hippuric acid even when benzoic acid has not been administered. Although certain forms of bacteria are known to have the ability to produce benzoic acid, this substance has not been described as a normal metabolite in vertebrates. Current isotope studies together with the observations reported in this paper provide evidence for believing that there is an endogenous source of benzoic acid which is not dependent upon food intake or on the activity of intestinal bacteria. The most likely precursor is one of the aromatic acids and, indeed, preliminary studies suggest that phenylalanine is one of the amino acids involved(5).

Summary. 1. The isotope concentration of the urinary hippuric acid of starved irradiated or non-irradiated rats given benzoic acid-carboxyl-C^{14} is much less than the dilution expected from the conjugation of the injected labeled benzoic acid with inert carbon atoms of the glycyl residue. 2. Evidence is presented that benzoic acid is a normally occurring metabolite in rats. 3. The effect of x-radiation on the ability of starved rats to conjugate benzoic acid-carboxyl-C^{14} has been tested and no defect detected under the experimental conditions used.

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1. Schreier, K., DiFerrante, N., Gaffney, G. W., Hempelmann, L. H., and Altman, K. I., *Arch. Biochem. Biophys.*, 1954, v50, 417.

2. Schachter, D., and Taggart, J. V., *J. Biol. Chem.*, 1953, v203, 925.

3. Gaffney, G. W., Schreier, K., DiFerrante, N., and Altman, K. I., *ibid.*, 1954, v206, 695.

4. Schreier, K., unpublished observations.

5. Bruns, F., Haberland, G. L., and Altman, K. I., experiments to be published.

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Action of Poly-α-Amino Acids on Clotting of Fibrinogen by Thrombin.* (21286)

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It was shown previously that basic polyamino acids inhibit blood thromboplastic activity(1), fibrinolysis(2), proteolytic activity of pepsin(3), clearing of alimentary lipemia by heparin(4), as well as bacterial(5) and viral growth(6). Acidic polyamino acids neutralized the inhibitory actions of basic polyamino acids.

In this communication it is reported that the basic polyamino acids, polylysine, polyornithine and polyarginine, accelerated the clotting of fibrinogen by thrombin, while the acidic polyamino acids, polyaspartic, polyglutamic and polycysteic acids retard the clotting.

Materials and methods. Fibrinogen. Bovine Fraction I† was used or purified by the procedure of Laki(7). The final preparation contained 88-93% clottable protein and was electrophoretically homogenous. Mobility calculated from the descending boundary pattern was 1.8×10^{-5} cm^2 V^{-1} $sec.^{-1}$ at pH 7.4, ionic strength 0.15 and t = 4°C. Determinations of fibrinogen were made by the procedure of Ware *et al.*(8).

Thrombin. A thrombin solution was obtained by adding 19 ml of isotonic buffer solution (see below) and 1 ml of 0.1 M sodium oxalate to a 1000 unit vial of Bovine Thrombin (Upjohn). One g of $BaSO_4$ (Merck) was added, the suspension was stirred for 15 minutes, centrifuged and the sediment discarded. This treatment reduced the protein content

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† Obtained through the courtesy of Mr. Lawrence L. Lachat of Armour & Co., Chicago, Ill.

of the thrombin solution by approximately 20% without affecting the total thrombin activity. The solutions obtained showed an activity of approximately one unit of thrombin per 0.04 mg protein. Electrophoretic examination of the untreated thrombin solution carried out at pH 7.4, ionic strength 0.15 and t = 4°C revealed 4 components with mobilities similar to those found by Seegers *et al.*(9). After treatment with $BaSO_4$ the fast moving component disappeared from the electrophoretic pattern.

Isotonic buffer solution. 10 ml of 0.1 M sodium veronal-HCl buffer (Michaelis) pH 7.4 were added to 90 ml of 0.9% NaCl solution. The ionic strength of the final buffer solution was 0.15. All substances were dissolved in and diluted with this medium.

Polyamino acids were obtained from the Department of Biophysics of the Weizmann Institute of Science. They included (n indicates average chain length): Poly-DL-lysine hydrobromide n=36(10), poly-DL-ornithine hydrochloride n=29(11), poly-DL-arginine sulphate n=20(11), poly-L-aspartic acid n=150(12), poly-glutamic acid n=49(13), poly-L-cysteic acid n=10(14) and poly-DL-alanine n=30(15). The basic polyamino acids were dissolved in the isotonic buffer solution. The acidic polyamino acids were first dissolved in 0.15 N NaOH, the solution was neutralized with 0.15 N HCl and 1/9 volume of 0.1 M veronal-HCl buffer pH 7.4 was added. The concentrations of the basic polyamino acids given in μg/ml refer to the concentration of the respective polycationic salts.

