/40 CaCl ₂ 0.2 Thrombin	1	4	5	7½	10	17	30
	2	4	4½	6¼	9½	15	28
	3	4	4½	7	9¾	16	28
	Avg.	4	4.6	6.9	9.7	16	29
0.2 Human pl. 0.1 Pyrocatechol 0.1 M/40 CaCl ₂ 0.2 Thrombin	1	3	4	5	7½	12	22
	2	3	3½	4½	7½	12	21
	3	3	3½	4¾	7½	12	21
	Avg.	3	3.6	4.7	7.5	12	21.3

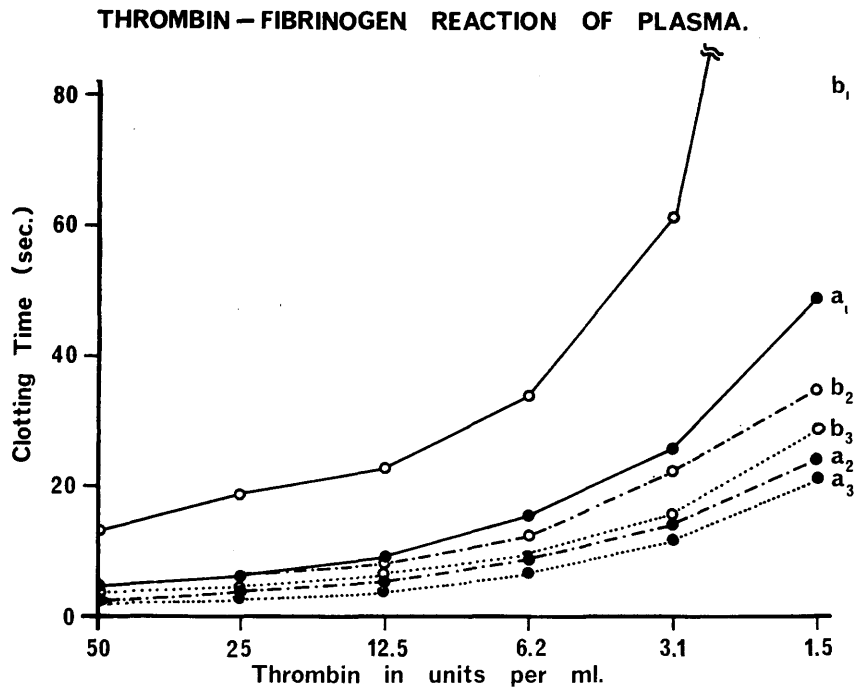


Figure 35. Thrombin-fibrinogen reaction of horse plasma.

Curve a₁ : human plasma + saline

Curve b₁ : horse plasma + saline

Curve a₂ : human plasma + saline + Pyrocatechol 1%

Curve b₂ : horse plasma + saline + Pyrocatechol 1%

Curve a₃ : human plasma + Pyrocatechol 1% + M/40 CaCl₂

Curve b₃ : horse plasma + Pyrocatechol 1% + M/40 CaCl₂

Table 63. Species specificity of the thrombin-fibrinogen reaction

(method described in appendix IV, 10)

	Thrombin solution (0.1 ml)		
	Human	Bovine 50 U/ml	Horse
	Clotting times (seconds)*		
0.4 ml Human fibrinogen 0.1 ml Saline	10	10	24
0.4 ml Horse fibrinogen 0.1 ml Saline	31	144	85
0.4 ml Human fibrinogen 0.1 ml Pyrocatechol 1%	7	8	20
0.4 ml Horse fibrinogen 0.1 ml Pyrocatechol 1%	12	15	26

* Average of two estimations.

The human fibrinogen solution contained 340 mg/100 mls solution.

The horse fibrinogen solution contained 240 mg/100 mls solution.

Table 64. Antithrombin assay in horse plasma

(method of Douglas and Biggs, 1953; appendix IV, 11, A)

Incubation mixture	Incubation time (minutes)							
	$\frac{1}{2}$	$\frac{3}{4}$	1	2	3	4	5	6
	Clotting times (seconds)							
0.5 ml horse pl. 0.5 ml thrombin	-	25	29	69	152	180+	180+	
0.5 ml human pl. 0.5 ml thrombin	15	-	18 $\frac{1}{2}$	35	70	135	180+	180+
	Units of residual thrombin							
Horse plasma	-	5.6	4.6	1.65	0.64	0	0	
Human plasma	10.3	-	8	3.7	1.63	0.75	0	0

The incubation times at which zero activity of thrombin was attained were read from curves a and b in figure 36.

Value for human plasma = 6.5 (curve a at 0.1 thrombin units/ml).

Value for horse plasma = 4.8 (curve b at 0.1 thrombin units /ml).

Horse plasma antithrombin = 6.5 x 100 ÷ 4.8

= 134.4 % of human plasma antithrombin.

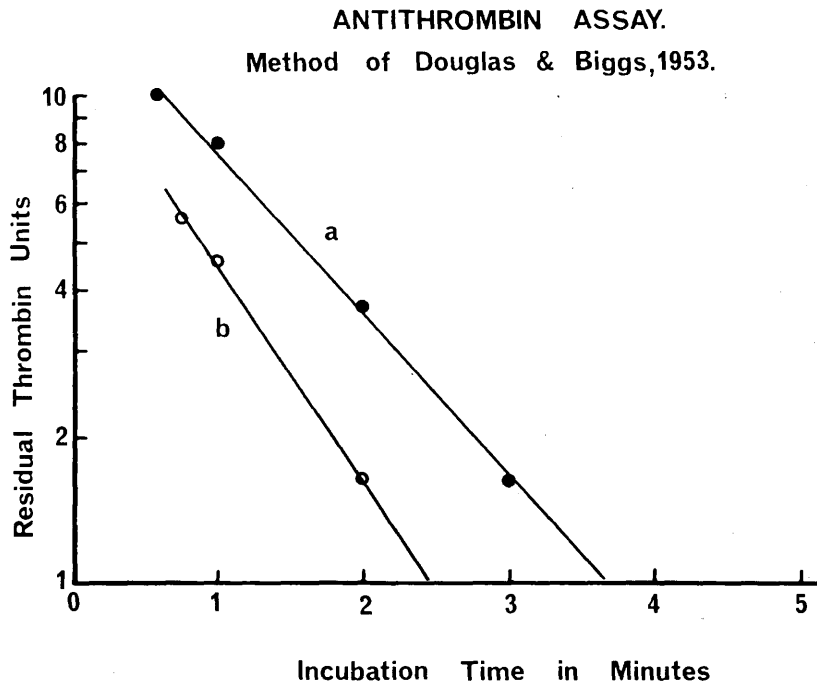


Figure 36. Antithrombin assay in horse plasma.

This figure was drawn from the results presented in table 64.

Curve a : human plasma.

Curve b : horse plasma.

Table 65. Antithrombin assay in horse plasma

(modified method of Astrup and Darling, 1942d; appendix IV, 11, B)

Mixture			A	B	C	D	
Saline	ml.		0.3	0.2	0.15	0.1	0.0
Plasma (dil. 1/5)	ml.		0.0	0.1	0.15	0.2	0.3
Thrombin*	ml.		1	1	1	1	1
		Expt.					
Clotting time of fibrinogen (seconds)	Human	1	11	13½	16	18	22
		2	16	22	25	28	32
		3	13	15	18	20	22
	Horse	1	11	15	16½	21	32
		2	16	26	37	41	56½
		3	13	17	23	25	35
T _o / T _n	Human	1	1	0.81	0.68	0.61	0.48
		2	1	0.72	0.64	0.57	0.50
		3	1	0.86	0.72	0.65	0.59
	Horse	1	1	0.73	0.62	0.52	0.34
		2	1	0.61	0.43	0.39	0.28
		3	1	0.76	0.56	0.52	0.37
Corrected T _o / T _n	Human	1	1	0.81	0.71	0.62	0.42
		2	1	0.80	0.70	0.60	0.40
		3	1	0.84	0.76	0.69	0.54
	Horse	1	1	0.74	0.62	0.50	0.26
		2	1	0.66	0.50	0.33	0.00
		3	1	0.76	0.64	0.52	0.28
Calculated units of antithrombin per ml plasma	Human	1	0	313	319	313	319
		2	0	300	300	300	300
		3	0	240	240	231	230
	Horse	1	0	429	418	412	407
		2	0	510	500	502	500
		3	0	360	360	360	360

* Thrombin solution used for experiment 1 contained 33 units/ml., while that used for experiments 2 and 3 contained 30 units/ml.

	<u>Expt. 1</u>	<u>Expt. 2</u>	<u>Expt. 3</u>
Average value for human plasma (units/ml) =	316	300	235
Average value for horse plasma (units/ml) =	416	503	360
Horse plasma antithrombin activity in percentage of human plasma antithrombin =	131.6%	167.6%	153 %
Average horse plasma antithrombin =	150.7 % of human plasma antithrombin.		

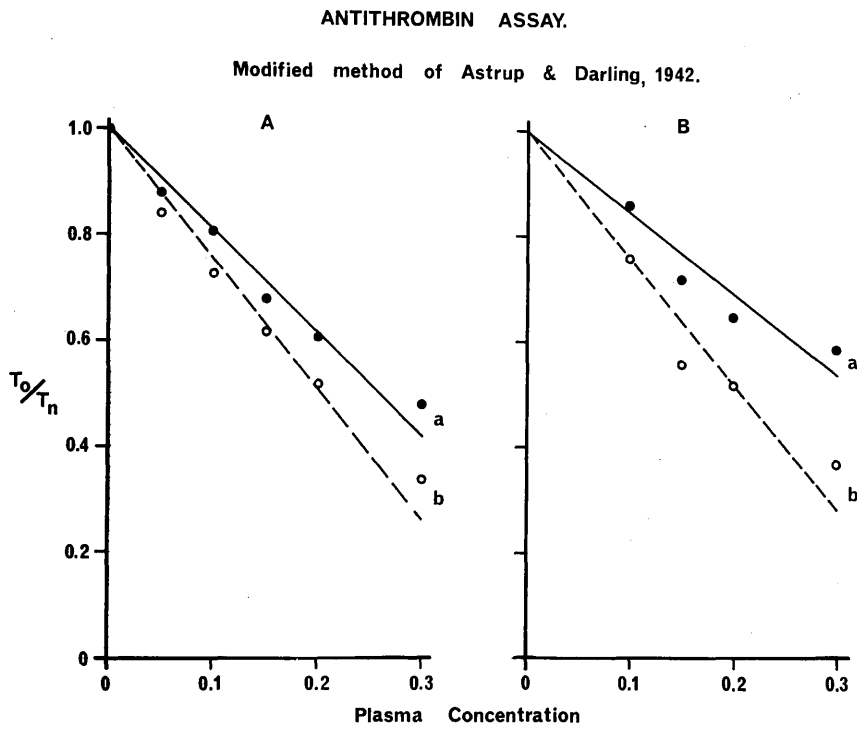


Figure 37. Antithrombin assay in horse plasma.

Figures A and B represent experiments 1 and 3 (table 65) respectively.

Curves a : human plasma.

Curves b : horse plasma.

The corrected values of T_0/T_n in table 65 were derived from these curves.

Table 66. Quick's one-stage prothrombin time test

Test plasma	Human brain	Horse brain
	Clotting times (seconds)	
Horse plasma	16	14
	21 (18)	12 (13)
	17	13
Human plasma	12	30
	17 (14.1)	27 (27)
	13½	24
Hse.pl. + Hum.pl.	14	15½
	19 (16)	13 (14.1)
	15	14
Hse.pl. + Saline	15½	17
	20½(17.3)	15 (15.3)
	16	14
Hum.pl. + Saline	14	44
	21 (17.5)	37½(37.9)
	17½	32

Figures between brackets are the mean for each group of three experiments carried out with different batches of brain thromboplastin and different plasma pools.

Table 67. Assay of factor V activity in horse plasma and serum

By correction of human stored factor V-deficient plasma, as described in appendix IV, 14. Human brain thromboplastin was used for all mixtures.

Plasma dilution	Factor V percentage	Clotting times (seconds)			
		Expt. 1	Expt. 2	Expt. 3	
		Human control plasma			
1/10	100 %	25	25	24	
1/20	50 %	30	29	29	
1/40	25 %	37	36	33	
1/80	12.5%	42	40	35	
1/160	6.2%	49	45	39	
1/320	3.1%	52	50	42	
1/640	1.5%	53	55½	44	
		Hse.pl.	Hse.pl.	Hse.ser.	Hse.pl.
1/10		15	15	25	19
1/20		16½	18	29	22
1/40		21	20	—	24
1/80		25	26	—	28
1/160		29	30	—	32
1/320		—	38	—	38
Calculated factor V		864 %	669 %	100 %	397 %

The factor V activity in horse plasma ranges from 397 to 864 per cent, with an average of 643 per cent of human plasma factor V activity.

Horse serum contained factor V activity equivalent to that of human plasma.

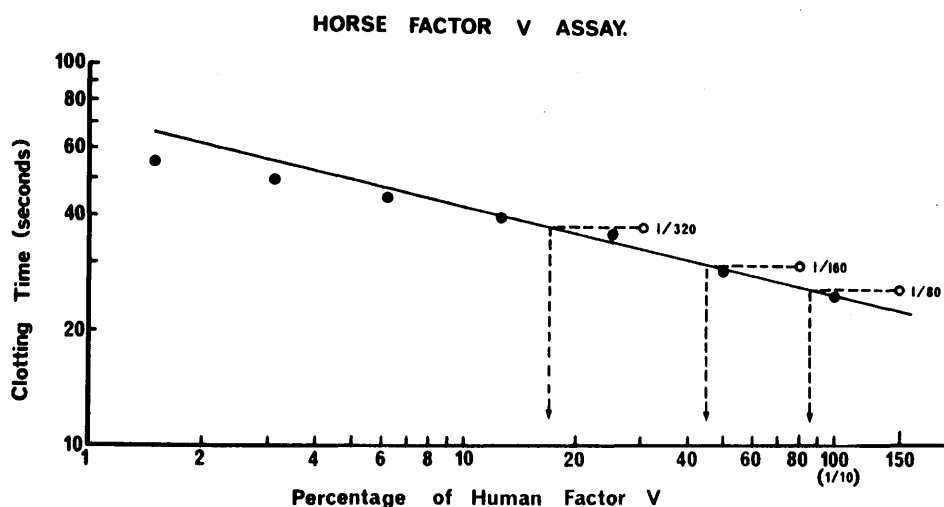


Figure 38. Factor V assay in horse plasma.

The dilution curve was drawn from the results obtained for human normal fresh plasma diluted in stored human plasma and clotted with human brain in the presence of CaCl_2 solution, experiment 2, table 67.

The dotted lines show how the clotting times obtained with various dilutions of test horse plasma were converted into percentage values of factor V activity, which were multiplied by the dilution factor to arrive at the final concentration of factor V in horse plasma in percentage of the human control plasma factor V content.

Table 68. Factors VII and X - brain thromboplastin species specificity

(method described in appendix IV, 17)

Incubation mixture :-

0.3 ml brain extract, diluted 1/4, variable,

0.3 ml purified factors VII and X, variable, (appendix III, 10)

0.3 ml M/40 CaCl₂ solution.

Variables	Substrate	Incubation time (minutes)						
		½	1	2	3	4	5	6
		Clotting times (seconds)						
Horse brain Hse. VII & X	Fresh hum.pl.	28	27½	29	33½	35½	39	41
	Fresh hse.pl.	23	23½	23	24½	26	27½	29
	Phenindione pl.	22	21½	22	23½	25	28½	30
Horse brain Hum. VII & X	Fresh hum.pl.	54	47	49½	47	47	49	47½
	Fresh hse.pl.	39	35	36½	37	40	38	40
	Phenindione pl.	70	65½	60	60	59	60	62
Horse brain Saline	Fresh hum.pl.	85	84	86	87	78	95	93
	Fresh hse.pl.	31	31½	32	30½	33	32½	33
	Phenindione pl.	156	155	150	153	152	157	156
Human brain Hse. VII & X	Fresh hum.pl.	29	28	30	30	32	33	32
	Fresh hse.pl.	22	18½	18½	21	23	23½	25
	Phenindione pl.	34	31	32	34	35	37	36
Human brain Hum. VII & X	Fresh hum.pl.	20	20	19	19	20	20½	22
	Fresh hse.pl.	18	18½	18½	18½	19	21	22
	Phenindione pl.	26	25	26	26	25½	24	25
Human brain Saline	Fresh hum.pl.	43	38	42	40	40½	42	41
	Fresh hse.pl.	38	40	40	41	40	42½	41
	Phenindione pl.	100	95	106	137	135	140	150

Table 69. Horse plasma factor VII assay

(method of Koller et al, 1951; appendix IV, 15, B)

Plasma dilution	Factor VII percentage	Clotting times (seconds)			
		Experiment 1		Experiment 2	
		Human brain	Horse brain	Human brain	Horse brain
		Hum.pl.	Hse.pl.	Hum.pl.	Hse.pl.
1/10	100 %	34	38	40	31
1/20	50 %	48	53	53	39
1/40	25 %	72	65	90	50
1/80	12.5 %	107	85	140	76
1/160	6.2 %	172	129	215	99
1/320	3.1 %	290	180	358	148
1/640	1.5 %	390	250	520	220
	0 %	740	643	765	780
		Hse.pl.	Hum.pl.	Hse.pl.	Hum.pl.
1/10		42	110	43½	101
1/20		68	154	67	136
1/40		100	216	108	201
Calculated F VII & X		63.3 %	6.9 %	80 %	5.6 %

Since this method is also sensitive to varying concentrations of factor X, the measured activity is due to the combined effect of both factors VII and X. Plasmas used in each of the experiments were the same, the various dilutions being tested with either type of brain extract.

With human brain extract, horse plasma factors VII and X activity averages 71 % of human plasma activity. While with horse brain extract, human plasma factors VII and X activity averaged 6.2 % of horse plasma activity. This discrepancy is thought to be due to species specificity between the brain extracts and the factors VII and X.

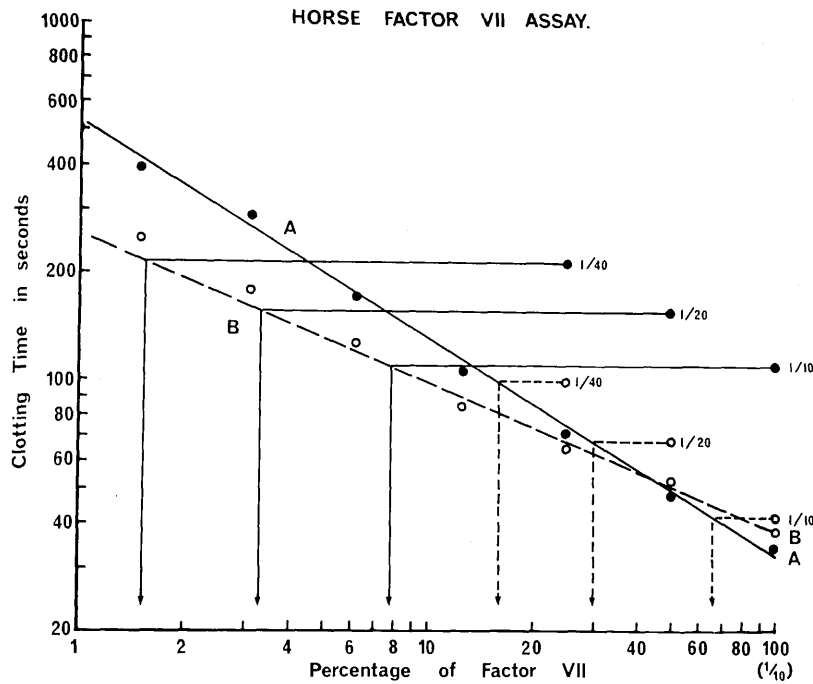


Figure 39. Horse plasma factors VII and X activity.

(Human plasma : black dots. Horse plasma : white dots)

Curve A : human plasma dilution curve, using human brain extract, experiment 1, table 69. The average of the three horse plasma dilutions reads 63.3 %.

Curve B : horse plasma dilution curve, using horse brain extract, experiment 1, table 69. The average of the three human plasma dilutions reads 6.9 %.

Table 70. Horse plasma factor X assay

(method of Bachmann, Duckert, and Koller, 1958; appendix IV, 18)

Plasma dilution	Factor X percentage	Clotting times (seconds)		
		Expt. 1	Expt. 2	Expt. 3
		Human	control	plasma
1/10	100 %	23	25	26
1/20	50 %	29	33	32½
1/40	25 %	39	38½	41½
1/80	12.5%	49	46½	46
1/160	6.2%	54½	55	63
1/320	3.1%	66	66	76
1/640	1.5%	81½	86	87
1/1280	0.7%	91½	93	101
	0 %	106	117	130
		Horse	Test	Plasma
1/10		25.8	25	31
1/20		31	29	40
1/40		39	38	44
1/80		—	—	54
Calculated factor X conc.		86 %	106 %	64.7 %

The average factor X concentration in horse plasma equals 85.5 % of human plasma factor X content.

