REFERENCES

Bailey, K., Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 360.
Boyd, M. J. & Logan, M. A. (1942). *J. biol. Chem.* **146**, 279.
Camien, M. N. & Dunn, M. S. (1950). *J. biol. Chem.* **185**, 553.
Dolby, D. E. & Waters, J. W. (1944). *Nature, Lond.*, **153**, 139.
Hutchings, B. L. & Peterson, W. H. (1943). *Proc. Soc. exp. Biol., N.Y.*, **52**, 36.
Kodicek, E. & Mistry, S. P. (1949). *Biochem. J.* **45**, xxxv.
Kodicek, E. & Pepper, C. R. (1948). *J. gen. Microbiol.* **2**, 292, 306.
Kreuger, K. & Peterson, W. H. (1945). *J. biol. Chem.* **158**, 145.
Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F. & Blotter, L. (1943). *J. biol. Chem.* **151**, 615.
Lugg, J. W. H. (1938). *Biochem. J.* **32**, 775.
Microbiological panel of the Society of Public Analysts and other Analytical Chemists (1946). *Analyst*, **71**, 397.
Snell, E. E. (1944). *Proc. Soc. exp. Biol., N.Y.*, **55**, 36.
Stokstad, E. L. R. (1941). *J. biol. Chem.* **139**, 475.
Stokstad, E. L. R. & Hutchings, B. L. (1947). *Biol. Symp.* **12**, 357. Lancaster, Pa.: Jaques Cattell Press.
Tepley, L. J. & Elvehjem, C. A. (1945). *J. biol. Chem.* **157**, 303.
Topley, K. & Wilson, G. S. (1945). *Principles of Bacteriology and Immunity*, vol. 1. London: Edward Arnold and Co.

The Action of Some Water-soluble Poly-α-amino-acids on Blood Clotting

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The action of naturally occurring acidic and basic substances of high molecular weight, such as heparin and protamine, on blood clotting is well known (for review see Chargaff, 1945) and the study of the anti-coagulant effect of these substances has added much to the understanding of the blood-clotting mechanism. The recent synthesis of water-soluble poly-α-amino-acids (for review see Katchalski, 1950) makes possible the study of the action of polymers of well defined structure on blood coagulation. As the poly-α-amino-acids represent materials of relatively high molecular weight, composed of amino-acid residues and linked by peptide bonds, the study of their action on blood coagulation may serve to elucidate some aspects of the mechanism of action of simple proteins, such as protamine, on blood coagulation.

The water-soluble poly-α-amino-acids may be classified according to their electrochemical properties into neutral, basic and acidic poly-amides. In the present study poly-DL-alanine (Astbury, Dalgliesh, Darmon & Sutherland, 1948), the only known neutral water-soluble poly-α-amino-acid, was chosen as representative of the neutral type; the basic poly-α-amino-acids are represented by synthetic poly-lysine (Katchalski, Grossfeld & Frankel, 1948) and poly-ornithine (Katchalski & Spitnik, 1949) and the acidic poly-α-amino-acids by the naturally occurring poly-D-glutamic acid (Hanby & Rydon, 1946) and the synthetic poly-aspartic acid (Frankel & Berger, 1949).

It has been found that neutral and acidic poly-amino-acids do not retard clotting of human blood at a concentration of about 100 μg./ml. blood. Basic poly-amino-acids, on the other hand, cause a marked prolongation of clotting time at the same concentration. The clot-decelerating effect of the basic poly-amino-acids, poly-lysine and poly-ornithine, can be neutralized by the acidic poly-amino-acids as well as by heparin. The basic poly-amino acids act as antiheparins *in vitro*, similarly to protamine. Experiments with poly-L-lysine and poly-DL-lysine showed that variation of the steric configuration does not change significantly their effect on blood clotting. Lysine, ornithine and aspartic acid monomers were found to have no influence on blood clotting. The mode of action of the basic poly-amino-acids on blood clotting was investigated. The similarity of their action to that of protamine will be pointed out.

EXPERIMENTAL

Materials

Poly-L-lysine hydrochloride. This was prepared from L-lysine hydrochloride according to Katchalski *et al.* (1948). Average degree of polymerization (n) = 35 lysine units. In order to determine whether the poly-lysine synthesized contained L-lysine units, the specific rotations of poly-lysine hydrochloride and of the lysine monomer formed on hydrolysis with HCl, were determined. Poly-lysine hydrochloride showed in an aqueous solution (1·79 g./100 ml.) a strong laevorotation $[\alpha]_D^{20°} - 65·4°$, whilst the lysine dihydrochloride monomer, derived from the polymer, showed the usual dextrorotation $[\alpha]_D^{20°} + 15·7°$ (determined at a concentration of 1·26 g. lysine/100 ml. aqueous solution containing 10 equiv. of HCl). Since the specific rotation of the lysine formed on hydrolysis of poly-lysine is normal, it is considered that the poly-lysine prepared from L-lysine is built of L-lysine units. The marked negative rotation of the polymer recalls the negative rotation of many natural proteins.

