restoring contractions. Here, ATP was presumably acting by virtue of its possession of "energy rich" phosphate bonds.

Summary. ATP, ADP, sodium tripolyphosphate and aluminum silicate restored the amplitude of contraction in hearts made hypodynamic by reduced calcium, excess potassium, or acetylmethylcholine chloride. In general, ATP was the most effective compound. ATP alone was effective in hearts depressed by N_2 plus IAA. ATP and ADP restored the excitability in hearts depressed by excess potassium. None of the 4 compounds had any effect on the freshly excised heart perfused with oxygenated Ringer.

1. Linder, F., and Rigler, R., *Pflüger's Arch. ges. Physiol.*, 1931, v226, 697.

2. Sheikhon, F. D., *Biulleten' eksperimental' noi Biologii i Meditsiny*, 1946, v21, 40.

3. Lichtneckert, I., and Straub, F. B., *Hungarica Acta Physiol.*, 1949, v2, 1.

4. Loewi, O., *J. Pharm. and Exp. Therap.*, 1949, v96, 295.

5. Wieland, H., *Arch. exp. Path. u. Pharmacol.*, 1921, v39, 46.

6. Clark, A. J., *J. Physiol.*, 1913, v47, 66.

7. The Metabolism of the Frog's Heart, Oliver and Boyd, London, 1938, p. 272.

8. Korey, S., *Biochem. et Biophys. Acta.*, 1950, v4, 58.

9. Greiner, T., *J. Pharm. and Exp. Therap.*, 1952, v105, 178.

10. Acierno, L., Burno, F., Burstein, F., and DiPalma, J. R., *J. Pharm. and Exp. Therap.*, 1952, v104, 264.

Received July 7, 1952. P.S.E.B.M., 1953, v82.

Effect of Synthetic Lysine Polypeptides on Rabbit Blood Coagulation.* (20075)

J. R. RUBINI,† R. R. BECKER, AND M. A. STAHMANN.

From the Department of Biochemistry, University of Wisconsin, Madison, Wisc.

As part of studies of the biological properties of lysine polypeptides(1-5), an investigation of their effect upon blood coagulation was undertaken in 1950. Since the basic polypeptides of lysine had been shown to resemble the protamines in many of their chemical and biological properties, it was expected that they would also have an effect on blood coagulation similar to that of the protamines. During the course of our investigations, de Vries, Schwager, and Katchalski reported on their observations of the action of some water-soluble poly-α-amino acids on blood clotting (6). In view of the continued interest in synthetic polypeptides and blood coagulation (7,8), it seems desirable to report some of our results.

Experiments with synthetic polypeptides of L-lysine and L-glutamic acid were carried out to study their effects on the coagulation of rabbit blood *in vitro* and *in vivo* and compared with protamine and heparin. While human and sheep blood cells have been found to be agglutinated by very low concentrations of lysine polypeptides(3) and also protamine (9), these substances do not agglutinate rabbit red cells. Rabbit blood was therefore used because it provides a good experimental system in which to study coagulation without any complicating effects of agglutination. The results of some of these experiments indicate that lysine polypeptides prolong the whole blood clotting time, increase the prothrombin time and neutralize the effect of heparin.

Materials and methods. The polypeptides were synthesized by polymerization of the N-carboxyamino acid anhydrides and analyzed by reaction with nitrous acid to determine the average number of amino acid residues (units) per polypeptide molecule(10,11). Stock solutions were made up in 0.85% saline,

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a research grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

† Present Address: Western Reserve University School of Medicine, Cleveland, O.

adjusted to pH 7.0 and stored at 4°C until needed. Clotting times were determined on blood obtained from fasted anesthetized (Nembutal) adult rabbits. Blood was taken by intracardiac puncture and immediately added in 0.8 ml volumes to test tubes (9 × 75 mm) containing 0.2 ml of the test solutions. The tubes were closed with paraffined corks and inverted every half minute during the first 15 minutes, and every one or 2 minutes thereafter. The clotting time was recorded as the time when all the blood first clotted and remained in place when the tube was inverted. Control whole blood clotting times generally ranged from 4-6 minutes. However, to facilitate comparison of all the results, the clotting times were calculated so that the control in each experiment had a clotting time of 5 minutes, and the experimental clotting times were proportionally adjusted. The *prothrombin time* determination (12,13), was carried out at 37°C. Oxalated rabbit blood was centrifuged, and the plasma diluted to 12.5% by adding physiological saline and the test substances. Calcium chloride solution (0.02 M) was mixed with a thromboplastin preparation (Difco Bacto-Thromboplastin) and 0.2 ml of this mixture was added to 0.1 ml of the diluted plasma. The time from this addition to the formation of a visible fibrin clot was recorded as the prothrombin time.

Results. The effect of the 19 unit L-lysine polypeptide *in vitro* on the clotting time of rabbit blood is shown in Table I. These data show that 125 μg of the L-lysine polypeptide prevented clot formation and as little as 31 μg increased the time of clotting from 5 to 13 minutes. The lysine polypeptide was somewhat more effective than protamine. The amino acid L-lysine and the L-glutamic acid polypeptide had no effect on the clotting time.