Table 71. Horse plasma prothrombin assay

(Two-stage area method of Biggs and Douglas, 1953a; appendix IV, 12, C)

Incubation mixture	Expt.	Incubation time (minutes)							Weight of area under curve & its percentage		
		$\frac{1}{4}$	$\frac{1}{2}$	$\frac{3}{4}$	1	2	3	4			5
		Clotting times (seconds)									
Hum.pl. + Hum.Br.	1A	--	15	18	25	59	180+	180+			
Hse.pl. + Hum.Br.	1B	--	--	13	14	31	80	180+			
Hum.pl. + Hse.Br.	1C	--	35	--	22	48	112	180+			
Hse.pl. + Hse.Br.	1D	--	9	9 $\frac{1}{2}$	11 $\frac{1}{2}$	45	101	180+			
Hum.pl. + Hum.Br.	2A	--	13	20	26	78	180+				
Hse.pl. + Hum.Br.	2B	16	--	14	15	30	75	125	180+		
Hum.pl. + Hse.Br.	2C	79	28	--	19	45	119	180+			
Hse.pl. + Hse.Br.	2D	--	9	11	15 $\frac{1}{2}$	65	175	180+			
Hum.pl. + Hum.Br.	3A	--	15	17	25	48	154	180+			
Hse.pl. + Hum.Br.	3B	18	--	16	21	46	111	147	180+		
Hum.pl. + Hse.Br.	3C	--	25	17 $\frac{1}{2}$	29	98	180+				
Hse.pl. + Hse.Br.	3D	--	10	13 $\frac{1}{2}$	16	51	134	175	180+		
			Thrombin units / ml.							mg	%
	1A	--	10.2	8.2	5.6	2	0		156.5		
	1B	--	--	12.2	11.2	4.6	1.4	0	269.0	171.8 %	
	1C	--	3.7	--	6.5	2.6	0.9	0	138.8		
	1D	--	19	17.5	14.5	2.7	1.6	0	344.0	247.8 %	
	2A	--	12.2	7.2	5.3	1.4	0		147.2		
	2B	9.6	--	11.2	10.2	4.5	1.5	0.8	0	284.2	193.0 %
	2C	1.4	4.9	--	7.8	2.7	0.8	0	156.5		
	2D	--	19	15	10	1.8	0.5	0	262.4	167.6 %	
	3A	--	10.2	8.8	5.6	2.6	0.6	0	175.0		
	3B	8.2	--	9.6	6.9	2.7	0.9	0.6	0	205.5	117.4 %
	3C	--	5.6	--	8.5	4.7	1.1	0	203.4		
	3D	--	16.8	11.5	9.6	2.4	0.7	0.5	0	254.0	124.8 %

Percentages of horse plasma prothrombin are related to the prothrombin content of human plasma tested with the same species of brain extract on the same day. With human brain, the average horse plasma prothrombin equals 160 % of the human, while with horse brain it averages 180 % of the human plasma prothrombin content.

Experiment 2 is illustrated in figure 40.

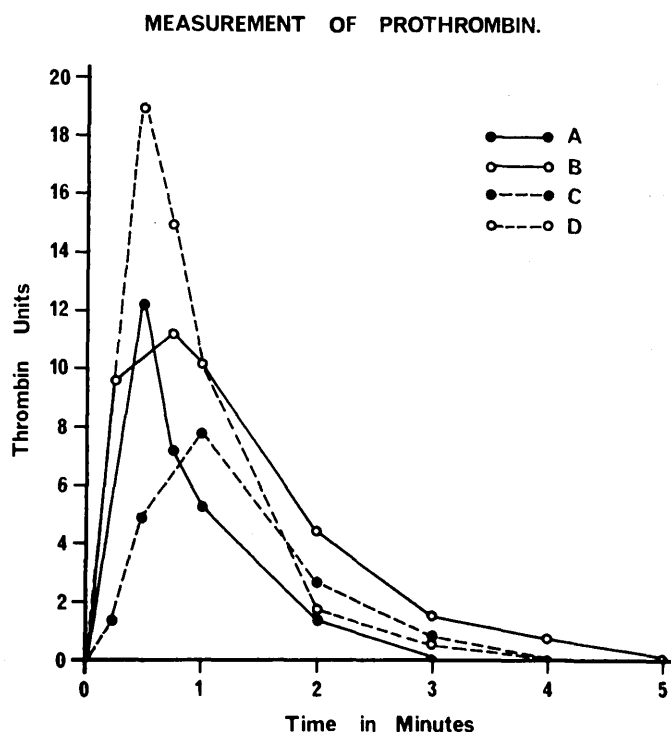


Figure 40. Measurement of horse plasma prothrombin.

- Curve A : obtained for human plasma using human brain. Weight of area under curve is 147.2 mg which is designated the 100% value.
- Curve B : obtained for horse plasma using human brain. Weight of area under curve is 284.2 mg which is equivalent to 193 % of human plasma prothrombin.
- Curve C : obtained for human plasma using horse brain. Weight of enclosed area is 156.5 mg, which reads 106.3% of human plasma prothrombin when estimated with human brain.
- Curve D : obtained for horse plasma using horse brain. Weight of enclosed area is 262.4 mg which gives a value of 167.6 % of the human plasma prothrombin when the latter was tested with horse brain.

(All data are from the results of experiment 2, table 71).

Table 72. Horse plasma prothrombin assay

(globulin fraction method of Douglas and Biggs, 1953; appendix IV, 12, B)

	Human brain		Horse brain	
	Hum.pl.	Hse.pl.	Hum.pl.	Hse.pl.
Clotting time of the substrate (seconds)	21	52	32	92
Thrombin units/ml.	7	2.3	4.1	1.2
Prothrombin concentration	100 %	32.8 %	100 %	29.2 %

Table 73. Horse prothrombin consumption index

A. Method of Biggs and Macfarlane, 1957, (appendix IV, 13, A) using horse brain extract.

	Expt. 1	Expt. 2	Expt. 3
Horse plasma time (seconds)	13½	13	14
Horse serum time (seconds)	180+	180+	180+
Prothrombin consumption index	- 7.5 %	- 7.2 %	- 7.7 %

B. Globulin fraction method of Douglas and Biggs, 1953, (appendix IV, 13, B), using human and horse brain extracts.

	Human brain		Horse brain	
	Hse.pl.	Hse.ser.	Hse.pl.	Hse.ser.
30 minute clotting time(sec)	52	180+	92	180+
Thrombin units/ml.	2.3	0.1	1.2	0.1
Prothrombin consumption I.	4.3 %		8.3 %	

Table 74. Horse blood platelets' count

(method described in appendix IV, 1, A)

	Total count / c.mm. whole blood*
Experiment 1	120,000
Experiment 2	97,000
Experiment 3	114,000
Experiment 4	105,000
Experiment 5	126,000
Experiment 6	133,000
Average	117,000

* Mean of duplicate estimations.

Table 75. Horse whole blood clotting time

(method of Lee and White, 1913; appendix IV, 3)

	Clotting times*	
	Ordinary glass	Siliconed glass
Experiment 1	7' 48"	11' 41"
Experiment 2	3' 40"	8' 10"
Experiment 3	9' 7"	20' 7"
Experiment 4	7' 30"	14' 34"
Experiment 5	9' 23"	17' 40"
Experiment 6	10' 5"	20' 46"
Average	7' 55"	15' 29"

* Mean of two tubes' readings.

Table 76. Recalcification time of plasma

(method described in appendix IV, 5)

	Clotting times (seconds)	
	Human plasma	Horse plasma
Experiment 1	167	337
Experiment 2	132	392
Experiment 3	141	375
Average	147	368

Table 77. Horse plasma Hageman factor assay

(method described in appendix IV, 24, II)

Plasma dilution in Hageman factor deficient plasma	Concentration of Hageman factor	Clotting times (seconds)
		Human plasma
1/5	100 %	142
1/15	33.3%	182
1/45	11.1%	257
1/135	3.7%	364
1/405	1.2%	540
		Horse plasma
1/15		180
1/45		241
Calculated Hageman factor concentration		118.5 %

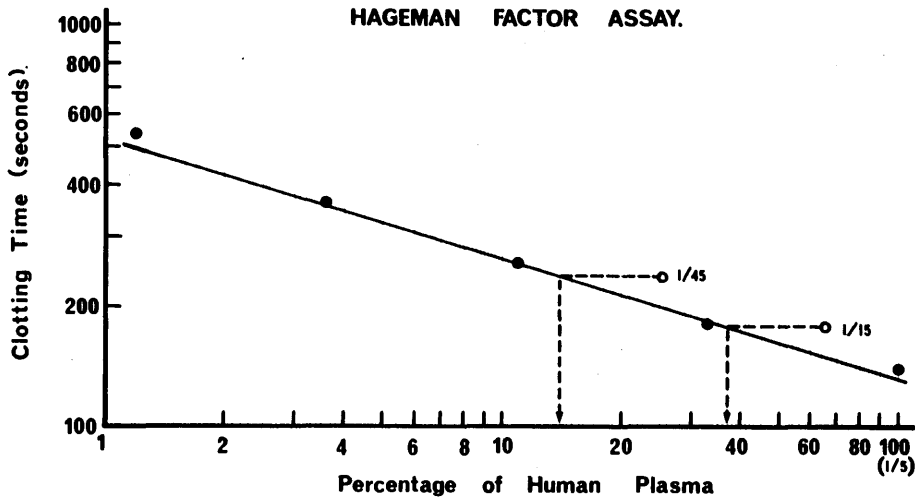


Figure 41. Horse plasma Hageman factor assay.

The above human plasma dilution curve was drawn from the results obtained in the experiment presented in table 77.

The dotted lines show how the clotting times obtained with the two dilutions of horse plasma tested, were converted into percentage values which when multiplied by the dilution factor give the final Hageman factor concentration in horse plasma in percentage of human plasma Hageman factor activity, since the human plasma dilution 1/5 was designated the 100 % value.

Table 78. Thromboplastin generation test

(method of Biggs and Douglas, 1953b; appendix IV, 20)

Incubation mixture : 0.3 ml. adsorbed plasma, variable, diluted 1/5,
 0.3 ml. serum, variable, diluted 1/10,
 0.3 ml. human platelets,
 0.3 ml. M/40 CaCl₂ solution.

Substrate : human high-spun plasma.

No.	Incubation mixture	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
116	Human plasma	1	30	9	8	9	10	9½
		2	49	13½	9	9	9	9
	Human serum	Avg.	39½	11¼	8½	9	9½	9¼
	%	5.6	78	145	128	110	120	%
117	Human plasma	1	15½	9	9½	9	10	9
		2	31	13	10	10	10	10
	Horse serum	Avg.	23¼	11	9¾	9½	10	9½
	%	17	84	104	110	100	110	%
118	Horse plasma	1	38	16½	16½	15	15	18
		2	52	24	14	14	13	12½
	Human serum	Avg.	45	20¼	15¼	14½	14	15¼
	%	4.2	23	42	47	50	42	%
119	Horse plasma	1	20	14	13	13	13	13½
		2	36½	19	12	12	13	13
	Horse serum	Avg.	28¼	16½	12½	12½	13	13¼
	%	11	36	64	64	59	56	%

Percentage active thromboplastin values were read from the human thromboplastin dilution curve A in figure 2.

Each of the two experiments was made with a different set of reagents.

Table 79. Thromboplastin generation test

(method of Biggs and Douglas, 1953b; appendix IV, 20)

Incubation mixture: 0.3 ml adsorbed plasma, variable, diluted 1/5,
 0.3 ml serum, variable, diluted 1/10,
 0.3 ml horse platelets,
 0.3 ml M/40 CaCl₂ solution.

Substrate : human high-spun plasma.

No.	Incubation mixture	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
120	Human plasma	1	127	105	68	25	11	12
		2	65	55	38	16½	9	8
	Human serum	Avg.	96	80	53	20¾	10	10
		%	0.8	1.2	3	22	100	100 %
121	Human plasma	1	48	13	11½	12	11½	12
		2	55	43	28	15	10	11
	Horse serum	Avg.	51½	28	19¾	13½	10¾	11½
		%	3.2	11.5	25	55	88	76 %
122	Horse plasma	1	137	127	111	60	37	29
		2	72	67	56	48	35	23
	Human serum	Avg.	104½	97	83½	54	36	26
		%	0.7	0.8	1.1	2.9	6.8	13.5%
123	Horse plasma	1	96	60	48	30	24	21
		2	62	50	41	24	20	19
	Horse serum	Avg.	79	55	44½	27	22	20
		%	1.3	2.7	4.3	12.5	19.5	23.5%

Percentage active thromboplastin values were read from the human thromboplastin dilution curve A in figure 2.

Experiment 2 was made with the same reagents used for experiment 2 in table 78 except for the different platelet preparation, and can therefore be directly compared with each other as to the thromboplastic

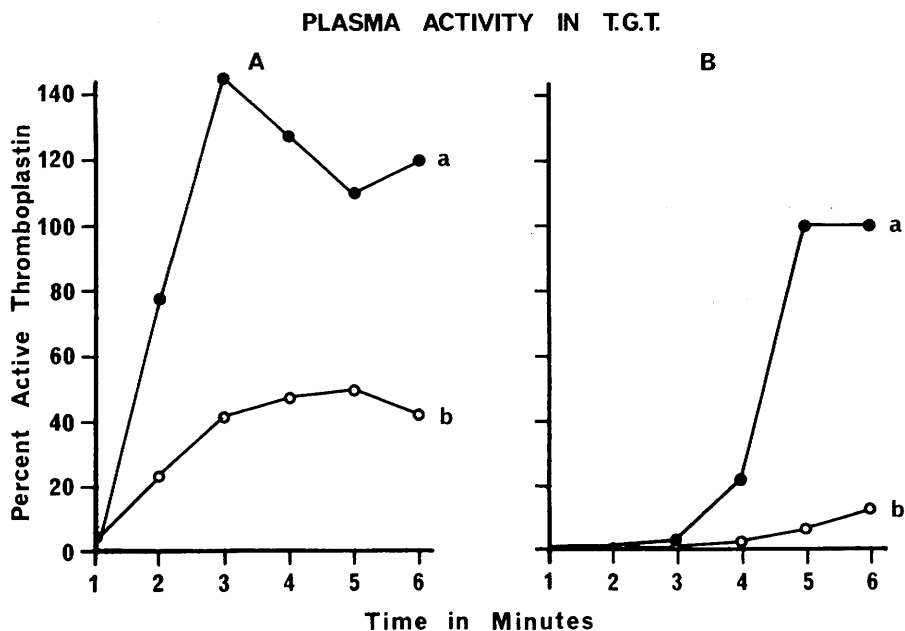


Figure 42. Horse plasma activity in the T.G.T.

Figure A (with human platelets)

Curve a : human ads.pl. + human serum (Mixture 116, table 78)

Curve b : horse ads.pl. + human serum (Mixture 118, table 78)

Figure B (with horse platelets)

Curve a : human ads.pl. + human serum (Mixture 120, table 79)

Curve b : horse ads.pl. + human serum (Mixture 122, table 79)

Table 80. A.H.G. assay in horse plasma

(method of Pitney, 1956; appendix IV, 21)

Plasma dilution	Concentration of A.H.G.	Clotting times (seconds)		
		Expt. 1	Expt. 2	Expt. 3
		Human control plasma		
1/20	100 %	16	19	20
1/40	50 %	19	22	23½
1/80	25 %	23	28	30
1/160	12.5 %	27	35	36
1/320	6.2 %	33	39	46
1/640	3.1 %	39	49	54
1/1280	1.5 %	—	56	—
		Horse test plasma		
1/20		16	17½	18
1/40		18	18½	20½
1/80		20	19	24½
1/160		—	—	31
Calculated A.H.G. concentration		137 %	310 %	180 %

The average A.H.G. concentration of the three horse plasma pools tested equals 209 % of the human control plasma A.H.G. content, with a range from 137 to 310 %.

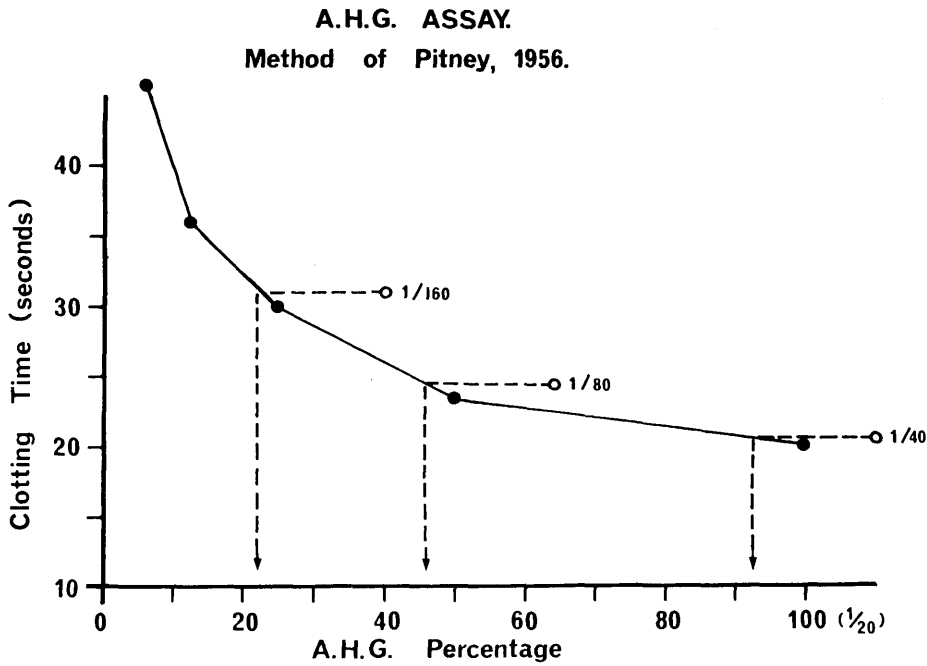


Figure 43. A.H.G. assay in horse plasma.

A diagrammatic presentation of experiment 3 from table 80.

Dilution 1/40 reads 94 % thus horse pl. contains $\frac{94 \times 40}{20} = 188 \%$

Dilution 1/80 reads 47 % thus horse pl. contains $\frac{47 \times 80}{20} = 188 \%$

Dilution 1/160 reads 20.7% thus horse pl. contains $\frac{20.7 \times 160}{20} = 166 \%$

The average A.H.G. concentration therefore in this horse plasma pool equals 180.6 % of the human control plasma A.H.G. content.

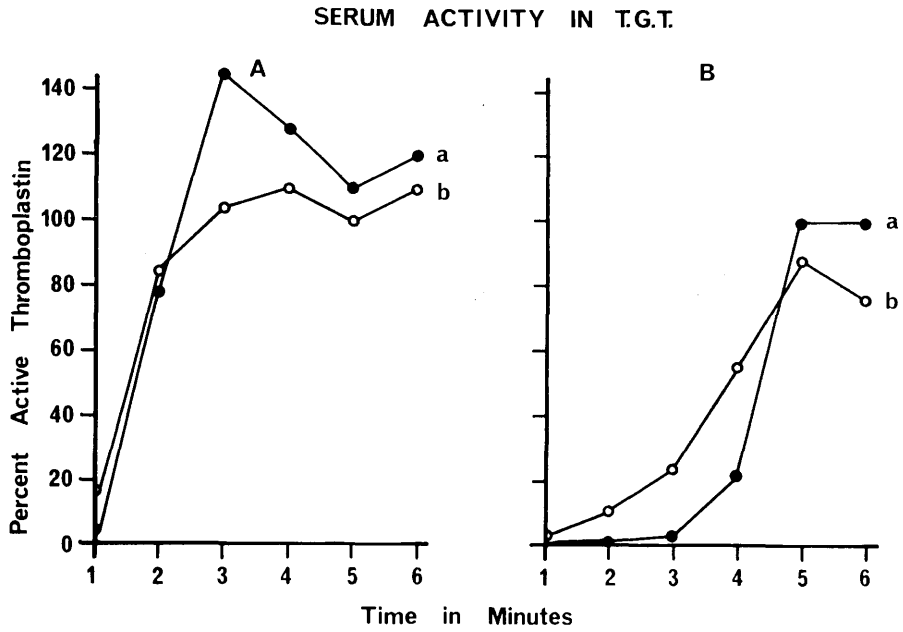


Figure 44. Horse serum activity in the T.G.T.

Figure A (using human platelets)

Curve a : human plasma + human serum (Mixture 116, table 78)

Curve b : human plasma + horse serum (Mixture 117, table 78)

Figure B (using horse platelets)

Curve a : human plasma + human serum (Mixture 120, table 79)

Curve b : human plasma + horse serum (Mixture 121, table 79)

Table 81. Christmas factor assay in horse serum

(based on method of Pitney, 1956; appendix IV, 22)

Serum dilution	X-mas factor concentration	Clotting times (seconds)			
		Expt. 1	Expt. 2	Expt. 3	Expt. 4
		Human control serum			
1/20	100 %	10	12	13½	13
1/40	50 %	11	13½	16	15
1/80	25 %	13	17	18	19
1/160	12.5%	15	26½	25	29
1/320	6.2%	18½	45	40	43
1/640	3.1%	24½	72	69	77
1/1280	1.5%	27	97	83	90
	0 %	32	118	142	152
		Horse test serum			
1/20		10	11	12½	12½
1/40		10½	12	14	13
1/80		11½	13½	16½	14½
1/160		13½	15	19	18½
Calculated F IX concentration		168 %	240 %	183 %	221 %

The average of the four horse serum pools tested is 203 % of the human serum factor IX concentration.

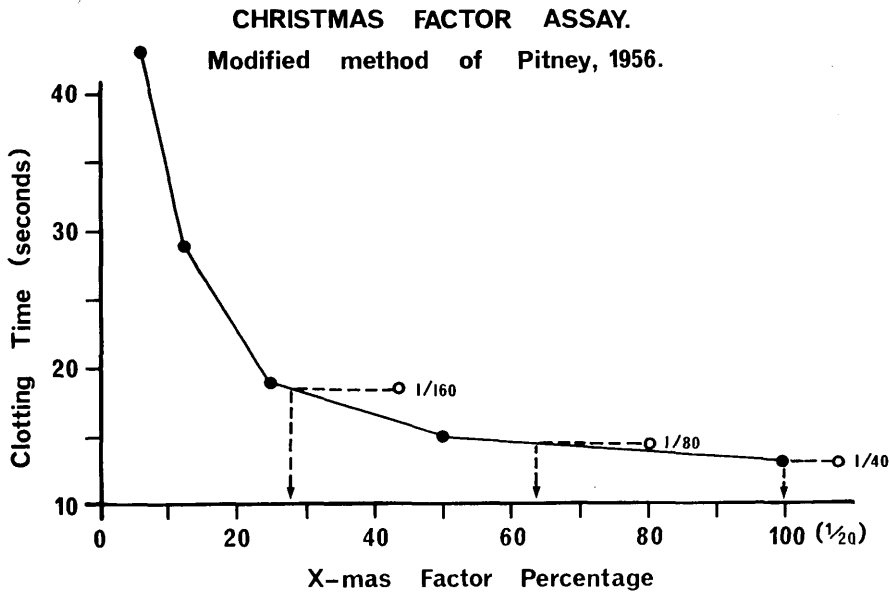


Figure 45. Christmas factor assay in horse serum.