Poly-DL-lysine hydrochloride. This was prepared from DL-lysine hydrochloride (Eck & Marvel, 1934, 1943), in the same way as the optically active poly-L-lysine. Average chain length (n) = 30 lysine units.

Poly-DL-ornithine hydriodide. This was prepared from DL-ornithine hydrobromide according to Katchalski & Spitnik (1949). The polymer synthesized contained an average of 26 ornithine residues.

Poly-D-glutamic acid. The poly-D-glutamic acid prepared from *Bacillus anthracis* was kindly supplied to us by Dr H. N. Rydon (cf. Hanby & Rydon, 1946).

Poly-aspartic acid. This was prepared from β-benzyl ester of *N*-carboxy-L-aspartic acid anhydride according to Frankel & Berger (1949). The polymer synthesized contained on the average 150 aspartic acid residues.

Poly-DL-alanine. This was prepared from *N*-carboxy-DL-alanine anhydride according to Astbury *et al.* (1948).

Protamine sulphate. This was prepared from ripe carp testicles according to Schmiedeberg (1899).

Heparin. A Lederle heparin solution containing 10 mg. Na salt of heparin/ml. was used. The solution had an anticoagulant potency of 1100 international units/ml.

Thrombin. A refined and dried preparation of bovine thrombin (Upjohn Co.) containing 1000 units/ampoule was used.

Bacto-thromboplastin. A dry preparation from rabbit brain (Difco Laboratories Inc.) was used. A saline suspension was prepared according to Quick (1942).

Blood. Human blood was used throughout.

Methods

Clotting time of venous human blood. This was measured on 2 ml. samples in glass test tubes at 37° by the method of Lee & White (1913) as modified by Pohl & Taylor (1937). Normal values are 5–11 min.

Clotting time of human blood treated with poly-α-amino-acids. To each of a series of test tubes containing 0·2 ml. of the poly-amino-acid solutions of various concentrations, 1·8 ml. venous blood were added immediately after venepuncture and the clotting time determined.

Plasma prothrombin time, one-stage procedure according to Quick (1942). Freshly prepared Difco rabbit-brain thromboplastin extract or human-brain extract prepared and preserved according to Aggeler, Howard, Lucia, Clark & Astaff (1946) was used. In this procedure clotting was induced in a mixture of oxalated plasma and thromboplastin by addition of $CaCl_2$. The prothrombin time is the time from the addition of $CaCl_2$ to the moment of clotting. The prothrombin time is influenced not only by variations in prothrombin content, but also by variations in the concentrations of accelerators of prothrombin conversion, antithrombin and fibrinogen (Alexander, de Vries & Goldstein, 1949).

Plasma and serum prothrombin content by the two-stage method of Sternberger (1947) *as applied to serum by Herz, de Vries & Heiman-Hollander* (1950). Plasma was defibrinated by addition of thrombin; the added thrombin was inactivated by incubation. In the first stage of this two-stage procedure prothrombin was completely converted to thrombin by the addition of thromboplastin and $CaCl_2$, while antithrombin activity was blocked with ethanol. In the second stage the thrombin formed was titrated with a fibrinogen substrate. With this method actual prothrombin content was measured and the effect of variations in prothrombin conversion accelerators, antithrombin and fibrinogen, was largely eliminated.

Prothrombin consumption. Prothrombin conversion in samples of serum obtained at various intervals after clotting was stopped by addition of 1 vol. of 0·1 M-sodium oxalate to 9 vol. of serum (de Vries, Alexander & Goldstein, 1949). The serum was incubated for 30 min. to inactivate the thrombin. The difference between the original plasma prothrombin and the serum prothrombin, as a measure of the thrombin formed, provided that all prothrombin consumed is converted to thrombin. In some experiments in which blood clotting was retarded by the addition of a poly-amino-acid, prothrombin consumption was determined in the plasma samples of the unclotted blood.

Antithrombic acitivity of serum. Bovine thrombin was added to the serum investigated and the mixture incubated at 37°. The amount of thrombin left in the mixture after increasing periods of incubation was measured by titration of a sample of the mixture on fibrinogen substrate ($BaSO_4$-treated normal oxalated plasma).

RESULTS

Anticoagulant action of basic poly-α-amino-acids on blood

Effect of water-soluble poly-α-amino-acids on clotting time of blood. Freshly drawn blood (1·8 ml.) was added to 0·2 ml. of aqueous poly-α-amino-acid solution; the clotting time was determined at 37°. The final concentrations of the preparations per ml. of blood mixture and the corresponding clotting times are recorded in Table 1. The effect of some amino-acids and of protamine sulphate on blood clotting is also shown.