Chargaff(14) has shown that protamine neutralizes the anticoagulant action of heparin. The data of Table II show that the lysine polypeptide also neutralizes heparin, for the clotting time was restored to normal when 62.5 μg of lysine polypeptide or protamine were added to blood containing 50 μg of heparin per ml. At higher levels, the clotting time was again prolonged. The amino acid L-lysine as well as the synthetic L-polyglutamic acid had no effect on heparin activity.

TABLE I. Effect of Lysine Polypeptide and Protamine on Blood Clotting Time.

Final conc., μg/ml	Clotting time in presence of— 19 unit polypeptide min.	Protamine min.
250	>40	>40
125	>40	22
62.5	18	9
31.2	13	8
0	5	5

TABLE II. Anti-Heparin Activity of Lysine Polypeptide and Protamine.

Final conc. — Heparin, μg/ml	Final conc. — Polypeptide or protamine, μg/ml	Clotting time in presence of — Polypeptide, min.	Clotting time in presence of — Protamine, min.
50	250	>40	14
50	125	11	—
50	62.5	6	5
50	31.5	>40	22
50	15.6	>40	>40
50	0	>40	>40
0	0	5	5

TABLE III. Effect of Synthetic Polypeptide and Protamine on Prothrombin Time.

Final conc. in .1 ml 12.5% plasma, μg	Prothrombin time, sec.— 19 unit polypeptide	Protamine
125	100	100
31.3	27.8	100
15.6	23.9	20.2
0	18.3	12

Previous work has shown that the optical configuration and the average chain length affects the hemagglutination(3) and anti-viral activity(2,4) of the lysine polypeptides. Chain length also influences their action on blood coagulation. The 19 unit L-lysine polypeptide at a concentration of 62.5 μg per ml prolonged the clotting time to 18 minutes, while the tube containing the 158 unit L-lysine polypeptide at the same concentration showed no clot formation for over 40 minutes. The effect of a lysine polypeptide *in vitro* upon the prothrombin time is shown in the data of Table III. These data show that both the 19 unit L-lysine polypeptide and protamine increase the prothrombin time when either is added to the oxalated plasma

before the addition of the thromboplastin-calcium chloride mixture. Both substances prolonged the clotting time beyond 100 seconds when present at a concentration of 125 μg per ml. Polyglutamic acid showed no effect.

When the glutamic acid polypeptide was added to the lysine polypeptide or to protamine, a turbid flocculent precipitate was formed which indicates combination. Sufficient polylysine or protamine was added to plasma to produce a doubling of the normal prothrombin time; the addition of the synthetic polyglutamic acid restored the prothrombin time to normal.

Protamine has been used to counteract over-heparinization and it was of interest to determine whether polylysine would also counteract the action of heparin *in vivo*. Ten mg of heparin in 2 ml of solution was injected intravenously into the ear vein of an adult rabbit and 7 minutes later a blood sample removed from the other ear failed to clot within 30 minutes. Then 5 mg of a 19 unit lysine polypeptide was injected into the heparinized rabbit. When the clotting time was tested 5 and again 150 minutes later it was normal. The order of injection was reversed in another rabbit. Here, 10 mg of the 19 unit polylysine in 2 ml was injected intravenously. This amount of polylysine did not alter the clotting time. Twenty minutes after the polypeptide injection, 10 mg of heparin was injected, yet the clotting time remained normal when tested 15 minutes afterwards.

The toxicities of the polypeptides were studied in mice and guinea pigs. Death usually followed the intravenous injection of from 0.5 to 2.0 mg of lysine polypeptides of various chain lengths per adult mouse. The mice appeared to have immediate respiratory difficulties and moved in a spasmodic manner. Death generally occurred in less than 2 minutes. Autopsies revealed some vasodilation of the lungs and the spleen appeared congested. Guinea pigs generally tolerated 6 mg of the lysine polypeptides but died when injected intravenously with 10 mg.

Discussion. Our results confirm the report by de Vries, Schwager, and Katchalski(6) that the basic lysine polypeptides prolong blood coagulation, increase the prothrombin time and counteract heparin activity. In this and, as well as in their toxicities, the synthetic lysine polypeptides closely resemble the naturally occurring basic protamines. Both readily combine with a variety of proteins through the formation of many ionic bonds between the basic groups of the polypeptide or protamine and acidic groups at the surface of the protein. The basic amino groups of the lysine polypeptide also combine with the acidic sulfonic acid groups of the heparin molecule, for on mixing solutions of polylysine and heparin a turbidity immediately develops. This neutralization of the negatively charged groups of heparin by the positively charged lysine polypeptide would seem to be sufficient to account for the anti-heparin activity of the polypeptide.