A diagrammatic illustration of experiment 4 (table 81) showing the human serum dilution curve obtained by correction of X-mas disease serum.

Dilution 1/40 reads 100% thus hse.ser. contains $\frac{100 \times 40}{20} = 200\%$

Dilution 1/80 reads 60% thus hse.ser. contains $\frac{60 \times 80}{20} = 240\%$

Dilution 1/160 reads 28% thus hse.ser. contains $\frac{28 \times 160}{20} = 224\%$

The average Christmas factor concentration for this serum pool is therefore 221.3 % of human serum Christmas factor activity.

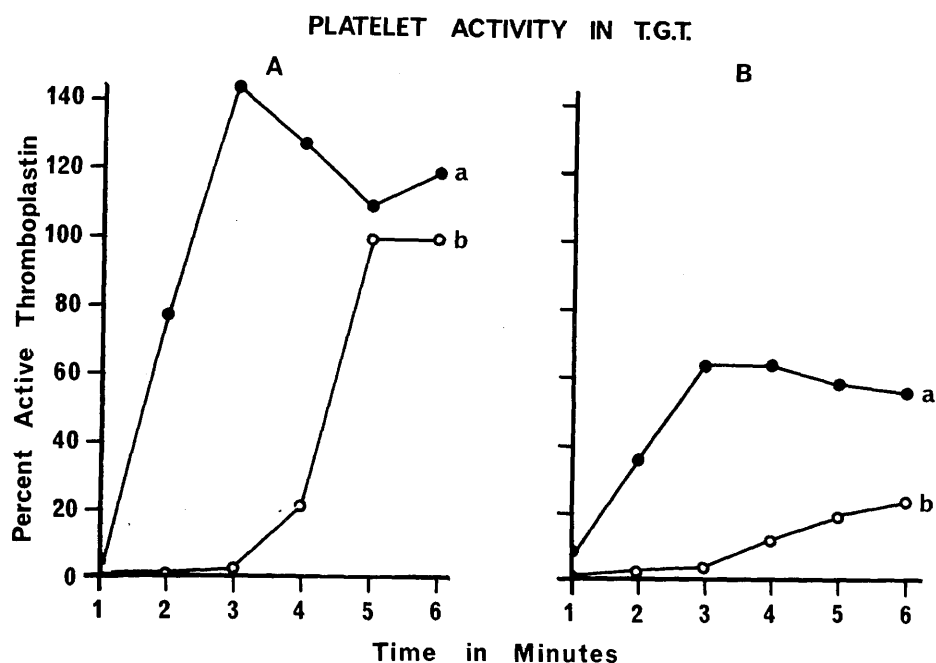


Figure 46. Thromboplastic activity of horse platelets in T.G.T.

Figure A (human ads.pl. and serum are constant)

Curve a : with human platelets (Mixture 116, table 78)

Curve b : with horse platelets (Mixture 120, table 79)

Figure B (horse ads.pl. and serum are constant)

Curve a : with human platelets (Mixture 119, table 78)

Curve b : with horse platelets (Mixture 123, table 79)

It is noted that the horse platelets' suspension used is weaker than the human preparation prepared from equal volumes of plasma.

Table 82. Clot retraction of horse blood

(method of Macfarlane, 1938; appendix IV, 4)

	Hematocrit value of sequestrene blood	Percentage of clot retraction in one hour*
Expt. 1	50 %	38 %
Expt. 2	39 %	34 %
Expt. 3	44 %	36 %
Expt. 4	40 %	36 %
Expt. 5	40 %	44 %
Average		37.6 %

* Average of two tubes' readings.

Table 83. Horse plasma substrate reactivity in the T.G.T.

Incubation mixture: 0.3 ml adsorbed plasma, diluted 1/5, variable,
 0.3 ml serum, diluted 1/10, variable,
 0.3 ml human platelets,
 0.3 ml M/40 CaCl₂ solution.

Substrate : horse high-spun plasma.

No.	Incubation mixture	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
124	Human ads.pl.	1	37	12	12½	14	13	13
		2	51	17	10½	11	12	12
	Human serum	Avg.	44	14½	11½	12½	12½	12½
	%	4.4	47	76	64	64	64 %	
125	Human ads.pl.	1	17	11	12½	12	13	13
		2	35	17	13	13	14	16
	Horse serum	Avg.	26	14	12¾	12½	13½	14½
	%	13.5	50	62	64	55	47 %	
126	Horse ads,pl.	1	56	21	22	22	24	22
		2	70	32	23	24	22½	20
	Human serum	Avg.	63	26½	22½	23	23¼	21
	%	2	13	18.5	17.5	17	21 %	
127	Horse ads.pl.	1	20	15	15	15	16	16
		2	40	22½	15	15	15	17
	Horse serum	Avg.	30	18¾	15	15	15½	16½
	%	10	27	43.5	43.5	41	36 %	

Percentage active thromboplastin values were read from the human thromboplastin dilution curve A, figure 2.

Incubation mixtures 124, 125, 126, and 127 contained identical reagents as were used in mixtures 116, 117, 118, and 119 of table 78, respectively, and are directly comparable to each other as regards the effect of using horse high-spun plasma as substrate instead of human high-spun plasma.

Table 84. Horse plasma substrate reactivity in the T.G.T.

Incubation mixture: 0.3 ml adsorbed plasma, diluted 1/5, variable,
 0.3 ml serum, diluted 1/10, variable,
 0.3 ml horse platelets,
 0.3 ml M/40 CaCl₂ solution.

Substrate : horse high-spun plasma.

No.	Incubation mixture	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
128	Human ads.pl.	1	140	127	110	70	27	17
		2	80	82	46	19	14	14
	Human serum	Avg.	110	104½	78	44½	20½	15½
		%	0.6	0.7	1.3	4.3	22.5	41 %
129	Human ads.pl.	1	66½	22	17	17	21	20
		2	64	53	35	19	14½	15
	Horse serum	Avg.	65½	37½	26	18	17¾	17½
		%	1.9	6.2	13.5	30	31	32 %
130	Horse ads.pl.	1	150	165	156	67	50	50
		2	71	68	58	37	24	21
	Human serum	Avg.	110½	116½	107	52	37	35½
		%	0.6	0.3	0.6	3.1	6.4	7 %
131	Horse ads.pl.	1	120	101	92	58	43	35
		2	62	47	34	23	20	22
	Horse serum	Avg.	91	74	63	40½	31½	27½
		%	0.9	1.4	2.1	5.2	9	12 %

Percentage active thromboplastin values were read from the human thromboplastin dilution curve A, figure 2.

Mixtures 128, 129, 130, and 131 contained identical reagents as were used in mixtures 120, 121, 122, and 123, table 79, respectively; and are therefore directly comparable with each other as regards the effect of using horse H.S.plasma as substrate, instead of the human.

Furthermore, results of experiments numbered 2 were obtained using identical reagents as in the same numbered experiments in tables 78 and 79 using human H.S.plasma substrate, with human and horse platelets respectively.

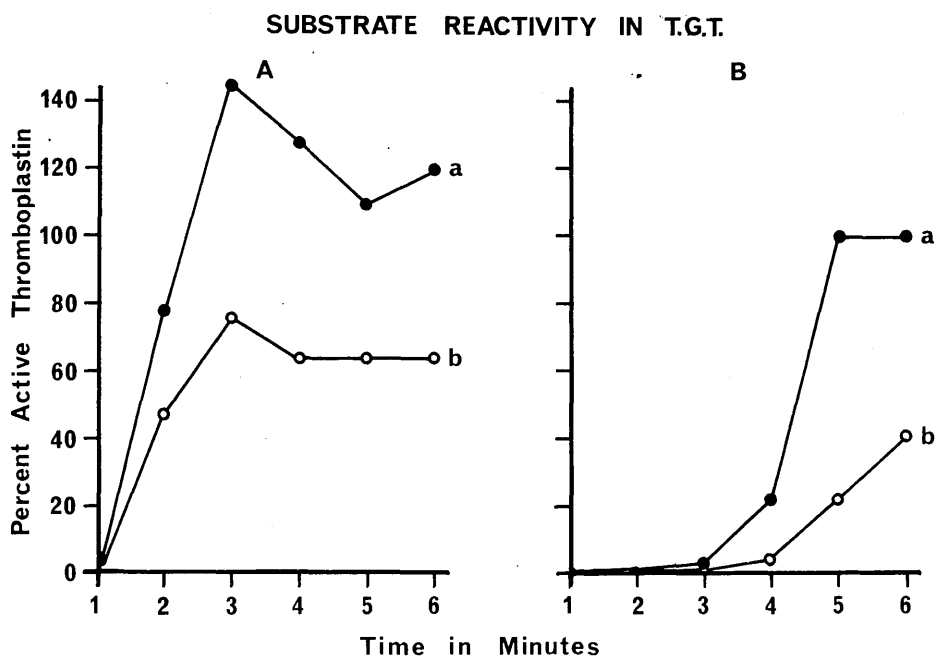


Figure 47. Horse plasma substrate reactivity in the T.G.T.

Figure A (human ads.pl., serum, and platelets are constant)

Curve a : using human H.S.pl. as substrate (Mixture 116, table 78)

Curve b : using horse H.S.pl. as substrate (Mixture 124, table 83)

Figure B (human ads.pl. and serum, with horse platelets are constant)

Curve a : using human H.S.pl. as substrate (Mixture 120, table 79)

Curve b : using horse H.S.pl. as substrate (Mixture 128, table 84)

The use of horse plasma as substrate does not reflect the true level of active thromboplastin generated as in the control mixtures, but shows a delay in its reactivity as compared to the human substrate plasma.

HORSE BLOOD THROMBOPLASTIN GENERATION.

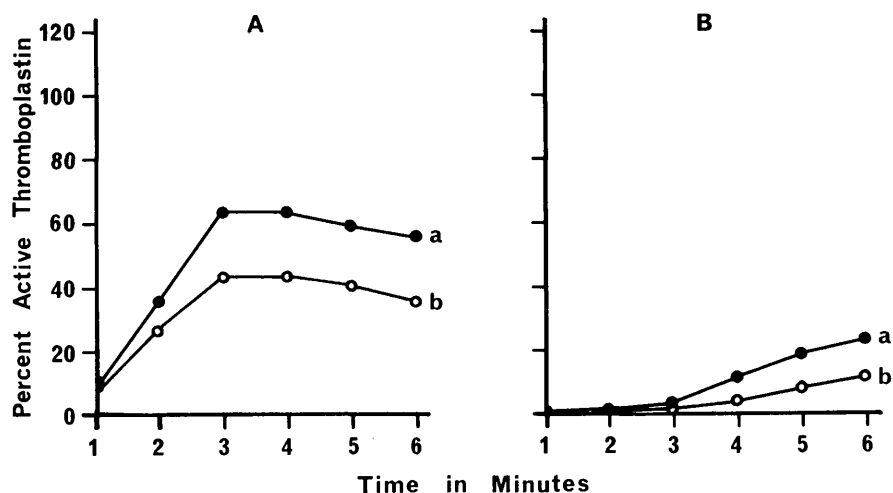


Figure 48. Horse plasma substrate reactivity in the T.G.T.

Figure A (horse ads.pl. and serum, with human platelets are constant)

Curve a : using human H.S.pl. as substrate (Mixture 119, table 78)

Curve b : using horse H.S.pl. as substrate (Mixture 127, table 83)

Figure B (horse ads.pl., serum, and platelets are constant)

Curve a : using human H.S.pl. as substrate (Mixture 123, table 79)

Curve b : using horse H.S.pl. as substrate (Mixture 131, table 84)

These curves again show the same effect of delayed horse plasma reactivity to active thromboplastin.

Attention is drawn to curve b, figure B, which represents a completely homologous horse thromboplastin generation system. It shows much weaker thromboplastin generation when compared to a normal human system.

Table 85. Investigation of inhibitors against human intrinsic thromboplastin in horse plasma and serum in the T.G.T.

(method described in appendix IV, 23)

Incubation mixture: 0.3 ml adsorbed plasma, diluted 1/5, variable,
 0.3 ml serum, diluted 1/10, variable,
 0.3 ml human platelets,
 0.3 ml M/40 CaCl₂ solution.

Substrate : human H.S.plasma.

A- Before incubation

No.	Incubation mixture (ml)	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
132	0.3 human ads.pl.	1	90	44	11½	12	11½	11½
		2	60	20	11	10	10½	10
	Avg.	75	32	11¼	11	11	10¾	10¾
	0.3 human serum	%	1.4	8.6	80	84	84	88 %
133	0.15 human ads.pl.	1	106	81	40	16	16	16
		2	92	69	23	13	13	13½
	Avg.	99	75	31½	14½	14½	14¾	14¾
	0.15 horse ads.pl.	%	0.7	1.4	9	47	47	46 %
134	0.3 human ads.pl.	1	30	13	12	13	13	15
		2	73	21	14	13	12	12
	Avg.	51½	17	13	13	12½	13½	13½
	0.15 human serum	%	3	33	59	59	65	55 %
135	0.15 human ads.pl.	1	78	55	38	16	12	12½
		2	83	42	21	12	10½	11½
	Avg.	80½	48½	29½	14	11¼	12	12
	0.15 heated Hse.pl.*	%	1.2	3.6	10.5	50	80	70 %
	0.30 human serum	%						

* Heated horse plasma was prepared by heating the adsorbed plasma for 15 minutes at 56°C. , centrifuging, and using the supernatant.

Table 85, continued

B- After incubation at 37°C. for 2 hours

No.	Incubation mixture (ml)	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
136	0.3 human ads.pl.	1	60	31	13	14	14	13
		2	67	22	12	11	11½	12
	0.3 human serum	Avg.	63½	26½	12½	12½	12¾	12½
		%	2	13	65	65	62	65 %
137	0.15 human ads.pl. 0.15 horse ads.pl.	1	80	50	22	17	18	19
		2	76	62	17½	14	14	15
	0.30 human serum	Avg.	78	56	14¾	15½	16	17
		%	1.3	2.6	46	41	38	33 %
138	0.30 human ads.pl. 0.15 human serum	1	48	12	14	13	13½	14
		2	80	15	13	13½	13	13
	0.15 horse serum	Avg.	64	13½	13½	13¼	13¼	13½
		%	2	55	55	56	56	55 %
139	0.15 human ads.pl. 0.15 heated Hse.pl.	1	76	45	21	15	18	21
		2	69	38½	18	13	15	13½
	0.30 human serum	Avg.	72½	41¾	19½	14	16½	17¼
		%	1.5	5	25	50	36	32 %

The above incubation mixtures were aliquots from mixtures used in table 85, A, after incubating the adsorbed plasmas or sera mixtures separately at 37°C. for 2 hours, then similarly tested.

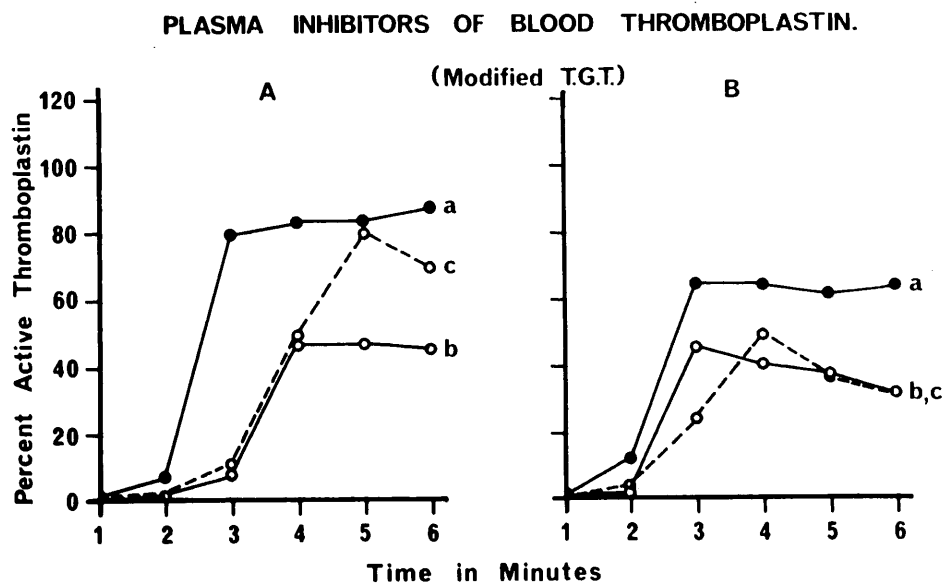


Figure 49. Horse plasma inhibitors of human intrinsic thromboplastin.

Figure A Before incubation (human serum constant)

Curve a : human ads.pl. alone (Mixture 132, table 85, A)

Curve b : human ads.pl. + horse ads.pl. (Mixture 133, table 85, A)

Curve c : human ads.pl. + heated Hse.pl. (Mixture 135, table 85, A)

Figure B After incubation at 37°C. for 2 hours (human serum constant)

Curve a : human ads.pl. alone (Mixture 136, table 85, B)

Curve b : human ads.pl. + horse ads.pl. (Mixture 137, table 85, B)

Curve c : human ads.pl. + heated Hse.pl. (Mixture 139, table 85, B)

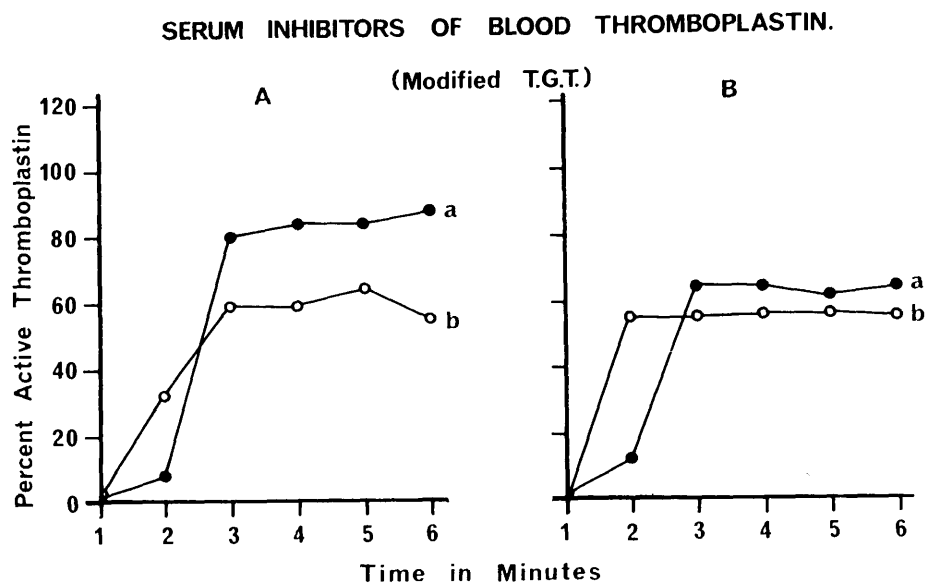


Figure 50. Horse serum inhibitors of human intrinsic thromboplastin.

Figure A Before incubation (human ads.pl. constant)

Curve a : human serum alone (Mixture 132, table 85, A)

Curve b : human serum + horse serum (Mixture 134, table 85, A)

Figure B After incubation at 37°C. for 2 hours (human ads.pl. constant)

Curve a : human serum alone (Mixture 136, table 85, B)

Curve b : human serum + horse serum (Mixture 138, table 85, B)

APPENDIX V, D

RESULTS OF EXPERIMENTS
ON RABBIT BLOOD

RESULTS OF EXPERIMENTS
ON RABBIT BLOOD

All the following data were obtained from experiments carried out on blood samples obtained from adult rabbits of the Californian strain, using pooled plasma or serum samples from at least three animals for each experiment to obviate as much as possible any individual variations from affecting the final levels of the various coagulation factors.

Blood samples were obtained fresh on the day of the test concerned as described in appendix II, 1, v.

Table 86. Fibrinogen estimation in rabbit plasma

(modified Greenberg method, appendix IV, 2, II)

	mg fibrinogen per 100 mls absolute plasma
Experiment 1	260
Experiment 2	190
Experiment 3	216
Experiment 4	195.5
Experiment 5	220
Experiment 6	237
Experiment 7	281.2
Average	228.1

Table 87. Thrombin-fibrinogen reaction of plasma

(method described in appendix IV, 8 and 9)

Clotting mixture (ml)	Expt.	Thrombin topical (P.D.& Co.) in units / ml					
		50	25	12.5	6.25	3.1	1.55
		Clotting times (seconds)					
0.2 Rabbit pl. 0.2 Saline 0.2 Thrombin	1	5½	7	9¼	13½	22½	45
	2	5	6	7	12	22	39
	3	4½	7	9½	14	26½	47
	Avg.	5	6.6	8.6	13.1	23.6	44
0.2 Human pl. 0.2 Saline 0.2 Thrombin	1	5½	7	10	16	26	51
	2	5	6½	9	15	27	48
	3	5	6½	10	16	25	48
	Avg.	5.1	6.6	9.6	15.6	26	49
0.2 Rabbit pl. 0.1 Pyrocatechol 0.1 Saline 0.2 Thrombin	1	3	4	5½	9	14	30
	2	3	4½	5	8½	14	26
	3	3½	4	5	9	14	24
	Avg.	3.1	4.1	5.1	8.8	14	26.6
0.2 Human pl. 0.1 Pyrocatechol 0.1 Saline 0.2 Thrombin	1	3	4½	5½	9	14	26
	2	3	4½	5½	9	15	26
	3	3	3½	5½	9	14	22
	Avg.	3	4.1	5.5	9	14.1	24.6

THROMBIN - FIBRINOGEN REACTION OF PLASMA.