Table 1 shows that the basic poly-amino-acids, poly-L-lysine, poly-DL-lysine and poly-DL-ornithine, retard blood coagulation markedly in the concentration used. Their clot-retarding effect is more pronounced than that of protamine. The acidic poly-amino-acids, poly-aspartic acid and poly-D-glutamic acid, as well as the neutral poly-DL-alanine, do not

retard blood clotting in the same range of concentrations. No effect on blood coagulation was observed with the amino-acid monomers tested.

Table 1. *Effect of water-soluble poly-α-amino-acids on clotting time of blood*

Preparation added	Final concentration in blood mixture (μg./ml.)	Clotting time (min.)
Poly-L-lysine hydrochloride	133	>90
Poly-DL-lysine hydrochloride	130	>90
Poly-DL-ornithine hydriodide	134	>90
Poly-aspartic acid	120	6
Poly-D-glutamic acid	125	7
Poly-DL-alanine	120	9·5
Protamine sulphate	134	25
L-Lysine monohydrochloride	128	8·5
DL-Lysine monohydrochloride	130	9·5
DL-Ornithine monohydrochloride	120	7
L-Aspartic acid	120	7
Control*	—	5–11

* In the control experiment 1·8 ml. blood were added to 0·2 ml. 0·9% (w/v) NaCl. The experiments reported in this table were performed on different human bloods whose range of clotting times is given.

Influence of varying concentrations of basic poly-α-amino-acids on clotting time of blood. In several experiments it was observed that clotting time increased with increasing concentrations of the basic poly-amino-acids. In order to compare the activities of the different basic poly-amino-acids, concentrations containing the same number of polar amino groups were tested. The concentrations recorded in Table 2, as base mols, indicate the number of mols

Table 2. *The influence of basic poly-α-amino-acids in varying concentrations (base mols/ml. of blood mixture) on clotting of blood*

Concentration (base mols*/ml. × 10^8)	Clotting times (min.) at varying concentrations: Poly-L-lysine hydrochloride	Poly-DL-lysine hydrochloride	Poly-DL-ornithine hydriodide
2·3	15·5	15·5	11·5
4·6	17·5	21·0	16·0
9·2	30·0	35·0	30·0
18·4	50·0	120	120
36·8	113	120	120
73·6	120	120	120

* In the calculation of the number of base mols, present in 1 ml. of blood-poly-amino-acid mixture, 1 base mol of poly-L-lysine hydrochloride or poly-DL-lysine hydrochloride was taken as equivalent to 164·5 g. of the polymeric hydrochloride, while 1 base mol of poly-ornithine hydriodide was taken as equivalent to 242 g. of the hydriodide.

of amino-acid residues per ml. of blood mixture. In the calculation of the number of base mols, the molecular weight of a lysine hydrochloride residue was taken as 164·5 and that of ornithine hydriodide as 242·0.

Table 2 shows that the clotting time of blood is prolonged to approximately the same extent by equal base mol concentrations of poly-L-lysine, poly-DL-lysine and poly-DL-ornithine.

Mode of anticoagulant action of basic poly-α-amino-acids

Inhibition of thrombin formation by poly-L-lysine. Mixtures of 0·5 ml. poly-L-lysine solutions and 4·5 ml. blood were allowed to clot at 37°. The clotted blood samples were kept at 37° for an additional hour and centrifuged for 10 min. at 2000 rev./min.; 1/9 vol. of 0·1 M-sodium oxalate was added to the sera thus obtained and the prothrombin content determined by the two-stage method (cf. Table 3). The validity of the latter procedure in the present case will be the subject of subsequent discussion.

The data (Table 3) show that prolongation of clotting time by poly-L-lysine is associated with elevated prothrombin levels or incomplete thrombin formation.

Table 3. *Elevated prothrombin levels in serum from poly-L-lysine-treated blood*

Final concentration of poly-L-lysine hydrochloride in the blood mixture (μg./ml.)	Clotting time (min.)	Serum prothrombin, determined by the two-stage test (% of normal)
0	11	1
4	24	3
8	26	13
16	32	33
31	36	15
62	125	53
125	150	27

Inhibition of prothrombin conversion. In order to evaluate the amount of prothrombin consumed in blood treated with different polymers, it was desirable to compare the levels of serum prothrombin after incubation of the blood samples for a fixed period of time. Comparative data on the inhibition of prothrombin conversion by the various basic poly-amino-acids, protamine, and the α-amino-acid monomers, L- and DL-lysine, are presented in Table 4. In each of the blood samples prothrombin was determined 70 min. after preparation of blood mixture. The plasmas of the blood mixtures which did not clot within 70 min. were defibrinated by the addition of thrombin before the determination of prothrombin by the two-stage test.