The toxicities of the synthetic lysine polypeptides in mice, guinea pigs and rabbits closely resemble those of protamine(9) both in the anaphylactoid symptoms described and the approximate lethal dose ranges noted. The close similarity in the many biological effects of protamine and the lysine polypeptides suggests that high molecular weight synthetic polypeptides may be of value as models in studies which are concerned with an understanding of the basis for some of the biological and chemical properties of naturally occurring peptides and proteins.

Summary. Synthetic polypeptides were investigated for their effects on rabbit blood coagulation *in vitro* and *in vivo*. The basic L-lysine polypeptides prolonged whole blood clotting time, increased prothrombin time and neutralized the effect of heparin. A synthetic L-glutamic acid polypeptide inhibited these effects of polylysine and protamine. In mice, guinea pigs and rabbits the lysine polypeptides produced anaphylactoid symptoms which closely resembled those of protamine toxicity. A mechanism involving a combination of the basic polypeptides with acidic protein surfaces or with heparin is discussed to explain some of these results.

1. Stahmann, M. A., Graf, L. H., Patterson, E. L., Walker, J. C., and Watson, W. W., *J. Biol. Chem.*, 1951, v189, 45.

2. Burger, W. C., and Stahmann, M. A., *J. Biol.*

Chem., 1951, v193, 13.

3. Rubini, J. R., Stahmann, M. A., and Rasmussen, A. F., Jr., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 659.

4. Rubini, J. R., Rasmussen, A. F. Jr., and Stahmann, M. A., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 662.

5. Burger, W. C., and Stahmann, M. A., *Arch. Biochem.*, 1952, v39, 27.

6. de Vries, A., Schwager, A., and Katchalski, E., *Biochem. J.*, 1951, v49, 10.

7. Katchalski, E., *et al.*, *Bull. Research Council Israel*, 1951, v1, 152.

8. Grinsburg, L., de Vries, A., and Katchalski, E., *Science*, 1952, v116, 15.

9. Shelley, W. B., Hodgkins, M. P., Visscher, M. B., PROC. SOC. EXP. BIOL. AND MED., 1942, v50, 300.

10. Becker, R. R., and Stahmann, M. A., *J. Am. Chem. Soc.*, 1952, v74, 38.

11. Green, M., and Stahmann, M. A., *J. Biol. Chem.*, 1952, v197, 771.

12. Quick, A. J., *J. Biol. Chem.*, 1935, v109, 73.

13. Shapiro, S., Sherwin, B., Reddish, M., and Campbell, H. A., PROC. SOC. EXP. BIOL. AND MED., 1942, v50, 85.

14. Chargaff, E., and Olson, K. B., *J Biol. Chem*, 1937, v122, 153.

Received September 11, 1952. P.S.E.B.M., 1953, v82.

Relation of Relaxin to Steroid Ovarian Hormones on Production of Mammary Spreading Factor.* (20076)

J. R. ELLIOTT AND C. W. TURNER.

From the Department of Dairy Husbandry, University of Missouri, Columbia, Mo.

Hamolsky and Sparrow(1) reported that daily treatment for 13 days with a combination of 0.83 μg of estradiol benzoate, 1 mg of progesterone and 25 guinea pig units of relaxin produced greater mammary gland growth in immature ovariectomized female rats than treatment with any 2 of these hormones. Since it has been well established that estrogen and progesterone in sufficient quantities produce good mammary gland growth (see review by Folley and Malpress(2)) it was concluded by Garrett and Talmage(3) that relaxin must act as a potentiator of estrogen rather than producing specific changes attributable to relaxin. A recent investigation by Trentin(4) on the effect of relaxin on mammary gland growth in the mouse has shown that there was little or no increase in the percentage of positive mammary alveolar responses in the ovariectomized mice treated with estrogen, progesterone and relaxin as compared to those treated with estrogen and progesterone only. Earlier observations by Elliott and Turner (5) showed that the injection of estradiol benzoate and progesterone into castrate albino rats in the proper proportions caused maximum elaboration or activation of a spreading factor which could be extracted from the mammary gland.

It has been suggested that the spreading factor which can be extracted from the growing mammary gland makes possible the forward extension of the mammary duct system into the fatty pad. It was thought possible that relaxin might directly or indirectly influence the elaboration or activation of the spreading factor and thus contribute to the greater mammary gland extension observed by Hamolsky and Sparrow(1).

Methods and materials. Castrate female albino rats weighing from 150 to 250 g were employed starting on the eleventh day postoperatively. The estradiol benzoate and progesterone used was dissolved in olive oil while relaxin was prepared as an aqueous extract of swine ovaries.† Daily subcutaneous injections were continued for 10 days. On the eleventh day the animals were killed, the mammary glands removed, extracted, and the amount of spreading factor determined by a method previously described(6).

* Contribution from the Department of Dairy Husbandry, Mo. Agric. Exp. Station, Journal Series No. 1343. Approved by the Director.

† The relaxin used was generously provided by Dr. R. L. Kroc, Chilcott Laboratories, Morris Plains, N. J.