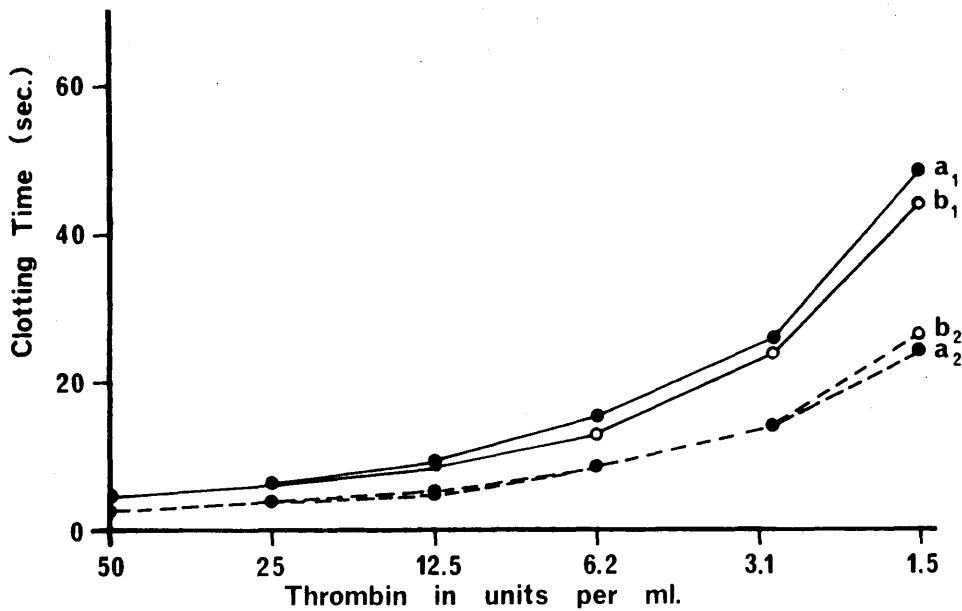


Figure 51. Thrombin-fibrinogen reaction of rabbit plasma.

Curve a₁ : human plasma + saline + thrombin.

Curve b₁ : rabbit plasma + saline + thrombin.

Curve a₂ : human plasma + pyrocatechol + saline + thrombin.

Curve b₂ : rabbit plasma + pyrocatechol + saline + thrombin.

(Illustration of results presented in table 87).

Table 88. Species specificity of the thrombin-fibrinogen reaction

(method described in appendix IV, 10)

	Thrombin solution (0.1 ml)		
	Human	Bovine 50 U/ml	Rabbit
	Clotting times (seconds)*		
0.4 ml human fibrinogen 0.1 ml saline	10½	5	14
0.4 ml rabbit fibrinogen 0.1 ml saline	11½	9	12½
0.4 ml human fibrinogen 0.1 ml pyrocatechol 1%	7		9
0.4 ml rabbit fibrinogen 0.1 ml pyrocatechol 1%	7½		7
0.4 ml human fibrinogen (1/2)	9		
0.4 ml rabbit fibrinogen(1/2)	9½		

* Average of duplicate estimations.

The human fibrinogen solution contained 340 mg/100 mls solution.

The rabbit fibrinogen solution contained 160 mg/100 mls solution.

In the last experiment the fibrinogen solutions were diluted in saline in the proportion of 1 : 1, thus the human fibrinogen solution (1/2) contained 170 mg fibrinogen /100 mls.

Table 89. Antithrombin assay in rabbit plasma

(method of Douglas and Biggs, 1953; appendix IV, 11, A)

Incubation mixture	Incubation time (minutes)						
	$\frac{1}{2}$	1	2	3	4	5	6
	Clotting times (seconds)						
0.5 ml rabbit plasma 0.5 ml thrombin	15	22	40	90	146	180+	180+
0.5 ml human plasma 0.5 ml thrombin	13 $\frac{1}{2}$	19	50	100	160	180+	180+
	Units of residual thrombin						
Rabbit plasma	10.2	6.5	3.2	1.2	0.6	0	0
Human plasma	11.6	7.8	2.4	1.0	0.6	0	0

The incubation times at which zero activity of thrombin was attained were read from curves a and b in figure 52.

Value for rabbit plasma = 6.5 (curve b at 0.1 thrombin units/ml).

Value for human plasma = 6.0 (curve a at 0.1 thrombin units/ml).

Rabbit plasma antithrombin = $6 \times 100 \div 6.5$

= 92.3 % of human plasma antithrombin.

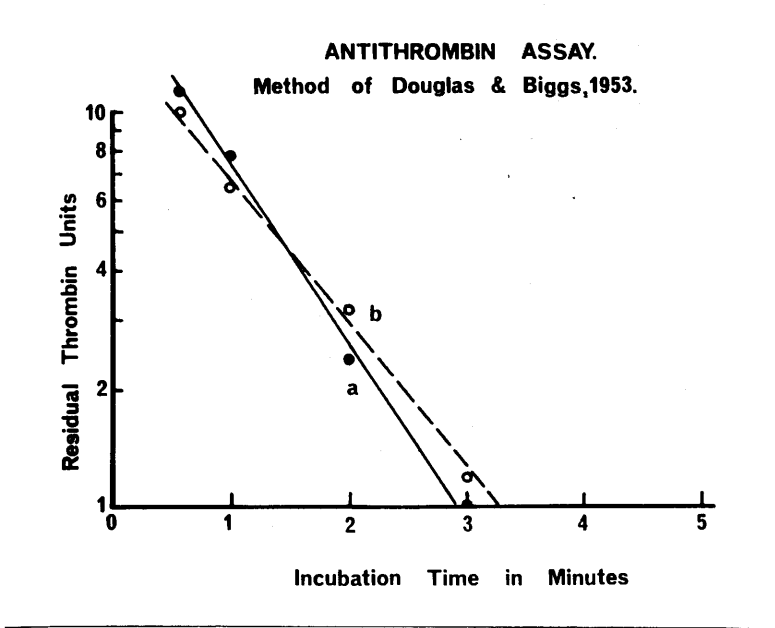


Figure 52. Antithrombin assay in rabbit plasma.

This figure was drawn from the results presented in table 89.

Curve a : human plasma.

Curve b : rabbit plasma.

Table 90. Antithrombin assay in rabbit plasma

(modified method of Astrup and Darling, 1942d; appendix IV, 11, B)

Mixture		Expt.	A	B	C	D	E	
Saline	ml.		0.3	0.25	0.20	0.15	0.10	0.0
Plasma (dil. 1/5)	ml.		0.0	0.05	0.10	0.15	0.20	0.3
Thrombin*	ml.		1	1	1	1	1	1
Clotting times of fibrinogen (seconds)	Human	1	16	19	22	25	28	32
		2	11	12½	13½	16	18	22½
		3	13	—	15	18	20	22
	Rabbit	1	16	19	21	25	26	33
		2	11	12½	15	17	20	22½
		3	13	—	16	17½	18½	22
T _o / T _n	Human	1	1	0.84	0.73	0.64	0.57	0.50
		2	1	0.88	0.81	0.68	0.61	0.48
		3	1	—	0.86	0.72	0.65	0.59
	Rabbit	1	1	0.84	0.76	0.64	0.61	0.48
		2	1	0.88	0.73	0.64	0.55	0.48
		3	1	—	0.81	0.74	0.70	0.59
Corrected T _o / T _n	Human	1	1	0.90	0.80	0.70	0.60	0.40
		2	1	0.90	0.81	0.71	0.62	0.42
		3	1	—	0.84	0.76	0.69	0.54
	Rabbit	1	1	0.90	0.80	0.70	0.60	0.40
		2	1	0.89	0.77	0.66	0.55	0.34
		3	1	—	0.84	0.76	0.69	0.54
Calculated units of antithrombin per ml plasma	Human	1	0	300	300	300	300	300
		2	0	330	313	319	313	319
		3	0	—	240	240	231	229.8
	Rabbit	1	0	300	300	300	300	300
		2	0	363	379	374	371	365
		3	0	—	240	240	231	229.8

Thrombin solution used for experiments 1 and 3 contained 30 units/ml., while that used for experiment 2 contained 33 units/ml.

	<u>Expt. 1</u>	<u>Expt. 2</u>	<u>Expt. 3</u>
Average value for human plasma (units/ml) =	300	315	235.2
Average value for rabbit plasma (units/ml) =	300	370	235.2
Rabbit plasma antithrombin activity in percentage of human plasma antithrombin =	100 %	117.4 %	100 %
Average rabbit plasma antithrombin =	105.8 % of human plasma antithrombin.		

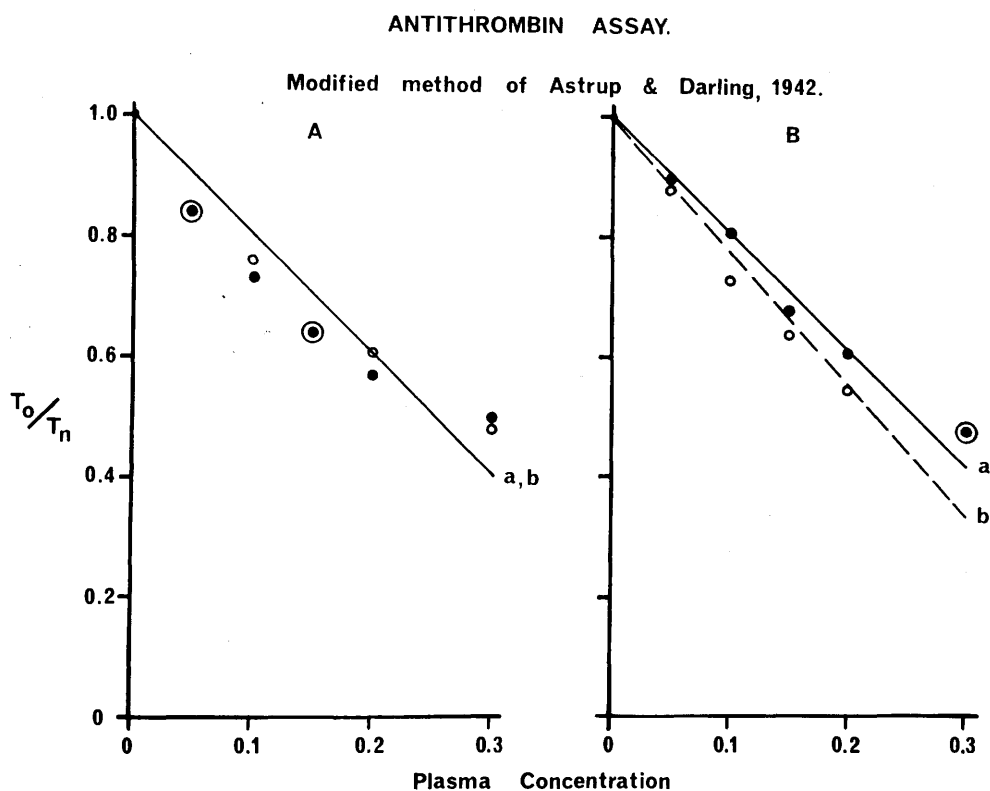


Figure 53. Antithrombin assay in rabbit plasma.

Figures A and B represent experiments 1 and 2 (table 90) respectively.

Curves a : human plasma.

Curves b : rabbit plasma.

The corrected values of T_0/T_n in table 90 were derived from these curves. For experiment 3, a similar curve was drawn to obtain the corrected values.

Table 91. Quick's one-stage prothrombin time test

Test plasma	Rabbit brain	Human brain
	Clotting times (seconds)	
Rabbit plasma	15	17
	16 (16.5)	17½(14.1)
	17	11
	18	11
Human plasma	22	17
	28 (28)	20 (17.7)
	30	17
	32	17
Rab.pl. + Hum.pl.	16½	17
	20 (19.6)	20 (16.1)
	20	13½
	22	14
Rab.pl. + saline	16½	19
	16 (18.1)	17 (17)
	19	16
	21	16
Hum.pl. + saline	26	20
	33 (32.5)	25 (21.2)
	37	20
	34	20

Figures between brackets are the mean for each group of four experiments carried out with different batches of brain thromboplastins and different plasma pools.

Table 92. Factor V - brain thromboplastin species specificity

(method described in appendix IV, 16)

Incubation mixture :-

0.3 ml brain extract, diluted 1/4 in saline, variable,

0.3 ml purified factor V (appendix III, 9), variable,

0.3 ml M/40 CaCl₂ solution.

Variables	Substrate	Incubation time (minutes)						
		$\frac{1}{2}$	1	2	3	4	5	6
		Clotting times (seconds)						
Human brain Human F V	Fresh hum.pl.	26	25	24	25	26	26 $\frac{1}{2}$	29
	Fresh rab.pl.	27	26 $\frac{1}{2}$	27	26 $\frac{1}{2}$	27	28	28
	F V-def.pl.	61	59	58	60	59	62	61 $\frac{1}{2}$
Human brain Rabbit F V	Fresh hum.pl.	26	26	24	24	24 $\frac{1}{2}$	25	26
	Fresh rab.pl.	28	28	27	27	30	30	28
	F V-def.pl.	30	30	30	30	31	32	32
Human brain Saline	Fresh hum.pl.	30	32	33	33	32	35	36
	Fresh rab.pl.	27	31	30	32	32	31	31
	F V-def.pl.	92	94	90	88	81	92	80
Rabbit brain Human F V	Fresh hum.pl.	36	36 $\frac{1}{2}$	34 $\frac{1}{2}$	36	37 $\frac{1}{2}$	39	38
	Fresh rab.pl.	27	26	24	24	23 $\frac{1}{2}$	22	21
	F V-def.pl.	79	77	77	78	77	78	75
Rabbit brain Rabbit F V	Fresh hum.pl.	33	32	31	33	33	33	31
	Fresh rab.pl.	28	26 $\frac{1}{2}$	25	24	23 $\frac{1}{2}$	19 $\frac{1}{2}$	19
	F V-def.pl.	40	40	41	41	41 $\frac{1}{2}$	44	45
Rabbit brain Saline	Fresh hum.pl.	40	40 $\frac{1}{2}$	43	40	39	40	40
	Fresh rab.pl.	32	31	28	27	26	25	24
	F V-def.pl.	98	97	93	95	93	95	97

Table 93. Rabbit plasma factor V assay

(method described in appendix IV, 14)

Plasma dilution	Factor V percentage	Clotting times (seconds)		
		Expt. 1	Expt. 2	Expt. 3
		Human	control	plasma
1/10	100 %	39	24	34
1/20	50 %	46	32	39
1/40	25 %	53	42	45
1/80	12.5%	60	48	54
1/160	6.2%	66	53	60
1/320	3.1%	69	60	68
1/640	1.5%	74	62	70
		Rabbit	test	plasma
1/160		25½	12	—
1/320		27	13½	—
1/500		—	—	25
1/800		38	22	—
1/1000		—	—	32
1/1600		41	29	—
1/2000		—	—	37½
1/3200		48	40	—
1/4000		—	—	43
1/6400		58	44	—
1/8000		—	—	51
1/12800		63	50	—
Calculated factor V conc.		9696 %	10120 %	11975 %

The average factor V content in rabbit plasma for the three experiments is 105.58 times that contained in the human control plasma.

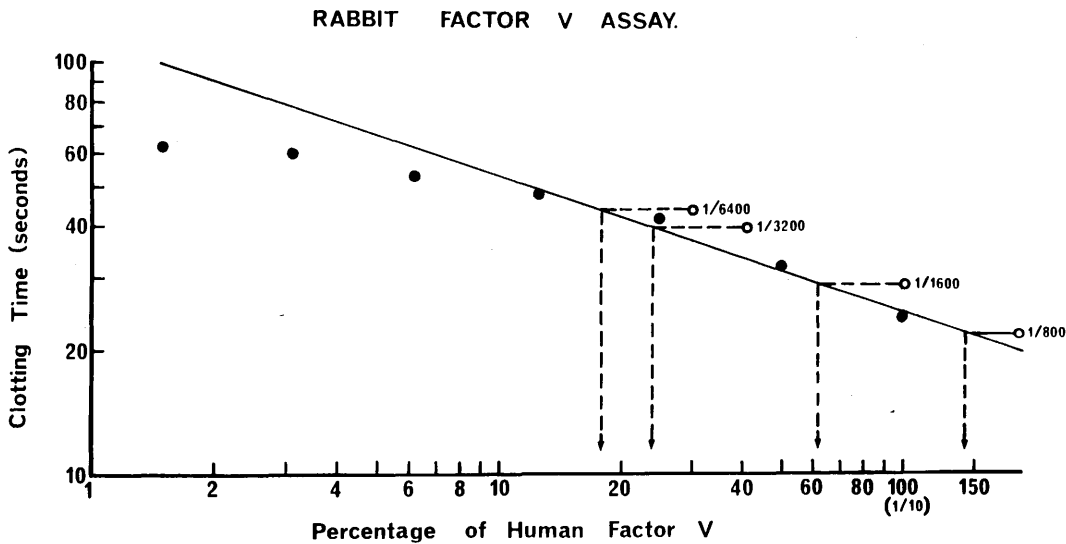


Figure 54. Rabbit plasma factor V assay.

The dilution curve drawn above for human normal plasma represents experiment 2, table 93.

Dilution 1/800 reads 142% thus rabbit plasma contains $\frac{142 \times 800}{10} = 11360 \%$

Dilution 1/1600 reads 62% thus rabbit plasma contains $\frac{62 \times 1600}{10} = 9920 \%$

Dilution 1/3200 reads 24% thus rabbit plasma contains $\frac{24 \times 3200}{10} = 7680 \%$

Dilution 1/6400 reads 18% thus rabbit plasma contains $\frac{18 \times 6400}{10} = 11520 \%$

The average of all readings for rabbit plasma factor V content therefore equals about 10120 % of the human control plasma factor V content, i.e. about 100 folds more.

Table 94. Factors VII and X - brain thromboplastin species specificity

(method described in appendix IV, 17)

Incubation mixture :-

0.3 ml brain extract, diluted 1/4 in saline, variable,

0.3 ml purified F VII and X solution, (appendix III, 10), variable,

0.3 ml M/40 CaCl₂ solution.

Variables	Substrate	Incubation time (minutes)						
		$\frac{1}{2}$	1	2	3	4	5	6
		Clotting times (seconds)						
Rabbit brain Rabbit VII & X	Fresh hum.pl.	39	32	28 $\frac{1}{2}$	29	26	24	25
	Fresh rab.pl.	19	15	13	13	13	13	14
	Phenindione pl.	32	27	25	24	23	24	24
Rabbit brain Human VII & X	Fresh hum.pl.	39	32	29	27 $\frac{1}{2}$	27 $\frac{1}{2}$	26	25
	Fresh rab.pl.	18	15	13	13	13	13	12
	Phenindione pl.	40	37	31	30	30	26	26
Rabbit brain Saline	Fresh hum.pl.	99	101	105	105	103	110	112
	Fresh rab.pl.	70	71	70	70	66	72	65
	Phenindione pl.	150	124	100	106	100	101	100
Human brain Rabbit VII & X	Fresh hum.pl.	13	12	12	12	11	11	12
	Fresh rab.pl.	6	5	5	5	5	5	5
	Phenindione pl.	15	14	14	14	14	13	13
Human brain Human VII & X	Fresh hum.pl.	14	12	13	12 $\frac{1}{2}$	12	12	12
	Fresh rab.pl.	6	5	4 $\frac{1}{2}$	4 $\frac{1}{2}$	4 $\frac{1}{2}$	5	5
	Phenindione pl.	16	15	15	15	15	15	15
Human brain Saline	Fresh hum.pl.	32 $\frac{1}{2}$	33	34	33	29	33	34
	Fresh rab.pl.	24	21	22	21 $\frac{1}{2}$	21 $\frac{1}{2}$	22 $\frac{1}{2}$	22
	Phenindione pl.	45	43	44	45	44	43	50

Table 95. Rabbit plasma factor VII assay

Method A : by correction of phenindione plasma, (appendix IV, 15, A).

Method B : by correction of Seitz-filtered bovine plasma, (method of Koller et al, 1951; appendix IV, 15, B).

Human brain thromboplastin was used in all experiments.

Plasma dilution	Factor VII percentage	Method A	Method B		
		Expt.1	Expt.2	Expt.3	Expt.4
Clotting times (seconds)					
Human control plasma					
1/10	100 %	25	42	39	40
1/20	50 %	30	56½	52	53
1/40	25 %	32	87	81	90
1/80	12.5%	35	132	124	140
1/160	6.2%	35½	195	200	215
1/320	3.1%	36	311	288	358
1/640	1.5%	36½	440	412	520
	0 %	38½	-	742	765
Rabbit test plasma					
1/10		20	41	38½	37
1/20		25	66	56	66
1/40		25	109	94	103
1/80		26	212	179	200
Calculated F VII & X conc. 820 %			86.6 %	94 %	87 %

The average of the last three experiments gives a value for rabbit factors VII and X activities in plasma of 89.2 % of the human control plasma content. It should be noted that the plasma pool tested in experiment 3 was the same one tested by method A in experiment 1, in which latter case it gave a very high value of factor VII activity, most probably due to the excess of factor V present in the test plasma relative to that in the substrate; while in method B the substrate bovine plasma is rich in factor V.