Poly-DL-lysine and poly-DL-ornithine inhibited prothrombin conversion similarly to poly-L-lysine; L- and DL-lysine monomers, on the other hand, did not interfere with thrombin formation. It is of interest that at a concentration of 4 μg./ml. blood (Table 3) poly-L-lysine hardly interfered with prothrombin conversion; at the higher concentra-

Table 4. *Inhibition of prothrombin conversion by various basic poly-α-amino-acids*

Preparation	Final concentration in blood mixture (μg./ml.)	Clotting time (min.)	Prothrombin remaining 70 min. after venepuncture; two-stage test (% of normal)
Poly-L-lysine hydrochloride	133	>70	50
Poly-DL-lysine hydrochloride	130	>70	19
Poly-DL-ornithine hydriodide	134	>70	45
Protamine sulphate	130	25	3
Protamine sulphate	1000	40	47
L-Lysine monohydrochloride	134	9·5	1
DL-Lysine monohydrochloride	128	8·5	1

Table 5. *Antithromboplastic activity of poly-L-lysine*

Exp. no.†	Thrombo-plastin extract (0·05 ml.)	Poly-L-lysine hydrochloride	Plasma (0·05 ml.)	Saline (0·05 ml.)	Saline (0·05 ml.)	Concentration of poly-L-lysine hydrochloride in the final one-stage mixture (μg./ml.)	Prothrombin time (sec.)
1	*	.	+	*	+	0	17·0
2	*	+	+	*	.	43	28·8
3	*	*	+	.	+	43	44·0

† In each experiment component pairs, indicated by asterisks or by crosses, were pre-incubated for 5 min. at 37°, before performance of the one-stage prothrombin procedure; 0·1 ml. of each of the two pre-incubated mixtures were mixed; 0·1 ml. 0·22 M-$CaCl_2$ was added and the clotting time determined (prothrombin time).

tions, however, of 125–130 μg./ml. blood, prothrombin conversion was markedly inhibited. In this respect, poly-lysine resembles protamine which manifests the same effect at relatively higher concentrations (Table 4).

Antithromboplastic action of poly-L-lysine. Incubation of thromboplastin with poly-L-lysine resulted in marked inactivation of the thromboplastic activity as demonstrated in the one-stage procedure for the determination of prothrombin.

Pre-incubation of thromboplastin extract with poly-L-lysine prolonged the one-stage prothrombin time considerably. The pre-incubation of the plasma with poly-L-lysine at the same concentration prolonged prothrombin time to a slighter extent. This points to an antithromboplastic activity of poly-L-lysine.

Sensitivity of fibrinogen to thrombin in blood treated with poly-L-lysine. Blood (1·8 ml.) was added to each of a series of test tubes containing 250 μg. poly-L-lysine hydrochloride in 0·2 ml. saline. Thrombin solutions of varying concentrations were added 5 min. after mixing. The clotting times of the mixtures were recorded and compared with those of normal blood added to thrombin directly (Table 6).

Table 6 shows that fibrinogen of blood, made incoagulable for more than 80 min. by poly-L-lysine, showed normal sensitivity to concentrations of 1–8 units of thrombin per ml. blood mixture. Similarly, it was shown that fibrinogen in oxalated plasma mixed with poly-L-lysine remained normally sensitive to thrombin.

Table 6. *Sensitivity of fibrinogen to thrombin in blood treated with poly-L-lysine*

Final concentration of thrombin in blood mixture (units/ml.)	Clotting times (sec.)	
	Blood treated with poly-L-lysine	Untreated blood
0	5000	540
1	35	40
2	35	40
4	25	35
8	15	15

Absence of effect of poly-L-lysine on the inactivation of thrombin in serum. A serum poly-L-lysine mixture and a serum-saline mixture were incubated with bovine thrombin at 37°. From time to time 0·2 ml. samples of the mixtures were withdrawn and their thrombin content determined by titration on 0·2 ml. plasma.

Table 7 shows that the antithrombic activity of serum is not influenced by poly-L-lysine in a concentration markedly retarding blood coagulation.

Effect of poly-L-lysine on the prothrombin determination by the two-stage tests. Since poly-L-lysine interferes with prothrombin conversion, it was necessary to check the reliability of the two-stage test for prothrombin determination, in the presence of basic poly-amino-acids. Poly-L-lysine was therefore added to defibrinated normal oxalated plasma and the amount of prothrombin determined by the usual two-stage procedure (Table 8).