Protamine sulfate from Nutritional Biochemicals Corp. was used.

Heparin (Upjohn) contained chlorobutanol as a preservative. Control tests revealed that this substance did not affect clotting of fibrinogen.

Platelet-free plasma. Human blood was collected by an all-silicone technique, mixed with 1/19 volume of 0.2 M sodium oxalate, centrifuged at 18,000 g for 15 minutes at 4°C and the supernatant separated.

Clotting times were measured at 37°C in glass tubes of 11 mm internal diameter. The first appearance of macroscopically visible fibrin strands was taken as the end point. Upon addition of basic polyamino acids and protamine to fibrinogen the solutions became turbid but this did not interfere with the reading of the end point.

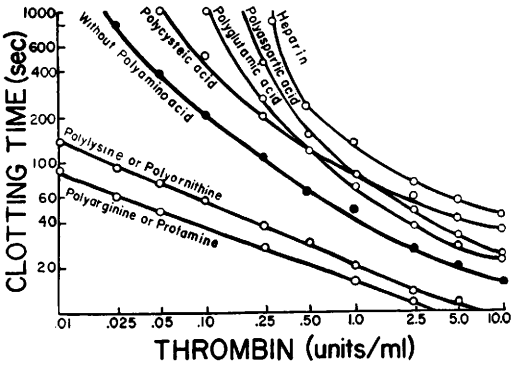

FIG. 1. Effect of 200 μg/ml of various poly-α-amino acids and of heparin on time of clotting of Fraction I by thrombin solutions of different strengths.

Clotting mixtures, when not stated otherwise, consisted of 0.2 ml of fibrinogen solution, 0.2 ml of solution of test substance or isotonic buffer solutions and 0.2 ml of thrombin solution which was added last. All concentrations are given per ml of final clotting mixture.

Experimental. The accelerating effect of the basic polyamino acids and protamine and the retarding effect of the acidic polyamino acids and heparin on the time of clotting of Fraction I by different strengths of thrombin is shown in Fig. 1. Similar concentrations of the neutral polyamino acid polyalanine and of the monomers of the respective polyamino

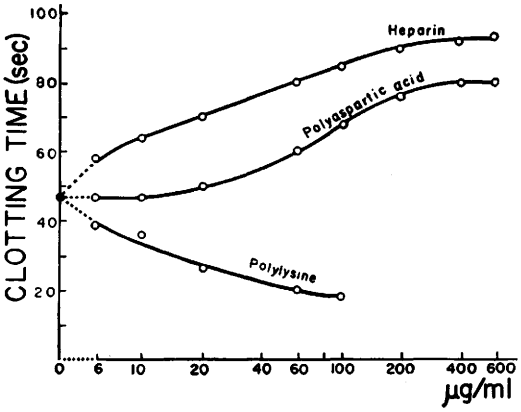

FIG. 2. Effect of polylysine, polyaspartic acid and of heparin in various concentrations on time of clotting of purified fibrinogen (2.5 mg/ml) by thrombin (1 unit/ml).

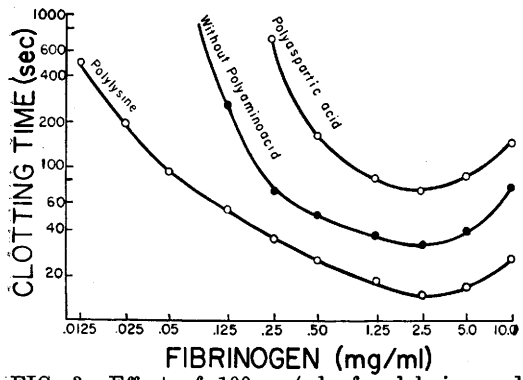

FIG. 3. Effect of 100 μg/ml of polylysine and polyaspartic acid on time of clotting of varying concentrations of purified fibrinogen by 1 unit/ml of thrombin.

acids as well as the basic dyes toluidine blue and diazine green did not influence the clotting time. The clot accelerating action of polylysine and the clot retarding action of polyaspartic acid and heparin increase with the rise in concentration (Fig. 2).

The effect on clotting time of varying fibrinogen concentration in the presence of a constant concentration of polylysine and polyaspartic acid respectively is shown in Fig. 3. Low concentrations of fibrinogen which were not clotted by thrombin after several hours were clotted within minutes in the presence of polylysine. This may be useful for the detection of very low concentrations of fibrinogen.

It is known that heparin combines with a plasma co-factor to give a complex with marked antithrombin activity. If the fibrinogen preparation used contained such a co-factor, an antithrombin complex between the co-factor and heparin would be formed (a similar complex might possibly be formed also in the case of polyaspartic acid). The clotting times in the presence of this complex would be significantly prolonged. The following experiment was designed to investigate this possibility.