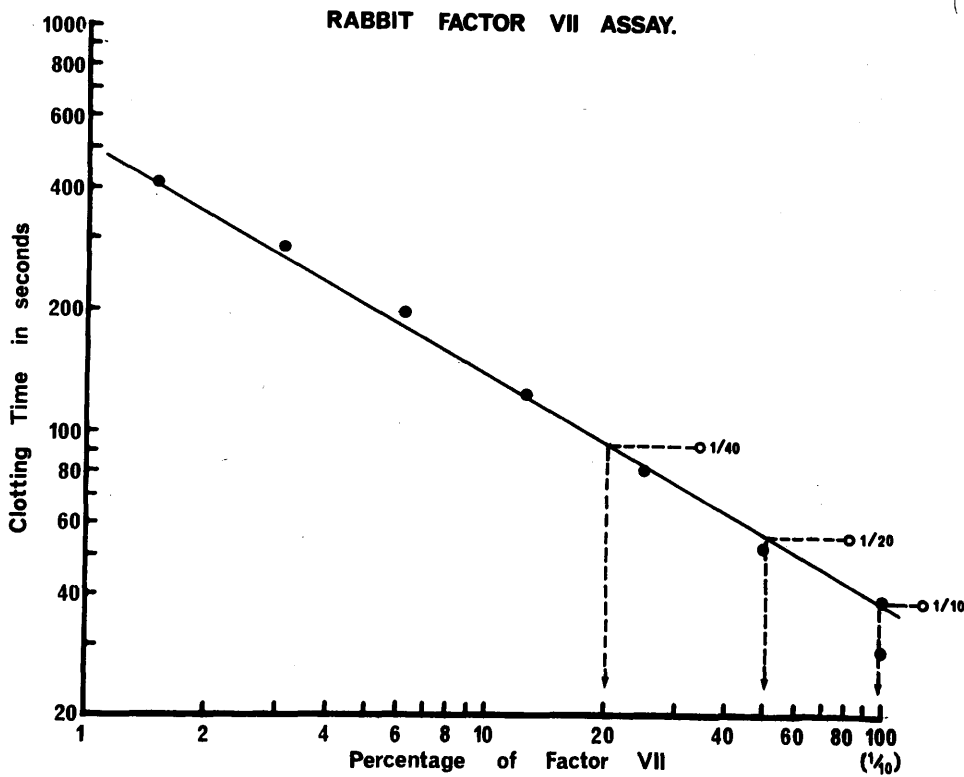


Figure 55. Rabbit plasma factors VII and X activity.

The above dilution curve of the normal human plasma represents experiment 3, table 95.

Dilution 1/10 reads 99 % thus rabbit plasma contains $\frac{99 \times 10}{10} = 99 \%$

Dilution 1/20 reads 51 % thus rabbit plasma contains $\frac{51 \times 20}{10} = 102 \%$

Dilution 1/40 reads 20.2% thus rabbit plasma contains $\frac{20.2 \times 40}{10} = 81 \%$

The average of the three dilutions = 94 % of the human plasma.

Table 96. Rabbit plasma factor X assay

(method of Bachmann, Duckert, and Koller, 1958; appendix IV, 18)

Plasma dilution	Factor X percentage	Clotting times (seconds)		
		Expt. 1	Expt. 2	Expt. 3
		Human control plasma		
1/10	100 %	20	23	22
1/20	50 %	26	29	29
1/40	25 %	32½	39	38
1/80	12.5%	40	49	48
1/160	6.2%	51	54½	54
1/320	3.1%	56	66	66
1/640	1.5%	73	81½	85
1/1280	0.75%	82	91½	89
	0 %	101	106	118
		Rabbit test plasma		
1/10		14	14	15
1/20		16	19	18
1/40		20	24	22
1/80		26	31	27
1/160		32	38½	32
1/320		41	--	41
Calculated factor X conc.		440.4%	388 %	479.2%

The average of the three experiments for rabbit plasma factor X content is 436.5 % of the human control plasma factor X content.

Table 97. Rabbit plasma prothrombin assay

(Two-stage area method of Biggs and Douglas, 1953a; appendix IV, 12, C)

Incubation mixture	Expt.	Incubation time (minutes)							Weight of area under curve & its percentage	
		$\frac{1}{2}$	$\frac{3}{4}$	1	2	3	4	5		
		Clotting times (seconds)								
Hum.pl. + Hum.Br.	1A	12	15	15	26	65	129	180+		
Rab.pl. + Hum.Br.	1B	12 $\frac{1}{2}$	16	20	64	108	170	180+		
Hum.pl. + Rab.Br.	1C	66	15	14	44	150	180+			
Rab.pl. + Rab.Br.	1D	—	17	40	84	131	180+			
Hum.pl. + Hum.Br.	2A	15	16	21	43	143	180+			
Rab.pl. + Hum.Br.	2B	16	18	29	96	180+	180+			
Hum.pl. + Rab.Br.	2C	45	17	18	36	112	180+			
Rab.pl. + Rab.Br.	2D	—	15	24	92	124	173 180+			
Hum.pl. + Hum.Br.	3A	12	16	18	70	180+				
Rab.pl. + Hum.Br.	3B	13 $\frac{1}{2}$	18	27	104	180+				
Hum.pl. + Rab.Br.	3C	55	—	10	20	80	180+			
Rab.pl. + Rab.Br.	3D	—	11	16	125	180+	180+			
		Thrombin units / ml.							mg	%
	1A	13.5	10.2	10.2	5.2	1.8	0.8	0	292.1	
	1B	13	9.5	7.2	1.8	1	0.5	0	196.1	67.1 %
	1C	1.7	10.2	11	2.8	0.6	0.0		188.3	
	1D	—	8.8	3.2	1.3	0.7	0.0		126.9	67.3 %
	2A	10.2	9.5	6.8	2.9	0.7	0		201.0	
	2B	9.5	8.2	4.6	1.1	0			134.6	66.9 %
	2C	2.7	8.9	8.2	3.6	0.9	0		185.6	
	2D	—	10.2	5.8	1.2	0.8	0.5	0	157.8	85.0 %
	3A	13.5	9.5	8.2	1.6	0			198.7	
	3B	11.5	8.2	5	1	0			136.4	68.6 %
	3C	2.2	—	16.5	7.2	1.4	0		297.8	
	3D	—	15	9.5	0.8	0.0	0		189.7	63.7 %

Percentage of rabbit plasma prothrombin is related to the prothrombin content of the human control plasma tested with the same species of brain extract. With human brain the average rabbit plasma prothrombin equals 67.5 % of the human, while with rabbit brain it averages 72 % of the human plasma prothrombin content.

Experiment 1 is illustrated in figure 56.

MEASUREMENT OF PROTHROMBIN.

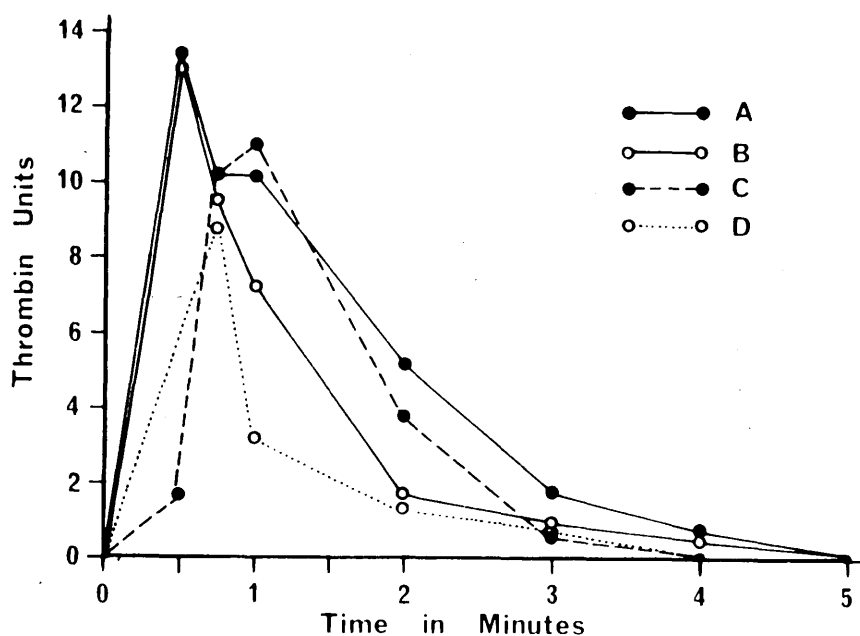


Figure 56. Measurement of rabbit plasma prothrombin.

Curve A : obtained for human plasma using human brain. Weight of area under curve is 292.1 mg which is designated the 100 % value.

Curve B : obtained for rabbit plasma using human brain. Weight of area enclosed is 196.1 mg which is equivalent to 67.1 % of human plasma prothrombin activity.

Curve C : obtained for human plasma using rabbit brain. Weight of enclosed area is 188.3 mg which is equivalent to 64.4 % of human plasma prothrombin when estimated with human brain.

Curve D : obtained for rabbit plasma using rabbit brain. Weight of enclosed area is 126.9 mg which gives a value of 67.3 % of the human plasma prothrombin when the latter was tested with rabbit brain.

(All the above data are from the results of experiment 1, table 97)

Table 98. Rabbit plasma prothrombin assay

(globulin fraction method of Douglas and Biggs, 1953; appendix IV, 12, B)

	Experiment 1		Experiment 2			
	Human brain		Human brain		Rabbit brain	
	Hum.pl.	Rab.pl.	Hum.pl.	Rab.pl.	Hum.pl.	Rab.pl.
Fibrinogen clotting times (seconds)*	20	63	24½	69	25½	71
Thrombin units/ml.	7.2	1.9	5.7	1.75	5.4	1.6
Prothrombin conc.	100 %	26.2 %	100 %	30.7 %	100 %	29.6 %

* Average of duplicate estimations.

With human brain as the thromboplastic agent, rabbit plasma was found to have an average prothrombin concentration of 28.4 % of human plasma prothrombin. With rabbit brain, it also contained 29.6 % of the human plasma prothrombin content.

It is noted that rabbit plasma used in experiment 2, having 30.7 % of the human plasma prothrombin, was also tested on the same day by the two-stage area method (experiment 2, table 97) using the same human brain extract. By the latter method it had 66.9 % of the human plasma prothrombin activity.

With rabbit brain extract, this method yielded a value of 29.6 % of the human, while the two-stage area method gave a value of 85 % of prothrombin for the same plasma pool against the same control human plasma.

Table 99. Rabbit plasma prothrombin consumption index

(method of Biggs and Macfarlane, 1957; appendix IV, 13, A)

	Incubation time (seconds)					
	45	90	45	90	45	90
	Expt. 1		Expt. 2		Expt. 3	
Rabbit plasma time(sec.)	13	26	17	37	18	37
Rabbit serum time (sec.)	52	90	72	180+	63	157
P.C.I.	25%	28.8%	23.6%	20.5%	28.5%	23.5%
Average P.C.I.	26.9 %		22 %		26 %	

The average P.C.I. of the three experiments = 24.9 %

Table 100. Rabbit blood platelets' count

(method described in appendix IV, 1, A)

	Total count/c.mm. whole blood*
Experiment 1	274,000
Experiment 2	422,000
Experiment 3	258,000
Experiment 4	360,000
Experiment 5	266,000
Experiment 6	335,000
Experiment 7	248,000
Experiment 8	225,000
Average	311,000

* Mean of duplicate counts.

Table 101. Rabbit whole blood clotting time

(method of Lee and White, 1913; appendix IV, 3)

	Clotting times*	
	Ordinary glass	Siliconed glass
Experiment 1	6' 30"	12' --
Experiment 2	7' 10"	13' 40"
Experiment 3	3' 45"	7' 58"
Experiment 4	6' 15"	7' 30"
Experiment 5	5' 52"	12' 2"
Experiment 6	6' 10"	13' 20"
Experiment 7	7' --	13' 29"
Average	6' 6"	12' 51"

* Mean of two tubes' readings.

Table 102. Recalcification time of plasma

(method described in appendix IV, 5)

	Clotting times (seconds)	
	Human plasma	Rabbit plasma
Experiment 1	148	106
Experiment 2	154	120
Experiment 3	143	116
Average	148	114

Table 103. Rabbit plasma Hageman factor assay

(method described in appendix IV, 24, II)

Plasma dilution in Hag.F.-def.pl.	Concentration of Hageman factor	Clotting times (seconds)	
		Expt. 1	Expt. 2
Human control plasma			
1/2	250 %	106	-
1/5	100 %	127	142
1/10	50 %	165	-
1/15	33.3%	-	182
1/20	25 %	191	-
1/40	12.5%	236	-
1/45	11.1%	-	257
1/80	6.2%	300	-
1/135	3.7%	-	364
1/405	1.2%	-	540
	0 %	1200+	1200+
Rabbit test plasma			
1/5		65	-
1/10		80	-
1/15		-	87
1/20		98	-
1/45		-	132
Calculated Hageman factor conc.		910 %	1140 %

The average of the two experiments equals 1025 %, i.e. rabbit plasma contains about tenfolds more of Hageman factor activity as compared to the human control plasma.

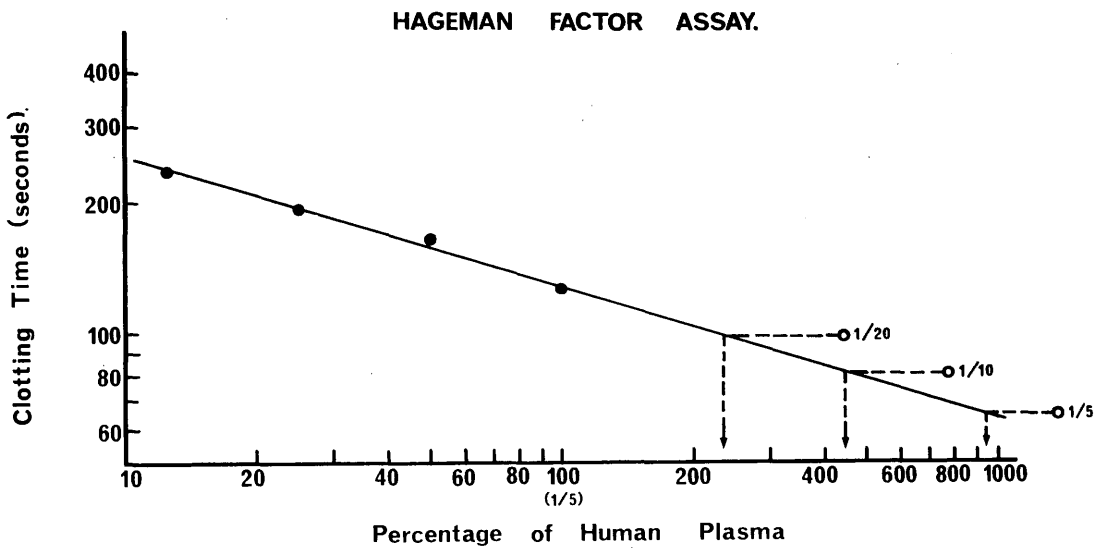


Figure 57. Hageman factor assay in rabbit plasma.

The above human plasma dilution curve was obtained from the results of experiment 1, table 103.

Dilution 1/5 reads 950% thus rabbit pl. contains $\frac{950 \times 5}{5} = 950 \%$

Dilution 1/10 reads 440% thus rabbit pl. contains $\frac{440 \times 10}{5} = 880 \%$

Dilution 1/20 reads 225% thus rabbit pl. contains $\frac{225 \times 20}{5} = 900 \%$

The average Hageman factor concentration in this plasma pool equals 910 per cent of human control plasma Hageman factor activity.

Table 104. Thromboplastin generation test

(method of Biggs and Douglas, 1953b; appendix IV, 20)

Incubation mixture : 0.3 ml adsorbed plasma, diluted 1/5, variable,
 0.3 ml serum, diluted 1/10, variable,
 0.3 ml human platelets,
 0.3 ml M/40 CaCl₂ solution.

Substrate : human high-spun plasma.

No.	Incubation mixture	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
140	Human plasma Human serum	1	52	15	12	11	10½	11
		2	65	15	10	9	10	10½
		3	60	12	10	9½	9½	9½
		Avg.	59	14	10.6	9.8	10	10.3
		%	2.4	50	90	106	100	94 %
141	Human plasma Rabbit serum	1	45	14	11	11	11	11
		2	68	22	17½	13	14	15
		3	63	14	12	12	11½	11½
		Avg.	58.6	16.6	13.5	12	12.1	12.5
		%	2.4	35	55	70	68	65 %
142	Rabbit plasma Rabbit serum	1	10	9½	9	8½	8½	8½
		2	18	11½	11	10½	11	10
		3	15	11	10	9	9	9
		Avg.	14.3	10.6	10	9.3	9.5	9.1
		%	48	90	100	117	112	122 %
143	Rabbit plasma Human serum	1	17	10	10	10	10	9½
		2	9	8	8	9	9	8½
		3	11	9	8½	8	8½	8½
		Avg.	12.3	9	9.5	9	9.1	8.8
		%	66	125	112	125	122	132 %

Percentage active thromboplastin values were read from the human thromboplastin dilution curve A, figure 2.

Table 105. Thromboplastin generation test

(method of Biggs and Douglas, 1953b; appendix IV, 20)

Incubation mixture : 0.3 ml adsorbed plasma, diluted 1/5, variable,
 0.3 ml serum, diluted 1/10, variable,
 0.3 ml rabbit platelets,
 0.3 ml M/40 CaCl₂ solution.

Substrate : human high-spun plasma.

No.	Incubation mixture	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
144	Human ads.pl. Human serum	1	64	30	14	11½	12	11½
		2	117	90	15	11½	12	12
		3	60	25	11	10½	10½	10
		Avg.	80.3	48.3	13.3	11.1	11.5	11.1
	%	1.1	3.6	56	82	76	82 %	
145	Human ads.pl. Rabbit serum	1	12	10	10	9	10	9
		2	70	40	20	16	14	13
		3	44	15	15	12	12	11
		Avg.	42	31.6	15	12.3	12	11
	%	4.9	9	43.5	66	70	84 %	
146	Rabbit ads.pl. Rabbit serum	1	8	8½	7	7½	7	7
		2	19	8½	9	9	9	9
		3	14	9	8	8	8	8
		Avg.	13.6	8.6	8	8.1	8	8
	%	54	138	160	158	160	160 %	
147	Rabbit ads.pl. Human serum	1	58	15	11½	11	10	10
		2	60	7½	7½	6½	6½	7½
		3	41	9½	7½	6½	7	7
		Avg.	53	10.6	8.7	8	7.8	8.1
	%	3	90	135	160	170	158 %	

Percentage active thromboplastin values were read from the human thromboplastin dilution curve A, figure 2. All the above experiments were conducted on the same batches of rabbit and human reagents as those used for experiments cited in table 104, and are therefore directly comparable with each other as regards the effect of using rabbit platelets' suspension instead of the human preparation.

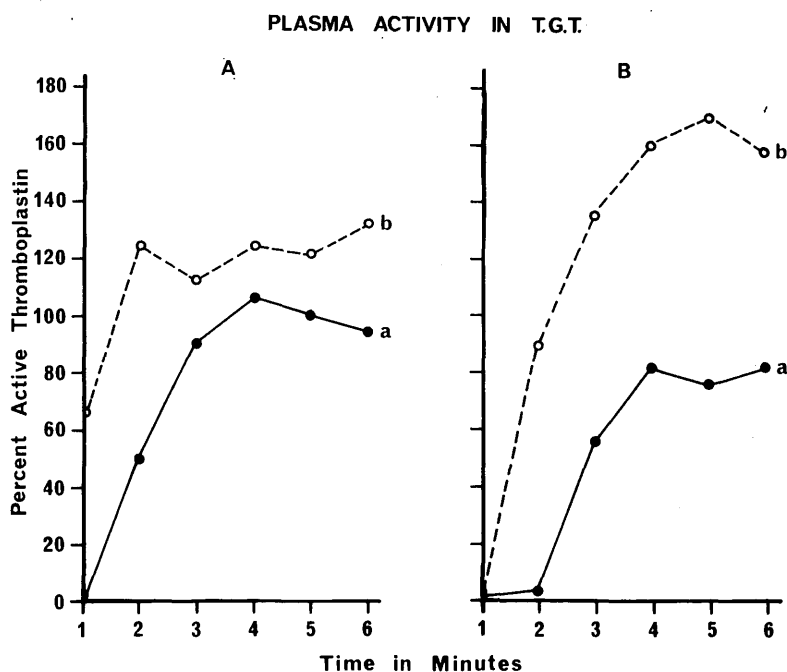


Figure 58. Rabbit plasma activity in the T.G.T.

Figure A (using human platelets)

Curve a : human ads.pl. + human serum (Mixture 140, table 104)

Curve b : rabbit ads.pl.+ human serum (Mixture 143, table 104)

Figure B (using rabbit platelets)

Curve a : human ads.pl. + human serum (Mixture 144, table 105)

Curve b : rabbit ads.pl. + human serum (Mixture 147, table 105)

Table 106. A.H.G. assay in rabbit plasma

(method of Pitney, 1956; appendix IV, 21)

Plasma dilution	Concentration of A.H.G.	Clotting times (seconds)		
		Expt. 1	Expt. 2	Expt. 3
		Human	control	plasma
1/20	100 %	20	20	19½
1/40	50 %	25½	25	24
1/80	25 %	33	33	33½
1/160	12.5 %	39	45	37
1/320	6.2 %	43	52	43
1/640	3.1 %	49	63	53
1/1280	1.5 %	50	67	63
		Rabbit	test	plasma
1/20		15½	13	12½
1/100		27	26	22
1/200		32	37	33
1/400		36	43	36
1/800		--	--	41
Calculated A.H.G. conc.		295 %	246.6 %	324 %

The average A.H.G. concentration in the three rabbit plasma pools equals 288.5 % of the human control plasma content, with a range from 246.6 to 324 per cent.