Table 7. *Inactivation of thrombin by serum in the presence of poly-L-lysine*

Time of incubation of thrombin with serum-saline or with serum poly-L-lysine mixture (min.)	Composition of the solutions: 1·6 ml. thrombin (200 units/ml.), 1·6 ml. serum, 0·16 ml. saline	Composition of the solutions: 1·6 ml. thrombin (200 units/ml.), 1·6 ml. serum, 0·16 ml. poly-L-lysine hydrochloride solution (1250 μg./ml.)
	Clotting times (min.)	
0·25	5	5
5	7	7
11	13·5	12·6
17	21	22·4
23	32	36·5
30	50	53
35	68	68
40	82	81

Table 8. *The disturbing effect of poly-L-lysine on the prothrombin determination by the two-stage test*

(In this experiment 0·2 ml. of saline or of poly-L-lysine solutions were added to 0·6 ml. of defibrinated normal oxalated plasma, and the usual two-stage procedure was applied.)

Poly-L-lysine hydrochloride; concentration in final mixture (μg./ml.)	Prothrombin found with the two-stage test (% of normal)
0	87
15·6	50
125·0	40
312·5	15

The data given in Table 8 show that the two-stage test yields low prothrombin values in the presence of poly-L-lysine. Thus, the serum prothrombin values given in Tables 3 and 4 are minimal values. The disturbance in thrombin formation caused by the basic poly-amino-acids must be more pronounced than is indicated by the data given.

As it has been proved that poly-lysine in the concentrations used has no effect on fibrinogen, it was of interest to investigate its action on each of the two stages of the two-stage method. It was found that the basic poly-amino-acids in final concentrations up to 300 μg./ml. do not interfere with the second stage, i.e. the titration of thrombin on fibrinogen. The first stage, i.e. the formation of thrombin from prothrombin, on the other hand, is markedly inhibited by poly-lysine and other basic poly-amino-acids in the concentration range used.

Antiheparin activity of poly-L-lysine, poly-DL-lysine and poly-DL-ornithine

In order to study the action of the different poly-amino-acids on heparinized blood, the following experiments were carried out: 1·6 ml. blood were added immediately after venepuncture to a mixture of 0·2 ml. heparin (80 μg./ml.) and 0·2 ml. poly-amino-acid solution (130 μg./ml.) and the clotting time recorded. Blood (1·8 ml.) added to 0·2 ml. heparin (80 μg./ml.) did not clot within 90 min. The clotting time of untreated blood was 7–8 min.

The clotting times observed indicate that the premixing of 16 μg. heparin with approx. 26 μg. of poly-L-lysine hydrochloride, poly-DL-lysine hydrochloride or poly-DL-ornithine hydriodide causes neutralization of the clot-retarding effect of heparin, and restoration of clotting time to normal. The acidic poly-amino-acids, poly-D-glutamic acid and poly-aspartic acid, as well as the neutral poly-α-amino-acid, poly-DL-alanine, had no demonstrable antiheparinic effect in these concentrations. The amino-acid monomers, L-lysine, DL-lysine, DL-ornithine and L-aspartic acid also did not show a significant antiheparinic action.

Neutralizing ratios of heparin to basic poly-α-amino-acids. Anti-poly-lysine activity of heparin

In the previous section it was pointed out that poly-L-lysine, in adequate concentration, may neutralize the clot-retarding activity of heparin. In a series of experiments the effect of mixtures of

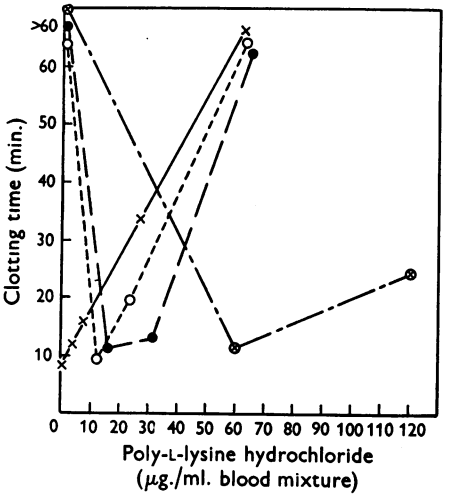

Fig. 1. The effect of mixtures of heparin and poly-L-lysine on the clotting time of human blood. Points representing blood mixtures containing the same concentration of heparin are joined by full or broken lines. Concentration of heparin in μg./ml. blood: –×—×–, 0·0; –○-----○–, 8·0; –●——●–, 20·0; ⊕—·—⊕–, 40·0.

heparin and poly-L-lysine in various concentrations, on blood coagulation was investigated. The experiments were carried out as follows:

1·6 ml. blood was added immediately after venepuncture to a mixture of 0·2 ml. heparin solution (or 0·2 ml. saline) and 0·2 ml. poly-L-lysine solution of various concentrations, and the clotting times recorded. The concentrations of

heparin and poly-L-lysine hydrochloride in the final blood mixtures and the corresponding clotting times are given in Fig. 1.

The clotting times recorded (Fig. 1) suggest that heparin and poly-L-lysine are mutually inactivated when they are present in suitable proportions. It was observed that the mixing of the basic poly-lysine with the acidic heparin leads to the formation of a precipitate, and it may be assumed that both components in the precipitate are rendered inactive. When poly-lysine is added in excess, some of it remains in solution and thus prolongs the clotting time. The antagonistic action of heparin and poly-L-lysine is demonstrable both when they are mixed previously to addition of blood, and when either of the components is added to the system sequentially. The addition of the antagonist in a neutralizing ratio, induced the appearance of a clot within a few minutes, even in blood which had been previously incubated with the coagulation inhibitor (poly-L-lysine, or heparin) for 5–20 min.

The action of acidic poly-α-amino-acids on blood treated with basic poly-α-amino-acids or with protamine

In some of the experiments recorded in Table 9, 1·6 ml. blood were added immediately after venepuncture to a mixture of 0·2 ml. of basic and 0·2 ml. acidic poly-amino-acid solution. In other experiments 1·6 ml. of blood were mixed immediately after venepuncture with 0·2 ml. of the basic poly-amino-acid solution, incubated for 5 min. and subsequently 0·2 ml. of acidic poly-amino-acid was added.

Table 9 shows that the acidic poly-α-amino acids, poly-aspartic acid and poly-D-glutamic acid in the concentration of 120 μg./ml. of blood mixture have no anticoagulant effect; they neutralize the anticoagulant activity of the basic poly-amino-acids. The anticoagulant activity of protamine is also antagonized by poly-aspartic acid.

DISCUSSION

From the data presented it follows that the basic poly-amino-acids, poly-L-lysine, poly-DL-lysine and poly-DL-ornithine, prolong the clotting time of human whole blood, when added immediately after venepuncture. The clot-decelerating activity of the basic poly-amino-acids was found considerably smaller than that of heparin and greater than that of the protamine preparation used. The basic poly-amino-acids also exhibit a distinct antiheparinic effect *in vitro* as demonstrated by the normalization of the clotting time of heparinized blood (Fig. 1).

The neutral water-soluble poly-amino-acid, poly-DL-alanine, and the acidic poly-amino-acids, poly-aspartic acid and poly-D-glutamic acid, showed no effect on blood clotting in the concentration of 120 μg./ml. blood mixture. The presence of basic amino groups in the poly-amino-acids studied seems to be essential for the anticoagulant action. This conclusion is supported by the observation that poly-lysine and poly-ornithine solutions containing equal molar concentrations of free amino groups (equal base mol concentrations) with approximately similar dissociation constants, possess equal anticoagulant activity. The fact that the anticoagulant and antiheparin activities of poly-L-lysine and poly-DL-lysine do not differ significantly indicates that the steric configuration of the amino-acid residues in the active polymer does not play a major part in the determination of the anticoagulant and antiheparin properties.

Since it was found that the corresponding basic amino-acid monomers do not influence blood coagulation and do not act as antiheparins in the investigated range of concentrations, it seems justified to assume that the chain length of the basic poly-amino-acids may be of importance in determining the anticoagulant and antiheparin properties of the basic polymers.

In the study of the mode of blood anticoagulant action of the basic poly-amino-acids, poly-L-lysine was chosen as representative of the group. It was shown that the clot-retarding effect of the poly-L-lysine in the concentrations used is not due to the inactivation of fibrinogen, nor to an increase in antithrombic activity of the blood. It could be demonstrated, that the clot retardation induced was associated with the presence of elevated prothrombin

Table 9. *Action of acidic poly-α-amino-acids on blood treated with basic poly-α-amino-acids or with protamine*

Poly-amino-acid (120 μg./ml.)	Components of test mixture (ml.)*													
Poly-L-lysine hydrochloride	--	0·2	—	0·2	0·2	—	—	—	—	—	—	—	—	—
Poly-DL-ornithine-hydriodide	—	—	—	—	—	0·2	0·2	0·2	—	0·2	0·2	—	—	—
Poly-aspartic acid	—	—	0·2	0·2	**0·2**	—	0·2	**0·2**	—	—	—	—	0·2	**0·2**
Poly-D-glutamic acid	—	—	—	—	—	—	—	—	0·2	0·2	**0·2**	—	—	—
Protamine sulphate	—	—	—	—	—	—	—	—	—	—	—	0·2	0·2	0·2
Saline	0·4	0·2	0·2	—	—	0·2	—	—	0·2	—	—	0·2	—	—
Blood	1·6	1·6	1·6	1·6	1·6	1·6	1·6	1·6	1·6	1·6	1·6	1·6	1·6	1·6
Clotting time (min.)	11	>90	8	11	11	90	14	8	7	20	5·5	22	12	8

* Poly-α-amino-acids other than those shown in black were put into the test tubes previously to the addition of the blood. Components shown in black were added to the test mixtures 5 min. after the addition of the blood.

levels in the serum of the slowly clotting blood. This finding indicates that poly-L-lysine interferes with the formation of thrombin from prothrombin in clotting blood. The experiment performed with thromboplastin (cf. Table 5) indicates that the inhibition of thrombin formation by poly-L-lysine is due to its antithromboplastic action rather than an antiprothrombic action. The possibility has not, however, been excluded that poly-L-lysine also exerts an inhibitory action on the formation of thromboplastin in shed blood, or that it inactivates prothrombin or one of the accelerators of prothrombin conversion (Owren, 1947; Ware, Guest & Seegers, 1947). Inhibition of thrombin formation was also demonstrated with poly-DL-lysine and poly-DL-ornithine; it seems fair to assume that the mechanism of clot-decelerating action of these polymers is similar to that of poly-L-lysine.