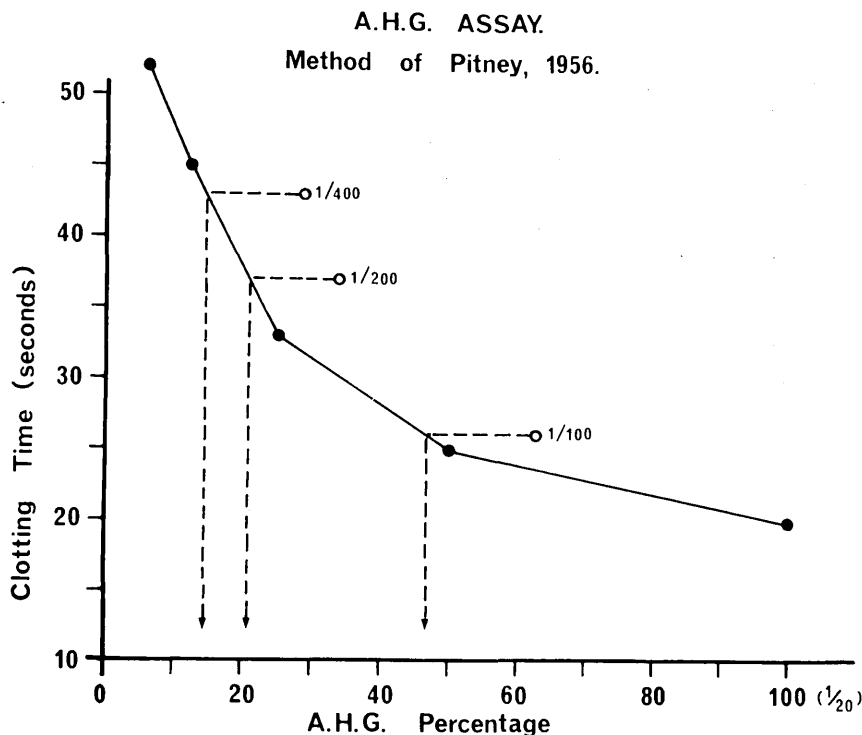


Figure 59. A.H.G. assay in rabbit plasma.

A diagrammatic presentation of experiment 2, table 106.

Dilution 1/100 reads 48.0% thus rabbit pl. contains $\frac{48 \times 100}{20} = 240 \%$

Dilution 1/200 reads 21.0% thus rabbit pl. contains $\frac{21 \times 200}{20} = 210 \%$

Dilution 1/400 reads 14.5% thus rabbit pl. contains $\frac{14.5 \times 400}{20} = 290 \%$

The average A.H.G. for this plasma pool therefore equals 246.6 %
of the human control plasma A.H.G. content.

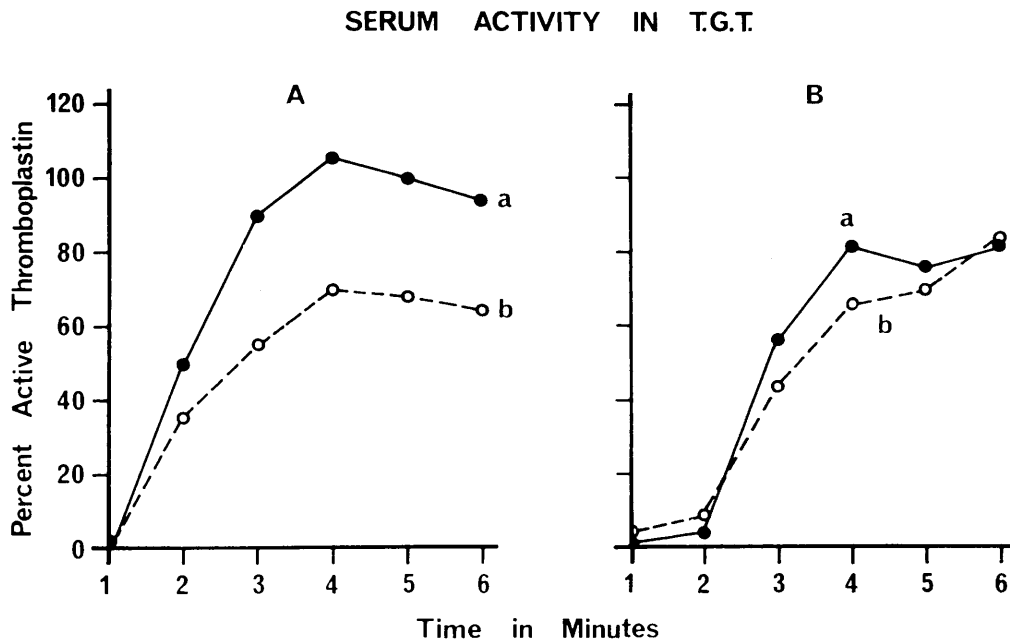


Figure 60. Rabbit serum activity in the T.G.T.

Figure A (using human platelets)

Curve a : hum.ads.pl. + human serum (Mixture 140, table 104)

Curve b : hum.ads.pl. + rabbit serum (Mixture 141, table 104)

Figure B (using rabbit platelets)

Curve a : hum.ads.pl. + human serum (Mixture 144, table 105)

Curve b : hum.ads.pl. + rabbit serum (Mixture 145, table 105)

Table 107. Christmas factor assay in rabbit serum

(based on method of Pitney, 1956; appendix IV, 22)

Serum dilution	X-mas factor concentration	Clotting times (seconds)		
		Expt. 1	Expt. 2	Expt. 3
		Human control serum		
1/20	100 %	10	15	15
1/40	50 %	11	20	18½
1/80	25 %	13	25	24
1/160	12.5%	15	34	28½
1/320	6.2%	18½	44	39
1/640	3.1%	24½	56	49
1/1280	1.5%	27	60	62
	0 %	32	65	74
		Rabbit test serum		
1/10		—	20	16
1/20		13½	26	18½
1/40		15	42	25
1/80		17	51	39½
Calculated X-mas factor		27.5 %	20.4 %	40 %

The average for the three rabbit serum pools equals 29.3 % of the human control serum Christmas factor activity.

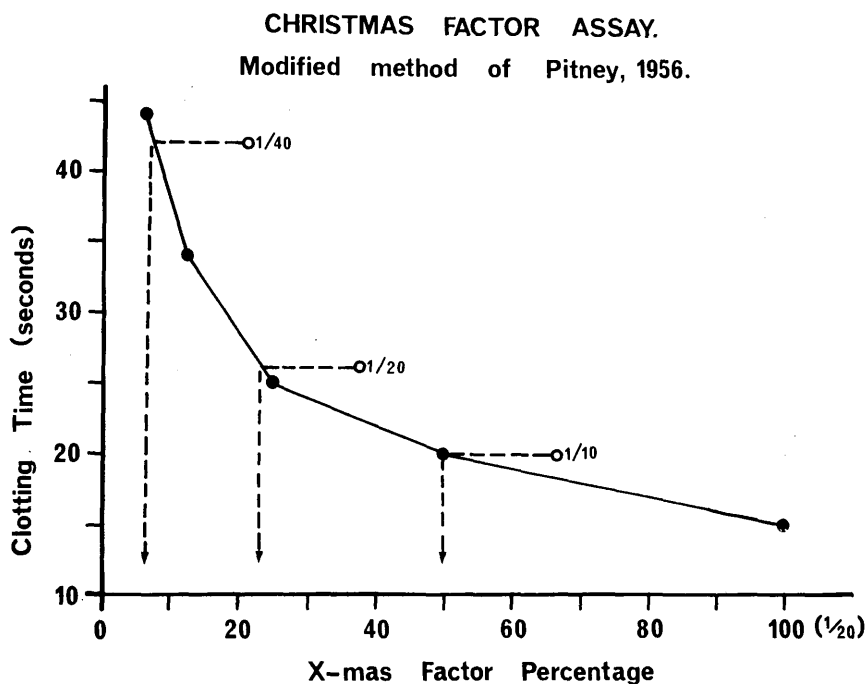


Figure 61. Christmas factor assay in rabbit serum.

Illustration of experiment 2, table 107, showing the human serum dilution curve by correction of Christmas disease serum.

Dilution 1/10 reads 50 % thus rabbit serum contains $\frac{50 \times 10}{20} = 25 \%$

Dilution 1/20 reads $23\frac{1}{2}\%$ thus rabbit serum contains $\frac{23\frac{1}{2} \times 20}{20} = 23\frac{1}{2}\%$

Dilution 1/40 reads $7\frac{1}{2}\%$ thus rabbit serum contains $\frac{7\frac{1}{2} \times 40}{20} = 15 \%$

Dilution 1/80 reads $4\frac{1}{2}\%$ thus rabbit serum contains $\frac{4\frac{1}{2} \times 80}{20} = 18 \%$

The average of Christmas factor content in this rabbit serum pool, therefore equals 20.4 % of the human control serum activity.

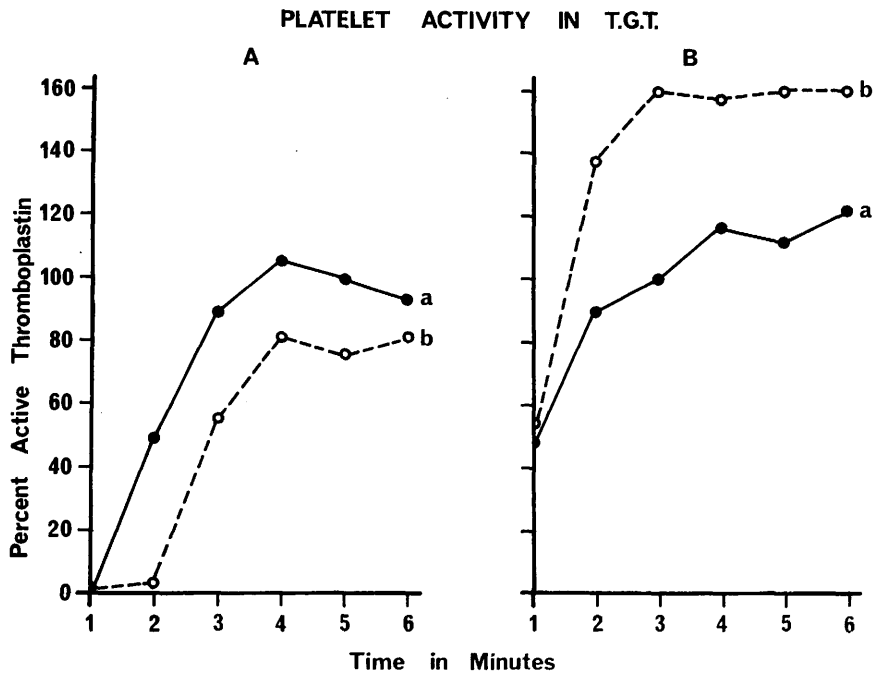


Figure 62. Thromboplastic activity of rabbit platelets in the T.G.T.

Figure A (human ads.pl. and serum are constants)

Curve a : using human platelets (Mixture 140, table 104)

Curve b : using rabbit platelets (Mixture 144, table 105)

Figure B (rabbit ads.pl. and serum are constants)

Curve a : using human platelets (Mixture 142, table 104)

Curve b : using rabbit platelets (Mixture 146, table 105)

Table 108. Clot retraction of rabbit blood

(method of Macfarlane, 1938; appendix IV, 4)

	Hematocrit value of sequestrene blood	Percentage of clot retraction in one hour*
Expt. 1	56 %	46 %
Expt. 2	49 %	58 %
Expt. 3	50 %	56 %
Expt. 4	52 %	56 %
Expt. 5	54 %	56 %
Expt. 6	55 %	56 %
Average		54.6 %

* Average of two tubes' readings.

Table 109. Rabbit plasma substrate reactivity in the T.G.T.

Incubation mixture : 0.3 ml adsorbed plasma, diluted 1/5, variable,
 0.3 ml serum, diluted 1/10, variable,
 0.3 ml human platelets,
 0.3 ml M/40 CaCl₂ solution.

Substrate : rabbit high-spun plasma.

No.	Incubation mixture	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
148	Human ads.pl. Human serum	1	35	12	10	10	10	10
		2	43	11	9	9	9½	9
		3	42	11	9	9	9	9
		Avg.	40	11.3	9.3	9.3	9.5	9.3
		%	5.4	79	117	117	112	117 %
149	Human ads.pl. Rabbit serum	1	30	11	10½	10	10½	10½
		2	42	23	12	11½	11½	11
		3	39	11½	11	11	11	11
		Avg.	37	15.1	11.1	10.8	11	10.8
		%	6.4	42	82	87	84	87 %
150	Rabbit ads.pl. Rabbit serum	1	20	10	9½	9	9	9½
		2	22	10	10	10	9½	10
		3	25	10	9	9	9½	9
		Avg.	22.3	10	9.5	9.3	9.3	9.5
		%	19	100	112	117	117	112 %
151	Rabbit ads.pl. Human serum	1	12	8½	8½	8½	8½	8
		2	9	7½	8	8	9	8½
		3	10	9	8	8	8	8
		Avg.	10.3	8.3	8.1	8.1	8.5	8.1
		%	94	150	158	158	140	158 %

Incubation mixtures 148, 149, 150, and 151 were made with the same solutions used for mixtures 140, 141, 142, and 143 respectively, and are therefore directly comparable with each other as regards the effect of using rabbit high-spun plasma as substrate instead of the human high-spun plasma.

Table 110. Rabbit plasma substrate reactivity in the T.G.T.

Incubation mixture : 0.3 ml adsorbed plasma, diluted 1/5, variable,
 0.3 ml serum, diluted 1/10, variable,
 0.3 ml rabbit platelets,
 0.3 ml M/40 CaCl₂ solution.

Substrate : rabbit high-spun plasma.

No.	Incubation mixture	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
152	Human ads.pl. Human serum	1	38	19	11	10	9	9
		2	54	33	12½	10	9	8
		3	32	21	9½	9	9	9
		Avg. %	41.3 5	24.3 15.7	11 84	9.6 110	9 125	8.6 138 %
153	Human ads.pl. Rabbit serum	1	8½	7½	6½	7	7	6½
		2	42	25	12	9	9	9
		3	25	13	10½	10	9	9
		Avg. %	25.1 14.4	15.1 42	9.6 110	8.6 138	8.3 150	8.1 158 %
154	Rabbit ads.pl. Rabbit serum	1	6½	6	6¼	6	6	6
		2	12½	5½	6	6	6	6
		3	13	7½	6	6	6½	6
		Avg. %	10.6 90	6.3 270	6 300	6 300	6.1 290	6 300 %
155	Rabbit ads.pl. Human serum	1	32½	10	9	7	7	8
		2	22	6	6½	6½	7	7
		3	30	6½	6½	6½	6½	6½
		Avg. %	28.1 11.2	7.5 185	7.3 200	6.6 245	6.8 230	7.1 210 %

Incubation mixtures 152, 153, 154, and 155 were made with the same reagents used for mixtures 144, 145, 146, and 147 respectively, with which they are therefore directly comparable as regards the effect of substituting rabbit high-spun plasma substrate instead of the human plasma substrate.

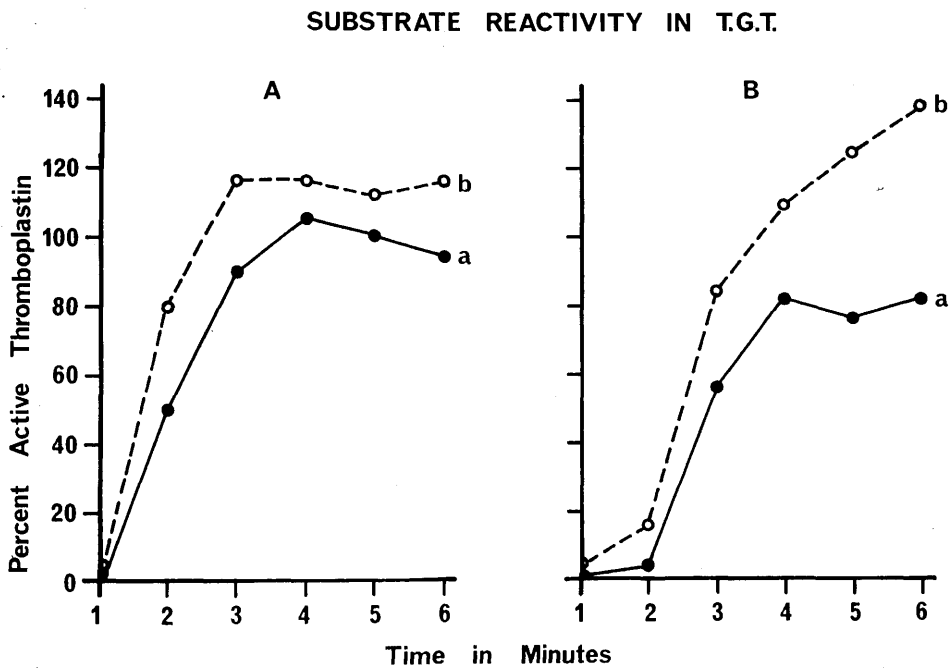


Figure 63. Rabbit plasma substrate reactivity in the T.G.T.

Figure A (human ads.pl., serum, and platelets are constants)

Curve a : on human H.S.plasma substrate (Mixture 140, table 104)

Curve b : on rabbit H.S.plasma substrate (Mixture 148, table 109)

Figure B (human ads.pl. and serum, with rabbit platelets are constants)

Curve a : on human H.S.plasma substrate (Mixture 144, table 105)

Curve b : on rabbit H.S.plasma substrate (Mixture 152, table 110)

SUBSTRATE REACTIVITY IN T.G.T.

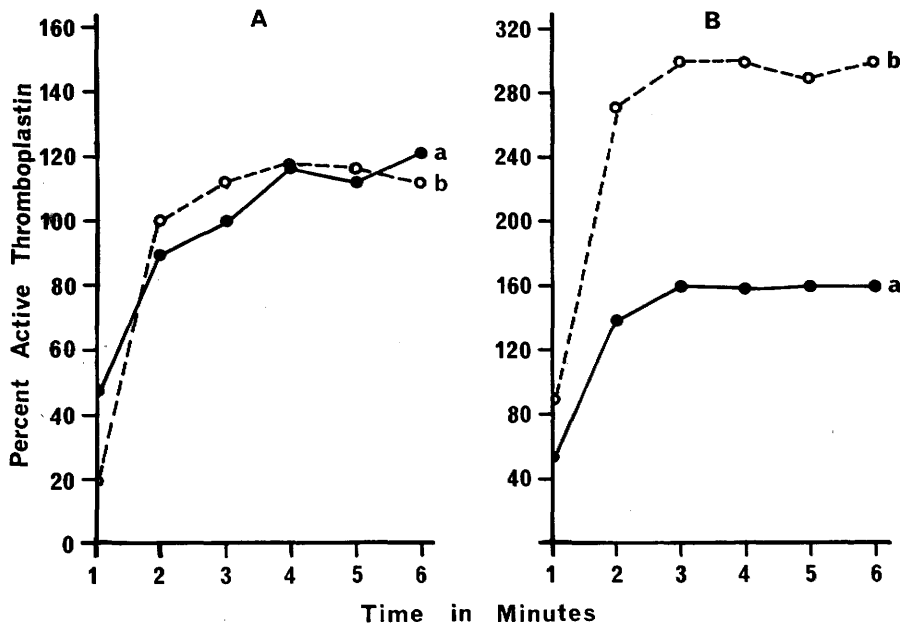


Figure 64. Rabbit plasma substrate reactivity in the T.G.T.

Figure A (rabbit ads.pl. and serum, with human platelets are constant)

Curve a : on human H.S.plasma substrate (Mixture 142, table 104)

Curve b : on rabbit H.S.plasma substrate (Mixture 150, table 109)

Figure B (rabbit ads.pl., serum, and platelets are constant)

Curve a : on human H.S.plasma substrate (Mixture 146, table 105)

Curve b : on rabbit H.S.plasma substrate (Mixture 154, table 110)

Curve b, figure B, represents a completely homologous rabbit thromboplastin generation system.

Table 111. Investigation of inhibitors against human intrinsic thromboplastin in rabbit plasma and serum in the T.G.T.

(method described in appendix IV, 23)

Incubation mixture : 0.3 ml adsorbed plasma, diluted 1/5, variable,
 0.3 ml serum, diluted 1/10, variable,
 0.3 ml human platelets,
 0.3 ml M/40 CaCl₂ solution.

Substrate : human high-spun plasma.

No.	Incubation mixture (ml)	Expt.	Incubation time (minutes)					
			1	2	3	4	5	6
			Clotting times (seconds)					
A - BEFORE INCUBATION								
156	0.30 Human ads.pl. 0.30 Human serum	1	58	26	12	10	10	10
		2	60	25	15	11	11	10
		Avg.	59	25½	13½	10½	10½	10
		%	2.4	14	55	92	92	100 %
157	0.15 Human ads.pl. 0.15 Rabbit ads.pl. 0.30 Human serum	1	42	10	10	9	9	9
		2	32	12	10	10	10	9½
		Avg.	37	11	10	9½	9½	9¼
		%	6.4	84	100	112	112	119 %
158	0.30 Human ads.pl. 0.15 Human serum 0.15 Rabbit serum	1	26	12½	12	12	12	13
		2	23	12	11	11	11	11
		Avg.	24½	12¼	11½	11½	11½	12
		%	15.5	67	76	76	76	70 %
B - AFTER INCUBATION AT 37°C. FOR 2 HOURS								
159	0.30 Human ads.pl. 0.30 Human serum	1	67	35	12½	11½	11½	12½
		2	87	36	14	11½	11½	11½
		Avg.	77	35½	13¼	11½	11½	12
		%	1.3	7	56	76	76	70 %
160	0.15 Human ads.pl. 0.15 Rabbit ads.pl. 0.30 Human serum	1	63	14	11	11	11½	11
		2	46	20	12	11	11½	11
		Avg.	54½	17	11½	11	11½	11
		%	2.7	33	76	84	76	84 %
161	0.30 Human ads.pl. 0.15 Human serum 0.15 Rabbit serum	1	40	12	12	12½	13½	12½
		2	21	11	10½	10½	11	10½
		Avg.	30½	11½	11¼	11½	12¼	11½
		%	9.6	76	80	76	67	76 %

PLASMA INHIBITORS OF BLOOD THROMBOPLASTIN.