The striking similarity of the anticoagulant and antiheparin activities of the basic poly-amino-acids to those of protamines is of interest. Thus, it has been shown by Chargaff (1938*b*) that the action of protamine on blood clotting is at least partially due to the inhibition of thromboplastin. This conclusion was supported by his finding (Chargaff, 1938*a*) that protamine precipitates kephalin in a pH range of 2 to 11, suggesting that the inactivation of thromboplastin by protamine is caused by its reaction with the lipid component of the thromboplastic lipoprotein. As we have found in some preliminary experiments that kephalin is precipitated by the basic poly-amino-acids used in the present work in the pH range of 2–10, the conclusion drawn by Chargaff as to the mode of action of protamine on thromboplastin seems to hold for the action of water-soluble basic poly-amino-acids as well. Inhibition of thrombin formation by protamine has recently also been shown by Portman & Holden (1949). In this connexion, it should be recalled that Mylon, Winternitz & de Sütö-Nagy (1942) and Portmann & Holden (1949) showed that protamine at relatively high concentrations precipitates and inactivates fibrinogen. The latter authors found, however, that at lower concentrations (1 mg. protamine sulphate per ml. blood) which still produce clot retardation, no inactivation of fibrinogen occurs.

Since heparin is precipitated by basic poly-amino-acids as well as by protamine (cf. Jaques, 1943), and since the precipitate formed has no anticoagulant action, it seems likely that inactivation of heparin in heparinized blood results from the formation of an inactive salt between the strongly acidic heparin and the basic poly-amino-acids. Our experiments with mixtures of heparin and poly-L-lysine in various proportions indicate that the anticoagulant activity of the mixtures is determined by the relative concentrations of the components. Excess of either one of the components leads to the prolongation of the clotting time. These results are in accordance with the findings of Jaques (1943) with heparin-protamine mixtures. The similarity between the effect on blood clotting of the synthetic basic poly-amino-acid, poly-L-lysine, with that of a naturally occurring basic protein, protamine (cf. Portmann & Holden, 1949) is of interest and may be summarized as follows: (*a*) Poly-L-lysine and protamine act as anticoagulants on whole blood *in vitro*. (*b*) Both inhibit thrombin formation. (*c*) Both show antithromboplastic activity. (*d*) In the concentrations investigated in the present study leading to prolongation of blood-clotting time, poly-lysine and protamine do not influence the sensitivity of fibrinogen towards thrombin. (*e*) In the effective anticoagulant concentrations studied they do not influence the antithrombic activity of serum. (*f*) Both act as antiheparins. (*g*) The anticoagulant effect of poly-lysine as well as that of protamine is neutralized by heparin and by the acidic poly-amino-acids, poly-aspartic acid and poly-D-glutamic acid.

SUMMARY

1. Synthetic basic poly-α-amino-acids (poly-L-lysine, poly-DL-lysine and poly-DL-ornithine) retard blood clotting markedly in a concentration of about 100 μg./ml. blood. The neutral water-soluble poly-α-amino-acid (poly-DL-alanine) and the acidic poly-α-amino-acids (poly-aspartic acid and poly-D-glutamic acid) show no anticoagulant activity in the concentration range studied.

2. The basic poly-amino-acids inhibit thrombin formation in blood and plasma. Incubation with poly-L-lysine markedly diminishes the ability of thromboplastin to convert prothrombin into thrombin. The sensitivity of fibrinogen to thrombin and the antithrombic activity of serum are not influenced by the basic poly-amino-acids.

3. The clot-retarding action of heparin is neutralized by the basic poly-amino-acids. The neutral and acidic poly-amino-acids do not manifest antiheparin activity.

4. The anticoagulant activity of the basic poly-amino-acids and of protamine may be netralized by heparin as well as by the acidic poly-amino-acids.

5. The similarity of the mode of action of synthetic water-soluble basic poly-amino-acids and of natural protamine on blood coagulation and heparin is pointed out.