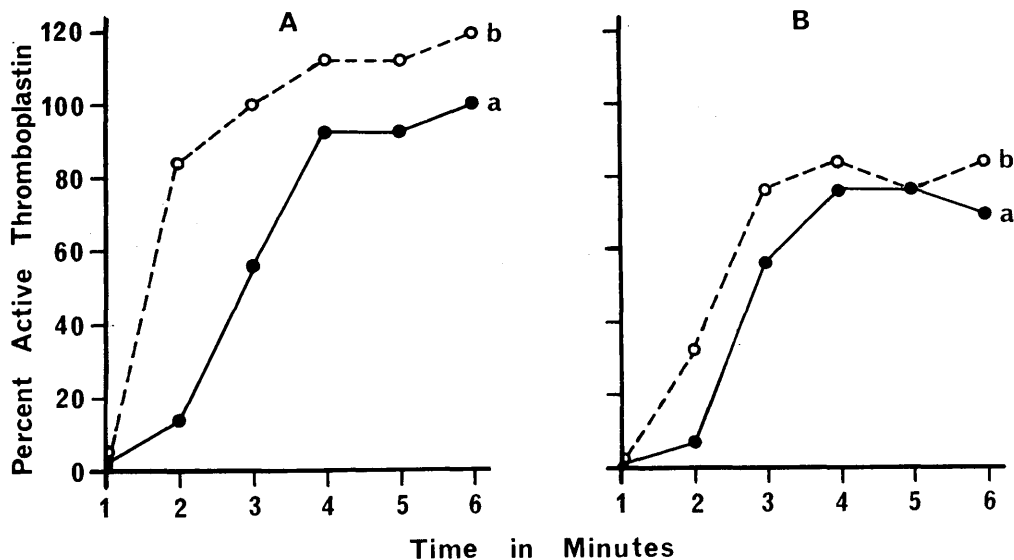


Figure 65. Rabbit plasma inhibitors of human intrinsic thromboplastin.

(Human serum and platelets are constant)

Figure A Before incubation

Curve a : human ads.pl. alone (Mixture 156, table 111)

Curve b : human ads.pl. + rabbit ads.pl. (Mixture 157, table 111)

Figure B After incubation at 37°C. for 2 hours

Curve a : human ads.pl. alone (Mixture 159, table 111)

Curve b : human ads.pl. + rabbit ads.pl. (Mixture 160, table 111)

SERUM INHIBITORS OF BLOOD THROMBOPLASTIN.

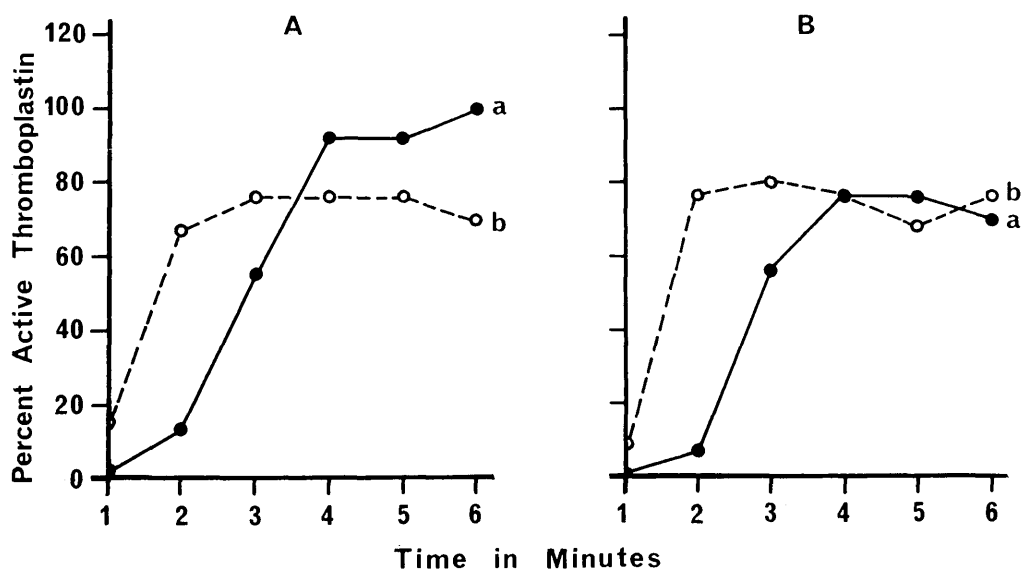


Figure 66. Rabbit serum inhibitors of human intrinsic thromboplastin.

(Human ads.pl. and platelets are constant)

Figure A Before incubation

Curve a : human serum alone (Mixture 156, table 111)

Curve b : human serum + rabbit serum (Mixture 158, table 111)

Figure B After incubation at 37°C. for 2 hours

Curve a : human serum alone (Mixture 159, table 111)

Curve b : human serum + rabbit serum (Mixture 161, table 111)

APPENDIX V, E

RESULTS OF EXPERIMENTS

ON LOBSTER BLOOD

RESULTS OF EXPERIMENTS

ON LOBSTER BLOOD

All the following data were obtained from experiments carried out on blood samples obtained from live lobsters of the *Nephrops norvegicus* strain, using pooled plasma or serum samples from at least three animals for each experiment to obviate as much as possible any individual variations from affecting the final levels of the various coagulation factors.

Blood samples were obtained fresh on the day of the test concerned as described in appendix II, 1, iii.

Table 112. Activity of lobster muscle extract

Clotting mixture	Expt. 1	Expt. 2	Expt. 3*
	Clotting times		
0.2 ml Lob.plasma 0.1 ml Lob.muscle extract 0.1 ml 3% CaCl ₂	3' 13"	5' —	10'+
0.2 ml Lob.plasma 0.1 ml Saline 0.1 ml 3% CaCl ₂	more than 10 minutes		
0.2 ml Lob.plasma 0.1 ml Lob.cell extract 0.1 ml 3% CaCl ₂	- 5"	- 6"	6"
0.5 ml Lob.plasma 0.1 ml Lob.muscle extract 0.1 ml 3% CaCl ₂	2' 15"	3' —	10'+
0.5 ml Lob.plasma 0.1 ml Saline 0.1 ml 3% CaCl ₂	more than 10 minutes		
0.2 ml Lob.fibrinogen 0.1 ml Lob.muscle extract 0.1 ml 3% CaCl ₂		1' 19"	10'+
0.2 ml Lob.fibrinogen 0.1 ml Saline 0.1 ml 3% CaCl ₂		10'+	10'+
0.2 ml Lob.fibrinogen 0.1 ml Lob.muscle extract diluted 1 in 2 0.1 ml 3% CaCl ₂		1' 23"	10'+

The test was performed at 37°C. in a similar manner to Quick's one-stage test, except for using the above specified reagents, and recording the mean clotting time of duplicate estimations.

* The lobster muscle extract used in experiment 2 was kept frozen at -20°C. for 72 hours and used fro experiment 3.

Table 113. Species specificity of lobster muscle extract

Equal volumes of plasma, tissue extract, and calcium chloride solution were tested by Quick's one-stage test (Appendix IV, 6). M/40 CaCl₂ solution was used for the human plasma and human brain mixture only. For all the other mixtures, different concentrations of CaCl₂ solution (M/40, 3%, and molar or 11%) were used and gave fairly similar clotting times.

	Human brain	Lob. muscle extract
	Clotting times	
Lobster plasma	10'+	4' 42"
	10'+	5' —
	10'+	3' 55"
Human plasma	15"	4' 20"
	14½"	2' 36"
	15"	3' 14"

Table 114. Factor V activity in lobster plasma

(by correction of factor V-deficient human plasma; appendix IV, 14)

Clotting mixture :-

0.1 ml test or control plasma 1/10 in factor V-deficient plasma,

0.1 ml human brain extract,

0.1 ml M/40 CaCl₂ solution.

Test substance (1/10)	Expt. 1	Expt. 2	Expt. 3
	Clotting times (seconds)		
Human plasma	19	20	22
Lobster plasma	80	78	91
Saline	67	63	82

Table 115. Factor VII activity in lobster serum

(by correction of phenindione human plasma; appendix IV, 15, A)

Clotting mixture :-

0.1 ml test or control serum 1/10 in phenindione plasma,

0.1 ml human brain extract,

0.1 ml M/40 CaCl₂ solution.

Test substance (1/10)	Expt. 1	Expt. 2	Expt. 3
	Clotting times (seconds)		
Human serum	21	20	19½
Lobster serum	50	52	49
Saline	48	53	47

Table 116. Factor X assay in lobster plasma

(method of Bachmann, Duckert, and Koller, 1958; appendix IV, 18)

Plasma dilution	Factor X percentage	Experiment 1	Experiment 2
		Clotting times (seconds)	
Human control plasma			
1/10	100 %	27	23
1/20	50 %	32	29
1/40	25 %	42	39
1/80	12.5%	47	49
1/160	6.2%	60	54½
1/320	3.1%	70	66
1/640	1.5%	90	81½
1/1280	0.7%	100	91½
Buffer	0 %	114	106
Lobster test plasma			
1/5		130	122
1/10		130	120
1/20		114	115
1/40		116	113
1/80		115	—
Calculated factor X conc.		0 %	0 %

Table 117. R.V.V.-cephalin clotting time of lobster plasma

(method described in appendix IV, 19)

Various concentrations of calcium chloride solution were used as shown. The different mixtures were also tested with lobster blood cell extract (last column) to prove suitability of the plasma.

Clotting mixture	Concentration of R.V.V.-cephalin		Lobster cell extract
	1/10,000 - 0.06%	1/40,000 - 0.06%	
Clotting times (seconds)			
Lobster plasma M/40 CaCl ₂	180+	180+	104
Lobster plasma 3 % CaCl ₂	180+	180+	6
Lobster plasma 11% CaCl ₂ (molar)	180+	180+	7½
Lob. fibrinogen 11% CaCl ₂ (molar)	180+	180+	5

Similar results were obtained on using R.V.V. reagent alone without cephalin.

Table 118. Thrombin clotting time of lobster plasma

Clotting mixture: 0.1 ml lobster plasma,
0.1 ml bovine thrombin, 100 units/ml.

	Thrombin clotting times (sec.)
Expt. 1	180+
Expt. 2	180+
Expt. 3	180+

These prolonged clotting times were not improved by the addition of a molar calcium chloride solution to the mixture.

Table 119. Antithrombin assay in lobster plasma

(modified method of Astrup and Darling, 1942d; appendix IV, 11, B)

Incubation mixture		A	B	C	D	
Saline	ml.	0.3	0.2	0.15	0.1	0.0
Plasma (diluted 1/5)	ml.	0.0	0.1	0.15	0.2	0.3
Thrombin (30 U/ml)	ml.	1	1	1	1	1
Clotting times of fibrinogen (seconds)	Hum.pl.	15	17	18	22	25
	Lob.pl.	15	15	15	15	15
T_o / T_n	Hum.pl.	1	0.88	0.83	0.68	0.60
	Lob.pl.	1	1	1	1	1
Corrected T_o / T_n	Hum.pl.	1	0.87	0.80	0.73	0.60
	Lob.pl.	1	1	1	1	1
Calculated units of antithrombin per ml. plasma	Hum.pl.	0	195	200	202	200
	Lob.pl.	0	0	0	0	0

Table 120. Lobster whole blood clotting times

(method of Lee and White, 1913; appendix IV, 3)

	Clotting times*	
	Ordinary glass	Siliconed glass
Expt. 1 at 37°C.	15' 12"	28' 30"
Expt. 2 at 37°C.	19' 20"	25' --
Expt. 3 at 15°C.	4' 16"	6' 33"
Expt. 4 at 15°C.	2' 50"	6' 20"
Expt. 5 at 15°C.	4' 42"	12' 30"
Expt. 6 at 15°C.	5' --	9' 10"
Expt. 7 at 15°C.	22' 40"	
Expt. 8 at 15°C.	20' 36"	
Expt. 9 at 15°C.	29' 10"	
Expt.10 at 15°C.	26' --	

* Average of two tubes' readings.

The slightest shaking of any tube resulted in instantaneous cellular agglutination and clotting of the blood, whether in ordinary glass or in siliconed tubes, at 37°C. or at 15°C. This necessitated very gentle and slow inversion of the tubes for the observation of the development of a firm clot.

Experiments 7, 8, 9, and 10 were made on lobster blood from the same animals used in experiments 3, 4, 5, and 6 respectively, after keeping them in captivity in the sea-water tank until they showed signs of devitalisation, which usually occurred within 24 to 48 hours.

Table 121. Hageman factor and plasma thromboplastin antecedent activity in lobster plasma

(method of Ratnoff and Rosenblum, 1958; appendix IV, 23, I)

Test plasma preparation	Clotting times (minutes) of human intact citrated platelet-deficient substrate plasma	
	Experiment 1	Experiment 2
Human, intact	90	110
Lobster, intact	150	120+
Saline	120+	120+
Human, glass activated	11	10
Lobster, glass activated	110	100

Table 122. Hageman factor assay in lobster plasma

(by correction of human Hageman-deficient plasma; appendix IV, 23, II)

Plasma dilution in Hag.-def. plasma	Concentration of Hageman factor	Clotting times (seconds)	
		Human control plasma	
1/5	100 %	142	107
1/15	33.3%	182	140
1/45	11.1%	257	186
1/135	3.7%	364	254
1/405	1.2%	540	320
	0 %	1200+	1200+
		Lobster test plasma	
1/5		2100+	1200+
1/15		1800+	1200+
Calculated Hageman factor conc.		0 %	0 %

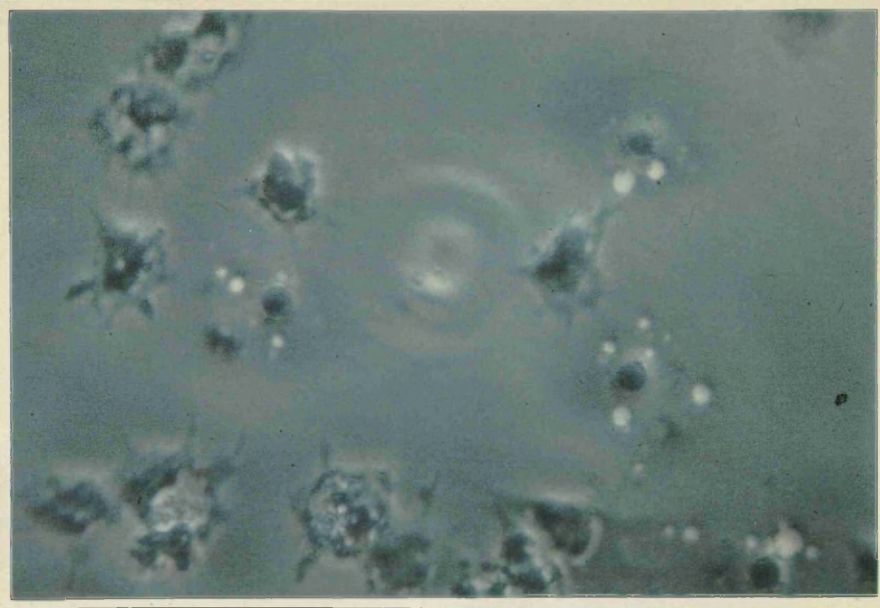


Figure 67. Phase contrast photomicrograph of lobster blood, one minute after withdrawal, showing the process of cell explosion. Magnification x170, enlargement x3.6, total x612.

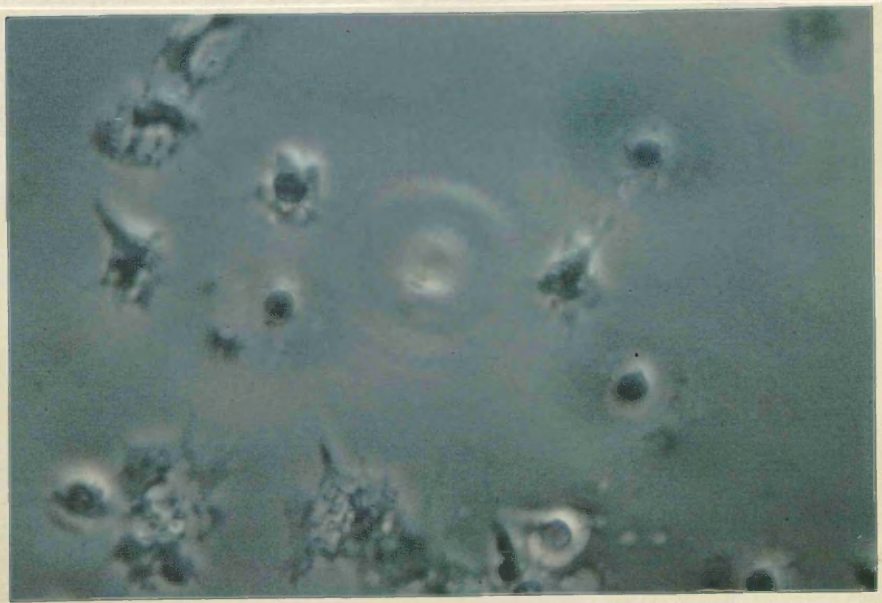


Figure 68. Phase contrast photomicrograph of the same field as above, two minutes later, showing the dense coagulation islands around the explosive cells. Magnification as above.

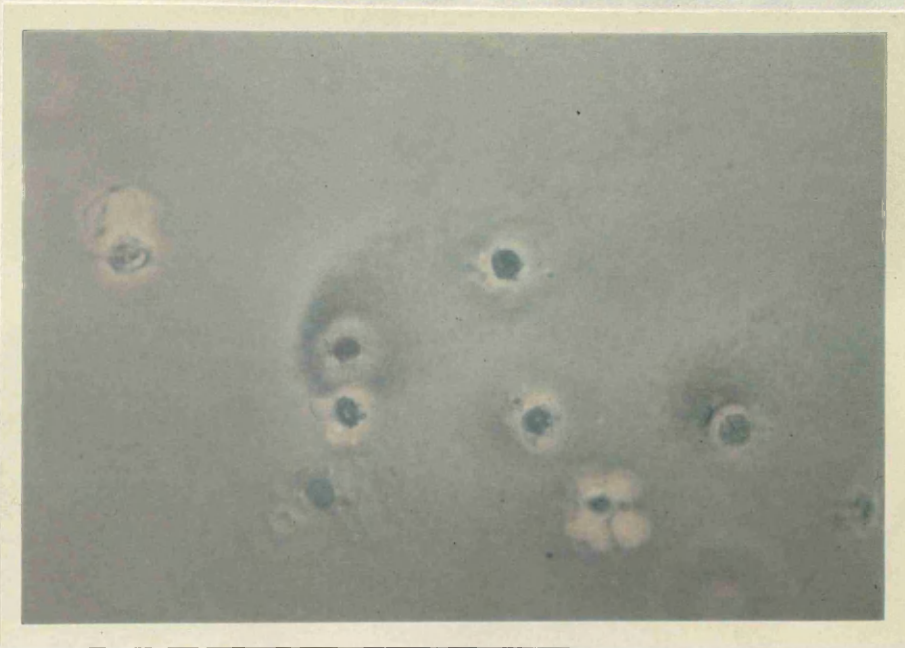


Figure 69. Phase contrast photomicrograph of lobster blood several minutes after withdrawal, showing two well developed coagulation islands. Magnification on film x170, enlargement x3.6, total x612.

Table 123. Lobster blood cells' count

(method described in appendix IV, 1, C)

	Total cell count/c.mm.	Percentage of the explosive cells
Experiment 1	30,400	23 %
Experiment 2	14,800	19 %
Experiment 3	22,800	20 %
Experiment 4	13,400	18 %
Experiment 5	15,000	24 %
Experiment 6	17,500	20 %
	10,800 *	15 %
Experiment 7	13,200	13 %
Experiment 8**	49,000	26 %
Experiment 9**	56,000	24 %
Experiment 10	27,000	20 %
Average	25,910	20.7%

* This count was made on blood of the same lobster after 24 hours in captivity, when it was found to be nearly dead.

** The two lobsters used for these two experiments were of unusually big size compared to the other several hundred specimens obtained.

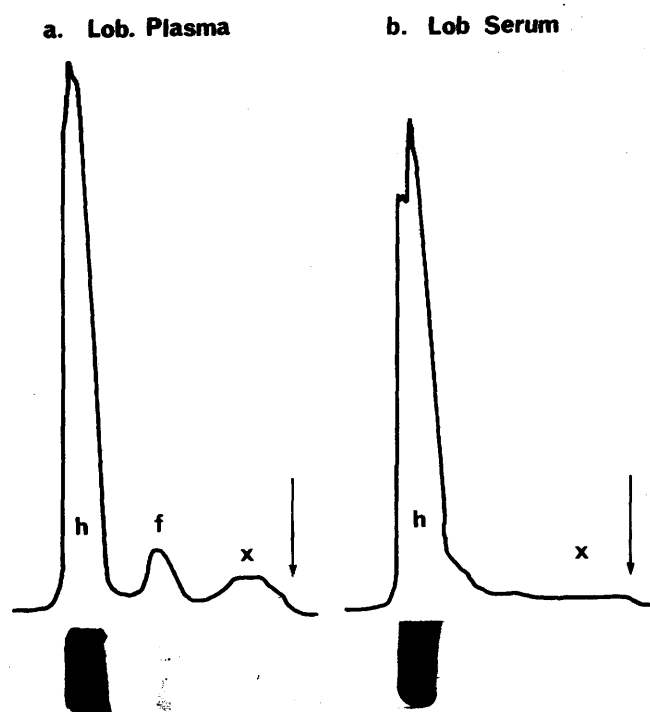


Figure 70. Electrophoresis pattern and its densitometric scanning of lobster plasma and serum.
(method described in appendix II, 7)

h : haemocyanin, a blue oxygen carrying pigment present free in the plasma.

f : fibrinogen.

x : unidentified protein component.