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REFERENCES

Aggeler, P. M., Howard, J., Lucia, S. P., Clark, W. & Astaff, A. (1946). *Blood*, **1**, 220.
Alexander, B., de Vries, A. & Goldstein, R. (1949). *New Engl. J. Med.* **240**, 403.
Astbury, W. T., Dalgliesh, C. E., Darmon, S. E. & Sutherland, G. B. B. M. (1948). *Nature, Lond.*, **162**, 596.
Chargaff, D. (1938*a*). *J. biol. Chem.* **125**, 661.
Chargaff, E. (1938*b*). *J. biol. Chem.* **125**, 671.
Chargaff, E. (1945). *Advanc. Enzymol.* **5**, 31.
Eck, J. C. & Marvel, C. S. (1934). *J. biol. Chem.* **106**, 387.
Eck, J. C. & Marvel, C. S. (1943). *Organic Synth.*, Collective vol. **2**, 374.
Frankel, M. & Berger, A. (1949). *Nature, Lond.*, **163**, 213.
Hanby, W. E. & Rydon, H. N. (1946). *Biochem. J.* **40**, 297.
Herz, N., de Vries, A. & Heiman-Hollander, E. (1950). *Acta med. scand.* **138**, 211.
Jaques, L. B. (1943). *Biochem. J.* **37**, 189.
Katchalski, E. (1950). *Advanc. prot. Chem.* **6**, 123.
Katchalski, E., Grossfeld, I. & Frankel, M. (1948). *J. Amer. chem. Soc.* **70**, 2094.
Katchalski, E. & Spitnik, P. (1949). *Nature, Lond.*, **164**, 1092.
Lee, R. I. & White, P. I. (1913). *Amer. J. med. Sci.* **145**, 495.
Mylon, E., Winternitz, M. C. & de Sütö-Nagy, G. J. (1942). *J. biol. Chem.* **143**, 21.
Owren, P. A. (1947). *Acta med. scand.* (Suppl), **194**.
Pohl, F. J. & Taylor, F. H. L. (1937). *J. clin. Invest.* **16**, 741.
Portmann, A. F. & Holden, W. D. (1949). *J. clin. Invest.* **28**, 1451.
Quick, A. J. (1942). *The Hemorrhagic Diseases and the Physiology of Hemostasis.* Springfield, Ill.: Ch. C. Thomas.
Schmiedeberg, O. (1899). *Arch. exp. Path. Pharmak.* (*D*), **43**, 67; cf. Bamann, E. & Myrbäck, K. (1941). *Die Methoden der Fermentforschung*, **1**, 377. Leipzig: Thieme.
Sternberger, L. A. (1947). *Brit. J. exp. Path.* **28**, 168.
de Vries, A., Alexander, B. & Goldstein, R. A. (1949). *Blood*, **4**, 247.
Ware, A. G., Guest, M. M. & Seegers, W. H. (1947). *J. biol. Chem.* **169**, 231.

The Isolation of $a\epsilon$-Diaminopimelic Acid from *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis*

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Hydrolysates of *Corynebacterium diphtheriae* have been shown by paper chromatography to contain an unknown neutral α-amino-acid (Work, 1949*a*, 1950*a*). Identical spots on chromatograms were also found by Klungsøyr & Synge (footnote, Work, 1950*b*) using acid hydrolysates of rumen contents of sheep, and by Asselineau, Choucroun & Lederer (1950) using an acid hydrolysate of an antigenic lipopolysaccharide from *Mycobacterium tuberculosis*. Preliminary reports have already been published on the isolation and identification of this amino-acid from *C. diphtheriae* (Work, 1949*b*, 1950*b*); the present paper gives details of its isolation, degradation and identification.

The behaviour of the unknown amino-acid on paper chromatograms has already been fully described ('neutral substance', Work, 1950*a*). Fig. 1 shows its position in relation to the commonly occurring amino-acids on the phenol-collidine two-dimensional chromatogram. The substance gives a spot which is practically identical in position with those given by ethanolamine-*O*-phosphoric acid and cystine. It can be distinguished from cystine by its stability to hydrogen peroxide, and from ethanolaminephosphoric acid by its behaviour during electrodialysis. Since there was no distinctive chemical or biochemical test by which the amino-acid could be recognized, its isolation was only possible through constant use of paper chromatography, each step being followed on two-dimensional chromatograms. The method finally used for the isolation from hydrolysates of *C. diphtheriae* depended on properties already reported (Work, 1950*a*), namely retention with the acidic amino-acids on acid-treated alumina and failure to migrate in an electric field. Acid-treated alumina was shown by Wieland (1942) to act as an anionic exchanger for acidic amino-acids; accordingly, in early attempts to purify the amino-acid, removal of the acidic constituents from the bacterial hydrolysate was investigated using columns of acid-treated alumina. When the amount of acid-treated alumina relative to the total acidic amino-acids of the hydrolysate was roughly of the order used by Wieland, only the acidic amino-acids were retained on the column; but when the proportion of alumina was increased three times, the unknown amino-acid also was retained, while the bases and the other neutral