The lobster serum was separated after spontaneous clotting of lobster whole blood by centrifugation.

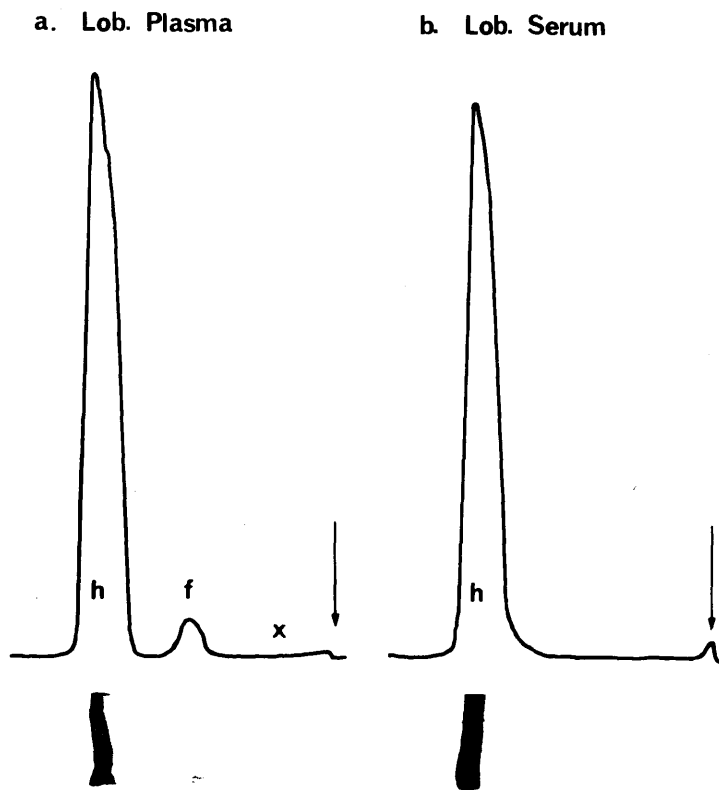


Figure 71. Electrophoresis pattern and its densitometric scanning of lobster plasma and serum.

(method described in appendix II, 7)

h : haemocyanin.

f : fibrinogen.

x : unidentified protein.

This is the more usual pattern obtained with most of the lobster plasma and serum samples.

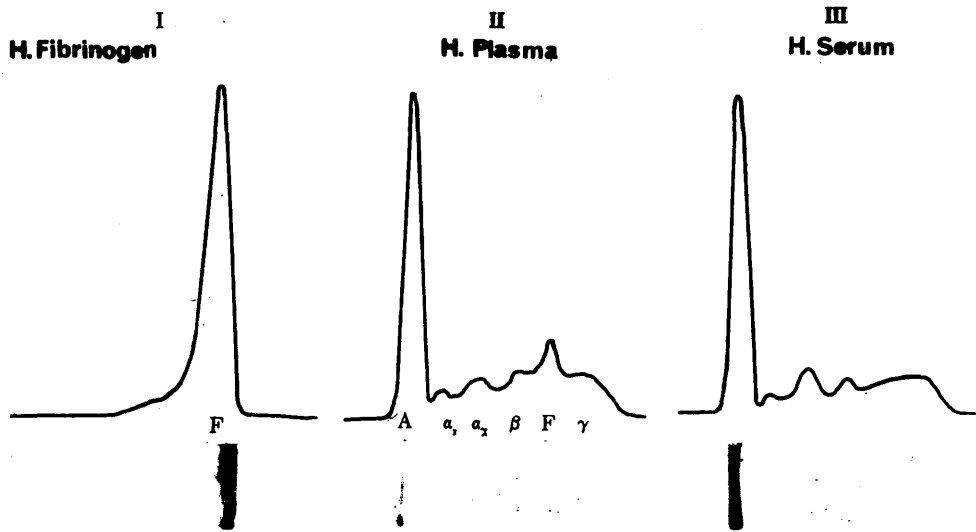


Figure 72. Electrophoresis patterns and their densitometric scannings for human plasma, serum, and purified fibrinogen.

(method described in appendix II, 7)

- A : albumin.
- α_1 : alpha-1-globulin.
- α_2 : alpha-2-globulin.
- β : beta-globulin.
- γ : gamma-globulin.
- F : fibrinogen.

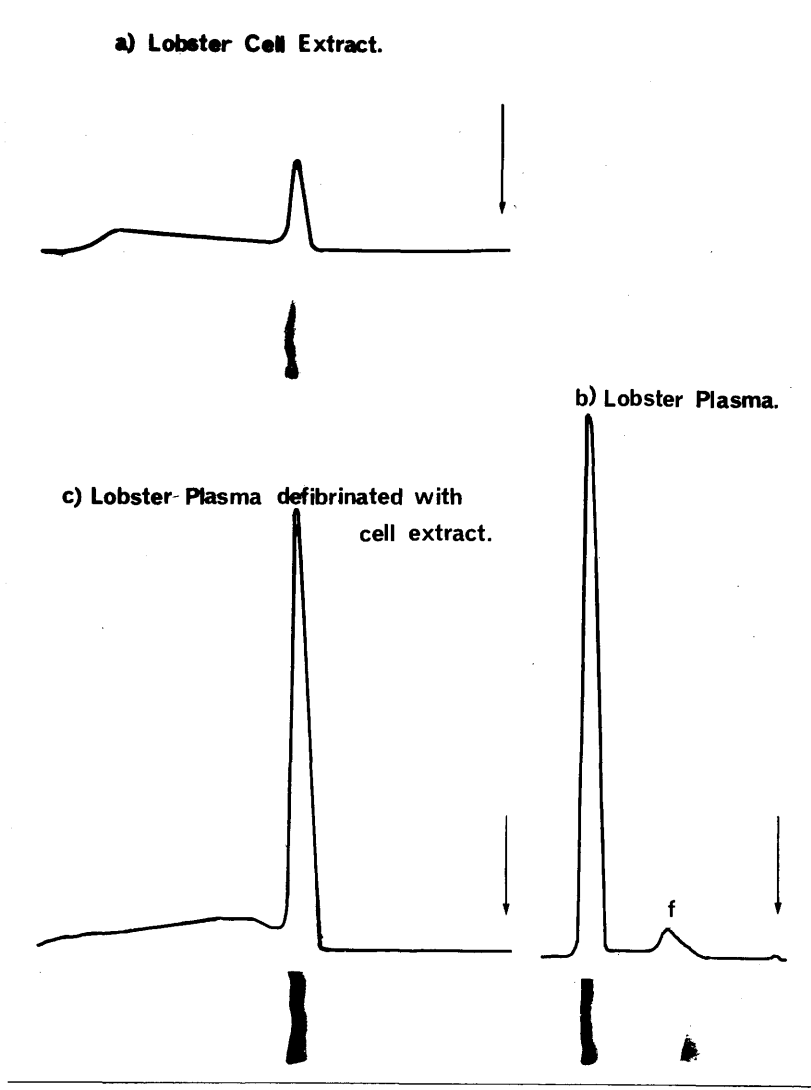


Figure 73. Electrophoresis pattern and densitometric scannings of lobster blood cells' extract, lobster plasma, and lobster plasma defibrinated with lobster cell extract. (method described in appendix II, 7)

The fibrinogen band f present in the plasma has been removed by the action of the cell extract in the form of fibrin which was separated by centrifugation.

Table 124. Fibrinogen assay in lobster plasma

(methods described in appendix IV, 2, I and II)

	mg fibrinogen per 100 mls absolute plasma
micro-Kjeldahl method	
Expt. 1	301
Expt. 2	202.2
Average	251.6
modified Greenberg method	
Expt. 3	138.8
Expt. 4	169.9
Expt. 5	186.6
Expt. 6 *	30
Expt. 7	330
Average	213.2

* Experiment 6 was made on blood from the same lobster used for experiment 5, after keeping it for a further 48 hours' period in captivity when it was found to be devitalised as judged by its sluggish movements and lack of fight on handling it.

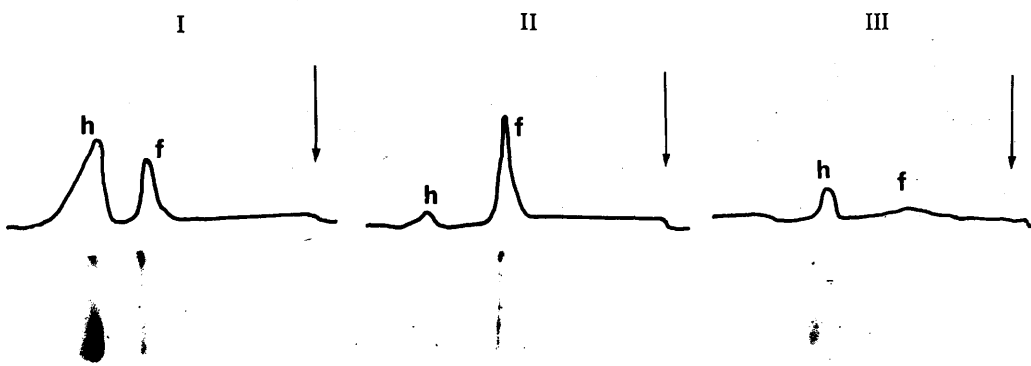


Figure 74. Electrophoresis patterns and their densitometric scannings of purified lobster fibrinogen preparations. (method described in appendix II, 7)

h : haemocyanin

f : fibrinogen

- I : precipitate 1 preparation, containing both haemocyanin and fibrinogen.
- II : precipitate 2 preparation, consisting mainly of fibrinogen.
- III : precipitate 3 preparation, containing a residual amount of haemocyanin and a trace of fibrinogen.

Table 125. Activity of lobster blood cells' extract

Clotting mixtures consisted of 0.1 ml amounts of the different reagents shown in the table, all tests were carried out at 37°C. The lobster blood cells' extract was prepared as described in appendix III, 16. The lobster fibrinogen preparations (ppt. 1, 2, and 3) were prepared as described in appendix III, 6, B.

Clotting mixture		M/40 CaCl ₂	3% CaCl ₂	11% CaCl ₂	Saline
		Clotting times (seconds)			
1	Lobster plasma	26	7	8½	180+
	Cell extract	36	5	6½	180+
		104	6	7½	180+
2	Lobster plasma	180+	180+	180+	180+
	Saline	180+	180+	180+	180+
		180+	180+	180+	180+
3	Cell extract	180+	180+	180+	180+
	Saline	180+	180+	180+	180+
		180+	180+	180+	180+
4	Human plasma	180	180+	180+	180+
	Cell extract	155	180+	180+	180+
		190	180+	180+	180+
5	Human plasma	170	180+	180+	180+
	Saline	120	180+	180+	180+
		165	180+	180+	180+
6	Lobster serum	180+	180+	180+	180+
	Cell extract	180+	180+	180+	180+
		180+	180+	180+	180+
7	Ads.lob.plasma *	180+	6	17	180+
	Cell extract	110	6½	8	180+
		134	6	9	180+

* Oxalated lobster plasma adsorbed with BaSO₄.

Table 125, continued

No.	Clotting mixture	M/40 CaCl ₂	3% CaCl ₂	11% CaCl ₂	Saline
		Clotting times (seconds)			
8	Undialysed ppt. 1 Cell extract	180+	72	180+	180+
		180+	76½	180+	180+
		180+	80	180+	180+
9	Dialysed ppt. 1 Cell extract	160	7	6½	180+
		28	8	7	180+
		31	7½	7	180+
10	Dialysed ppt. 2 Cell extract	170	6	6	180+
		23	7½	5	180+
		28½	6	6	180+
11	Dialysed ppt. 3 Cell extract	180+	120**	135**	180+
		180+	123**	134**	180+
		180+	160**	180+	180+
12	Human fibrinogen Cell extract	180+	180+	180+	180+
		180+	180+	180+	180+
		180+	180+	180+	180+
13	Dialysed ppt. 1 Saline	180+	180+	180+	180+
		180+	180+	180+	180+
		180+	180+	180+	180+
14	Dialysed ppt. 2 Saline	180+	180+	180+	180+
		180+	180+	180+	180+
		180+	180+	180+	180+

** Formation of a small weak gel.

Table 126. Measurement of the esteratic activity
of the lobster blood cells' extract

(method of Sherry and Troll, 1954; appendix IV, 24)

	Expt. 1	Expt. 2	Expt. 3	Expt. 4
	Bovine Thrombin*	Lobster cell extract		
mls. alkali consumed by 0.2 ml test solution	1.42	1.00	1.09	1.09
mls. alkali consumed by the blank	0.97	1.00	1.06	1.10
net mls. alkali consumed in test	0.45	0	0.03	0
TAME units in 0.2 ml of test solution	7.55	0	0.50	0

* Bovine thrombin (P.D. & Co.) containing nominal 50 N.I.H. units/ml.

Table 127. Storage lability of lobster blood cells' extract

Clotting mixture: 0.2 ml fresh lobster plasma,

0.1 ml lobster blood cells' extract, as specified,

0.1 ml 3% CaCl₂ solution.

	Expt. 1	Expt. 2	Expt. 3
	Clotting times (seconds)		
Fresh cell extract	4	6	5½
Cell extract stored 3 hours at 18°C.	240 +	240 +	240 +
Cell extract stored 3 hours at -20°C.	240 +	240 +	240 +

Table 128. Effect of heparin on lobster blood cells' extract activity

Lobster clotting mixtures:-

0.1 ml lobster plasma,
 0.1 ml heparin solution, various concentrations,
 0.1 ml saline or heparin antagonist, as shown,
 incubate above mixture for 3 minutes at 37°C. then add:-
 0.1 ml lobster blood cells' extract,
 0.1 ml 3% CaCl₂ solution.

Human clotting mixtures:-

0.1 ml human plasma,
 0.1 ml heparin solution, various concentrations,
 0.1 ml saline or heparin antagonist, as shown,
 incubate above mixture for 3 minutes at 37°C. then add:-
 0.1 ml bovine thrombin, 50 units/ml.

No.	Incubation mixture	Total heparin units present in each mixture						
		500	250	125	62.5	31.2	15.6	0
		Clotting times (seconds)						
1	Lobster plasma Saline	360	150	120	69	42	28	27
2	Human plasma Saline	240+	240+	240+	240+	240+	240+	17
3	Lobster plasma Toluidine blue 25 mg%	250	100	90	61	44	39	35
4	Human plasma Toluidine blue 25 mg%	240+	240+	240+	240+	240+	240+	15
5	Lobster plasma Toluidine blue 50 mg%	64	54	49	44	45	48	46
6	Human plasma Toluidine blue 50 mg%	240+	240+	240+	240+	240+	240+	25
7	Lobster plasma Polybrene (1 mg/0.1 ml)	192	51	52	52	53	48	56
8	Human plasma Polybrene (1 mg/0.1 ml)	240+	240+	22½	22½	22	21½	22

Table 129. Lobster blood cells' extract - fibrinogen reaction

(method described in appendix IV, 8 and 9)

Clotting mixture : 0.2 ml lobster plasma or fibrinogen preparation (ppt.2),
 0.1 ml lobster blood cells' extract,
 0.1 ml 3% CaCl₂ solution.

	Clotting times (seconds)					
	Dilution of variable in saline					
	1	1/2	1/4	1/8	1/16	1/32
Lobster plasma	Lobster blood cells' extract *					
	5	8	17½	250	560	1200+
	5	9½	23	240	600	1200+
Lobster fibrinogen	7½	18	51	113	300	900
	10	18	42	74	150	300
Lob. cell extract **	Lobster fibrinogen					
	6½	11	22½	77	189	360+
Lob. cell extract **	Lobster oxalated plasma					
	5	7	10	27	118	190

* Two different batches of fresh lobster blood cells' extract were used, the first experiments in each case was made with one preparation, while the second two experiments were made using the second batch.

** The same batch of this cell extract was used in clotting the various dilutions of lobster plasma or purified fibrinogen (ppt. 2).

Table 130. Antihæmophilic globulin activity in lobster plasma

(T.G.T. of Biggs and Douglas, 1953b; appendix IV, 20)

Incubation mixture :-

0.2 ml BaSO₄-adsorbed oxalated plasma, diluted 1/5, variable,

0.2 ml human serum, diluted 1/10,

0.2 ml human platelets,

0.2 ml M/40 CaCl₂ solution.

Substrate : human high-spun plasma.

Mixt. No.	Adsorbed test plasma mixture (ml)	Incubation time (minutes)					
		1	2	3	4	5	6
		Clotting times (seconds)					
162	0.2 ads.hum.pl.	24	13½	12	12	11	10
		43	15	12½	11½	11	10½
163	0.2 ads.lob.pl.	180+	180+	180+	180+	180+	180+
		180+	180+	180+	180+	180+	180+
164	0.1 ads.hum.pl.	180+	180	70	22	18	18
	0.1 ads.lob.pl.	180+	162	57	23	20	19
165	0.1 ads.hum.pl.	163	97	19	14	14	17
	0.1 saline	180+	102	44	30	19	16

The adsorbed lobster plasma used was clottable with lobster blood cells' extract and calcium chloride solution within 7 seconds.

From the above table it is obvious that lobster adsorbed plasma lacks completely any antihæmophilic globulin activity, nor does it possess any inhibitory activity on human plasma antihæmophilic globulin activity.

Table 131. Christmas factor activity in lobster serum

(T.G.T. of Biggs and Douglas, 1953b; appendix IV, 20)

Incubation mixture :-

0.2 ml adsorbed human normal plasma, diluted 1/5,

0.2 ml serum mixture, variable,

0.2 ml human platelets,

0.2 ml M/40 CaCl₂ solution.

Substrate : human high-spun plasma.

Mixt. No.	Serum mixture (ml)	Incubation time (minutes)					
		1	2	3	4	5	6
		Clotting times (seconds)					
166	0.2 hum.ser. 1/10	75	25	12½	12	11	11
		62	20	12	11	11	10
167	0.2 lob.ser. 1/10	180+	142	112	120	125	119
		180+	167	136	142	129	131
168	0.2 lob.ser. 1/5	115	126	104	147	145	147
		168	145	138	118	140	142
169	0.1 hum.ser. 1/10	66	25	12½	13	14	15
	0.1 lob.ser. 1/10	59	24½	12	12½	12	13
170	0.1 hum.ser. 1/10	145	30	12	11½	13	14
	0.1 saline	132	26	14	14	13½	15
171	0.2 Christmas ser.	103	91	109	98	59	45
		121	101	97½	86	56	48

Lobster serum had no Christmas factor activity at all in a human thromboplastin generation mixture. Also, it did not contain any inhibitor against human serum Christmas factor activity.

Table 132. Development of clotting activity
in native lobster whole blood

A technique essentially similar to the thrombin generation test of Macfarlane and Biggs (1953) was used. Immediately after withdrawal of 2 mls. lobster whole blood by a siliconed syringe, the blood is placed in a tube and a stop watch started. At one minute intervals 0.1 ml volumes are removed and added together with 0.1 ml volumes of 3% CaCl₂ solution to 0.2 ml amounts of purified lobster fibrinogen previously distributed in clotting tubes placed in the water bath at 37°C. The clotting times are recorded and are a measure of the rate and amount of clotting activity produced in the native lobster blood. When the native blood itself clots, the clot is removed by winding it on an applicator wooden stick and the test continued.

	Incubation time (minutes)										
	½	1	2	3	4	5	6	7	8	9	10
	Clotting times of lobster fibrinogen (seconds)										
Expt. 1 at 37°C.	42	27	34	49	57	50	57	65	78	74	92
Expt. 2 at 37°C.	30	23	27	35	42	57	55	75	70	76	74
Expt. 3 at 37°C.	37	13	18	30	36	42	38	50	57	64	68
Expt. 4 at 20°C.	37	27	32	32	35	30	33	54	52	58	73

Table 133. Clot retraction of lobster whole blood

(method of Macfarlane, 1938; appendix IV, 4)

	Percentage of retraction	
	in one hour	in 24 hours
Experiment 1	4 %	6 %
Experiment 2	2 %	2 %
Experiment 3	0 %	3 %

Due to the negligible retraction observed after one hour, identical aliquots of lobster whole blood were incubated at 37°C. for 24 hours before estimating the degree of retraction which was still very negligible.

Lobster blood clots are not firm, being in the form of clear gels that break easily on handling.

Table 134. General comparison of the levels of the blood
coagulation factors of the investigated species

The average levels of the blood coagulation factors shown below were compiled from the findings presented previously. Except in the case of platelets and fibrinogen, the levels were expressed in terms of percentage as compared to the reference control human plasma or serum.

	S P E C I E S				
	Lobster	Fowl	Cattle	Horse	Rabbit
Blood platelets /c.mm.whole blood	5,200*	19,650**	312,350	117,000	311,000
Fibrinogen in mgms. per 100 mls plasma	230	264	697	436	228
Prothrombin	Nil	13-23 %	100-300 %	160 %	70 %
Factor V	Nil	Present (sp.sp.)	268 %	643 %	10558 %
Factor VII	Nil	Present (sp.sp.)	Present (sp.sp.)	80 % (sp.sp.)	89 %
Factor VIII	Nil	Present (sp.sp.)	816 %	209 %	288 %
Factor IX	Nil	Nil	20 %	203 %	29 %
Factor X	Nil	Present (sp.sp.)	101 % (sp.sp.)	85 % (sp.sp.)	436 %
Factor XI	Nil	Nil	100 %	--	--
Factor XII	Nil	Nil	100 %	118 %	1025 %

* Explosive cells.

** Thrombocytes.

(sp.sp.) : species specific.

- 309 -

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- 313 -

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THE END.