Two series of experiments were performed in which either platelet-free plasma or purified fibrinogen were mixed with polylysine, polyaspartic acid or heparin. In each case thrombin was added either immediately or after preincubation of the mixture at 37°C for a period of 10 minutes, and clotting time was measured (Table I). Polylysine accelerated clotting of both plasma and purified fibrinogen solution. Polyaspartic acid retarded clotting of purified fibrinogen but had no significant effect on the plasma clotting times. In the case of polylysine and polyaspartic acid preincubation with purified fibrinogen as well as with plasma had no effect on clotting times. In the case of heparin however it can be seen that preincubation increased the clotting times of platelet-free plasma considerably, while it had no effect when purified fibrinogen was used. (Table I). It should be noted that at the

TABLE I. Effect of Preincubation of Platelet-Free Plasma or Purified Fibrinogen with Polylysine, Polyaspartic Acid or Heparin on Time of Clotting by Thrombin.

		Platelet-free plasma*		Purified fibrinogen*	
		Clotting times (sec)			
Test substance	Final conc., μg/ml	Without preincubation	After preincubation	Without preincubation	After preincubation
Isotonic buffer control		11	10	16	16
Polylysine	100	6	6	11	11
Polyaspartic acid	400	10	9	24	25
Heparin	.5	13	15	16	16
	1	26	30	17.5	17
	2	34	42	21	20
	3	77	95	23	22
	5	180	420	24	24
	7.5	380	960	25	26
	15	900	∞	27	28
	30	∞	∞	27	28

* Containing 4.5 mg fibrinogen/ml.

To .2 ml substrate .1 ml test substance was added. After 10 min. incubation at 37°C .2 ml of 9 units/ml thrombin together with another .1 ml test substance were added. The latter was added in order to keep its concentration constant throughout the experiment.

TABLE II. Neutralization of the Clot Accelerating Action of Polylysine by Polyaspartic Acid.

Polyamino acids in clotting mixture*	Clotting time (sec)
Control (isotonic buffer without polyamino acid)	42
Polylysine	21
Polyaspartic acid	60
Polylysine + polyaspartic acid premixed	43
Polyaspartic acid added to polylysine —fibrinogen mixture	41
Polylysine added to polyaspartic acid —fibrinogen mixture	43

* Containing .2 ml purified fibrinogen (7.5 mg/ml), .2 ml thrombin (3 units/ml) and .1 ml of polyamino acid solutions indicated in the table (polylysine 600 μg/ml, polyaspartic acid 1200 μg/ml); the mixtures were made up to a final volume of .6 ml.

same heparin concentration plasma clotting times were markedly longer than with purified fibrinogen.

The antithrombin activity of plasma in the presence of heparin is known to increase markedly during the first few minutes of incubation(16,17). As no such increase was noted in the case of the purified fibrinogen used it may be concluded that it is devoid of the plasma heparin co-factor. The clot retardation by polyaspartic acid and heparin seems therefore to be the result of a direct action on the conversion of fibrinogen to fibrin by thrombin. This is consistent with the observations of Klein and Seegers(18) on the clot retarding action of heparin.

The clot accelerating effect of polylysine was abolished by addition of polyaspartic acid, and the retarding effect of polyaspartic acid was abolished by addition of polylysine (Table II). This neutralization also took place even after the reaction was allowed to proceed (Table III).

Discussion. The clotting of fibrinogen by thrombin and by coagulase-thrombin(19) is the first enzymatic reaction found to be accelerated by the basic synthetic polyamino acids, already known to be inhibitors of a variety of biological systems. In preliminary experiments it was observed that the clotting of fibrinogen by papain is also accelerated by basic polyamino acids(20). Toluidine blue and diazine green, basic dyes with low molecular weight, which—like basic polyamino acids—neutralize heparin and inhibit thromboplastic activity, did not exhibit any activity in the above reaction. It appears therefore that the polycationic nature of the polymers employed plays an important role in their accelerating action. This is also apparent from the fact that the natural basic polypeptide, protamine, exhibits "fibrinoplastic" activity(21).

The clotting of fibrinogen by thrombin involves splitting off from fibrinogen of an acidic polypeptide rich in aspartic and glutamic acid residues(22). The thread-like basic polylysine molecules may combine with the acidic polypeptide either when released or while still attached to the fibrinogen molecule. Such a combination may reduce the repulsive forces between negatively charged thrombin and fibrinogen and facilitate the approach of the enzyme to the substrate. The possibility that basic polyamino acids combine with thrombin, or both thrombin and fibrinogen, should also be considered.

The neutralization of the clot accelerating action of the basic polyamino acid polylysine by the addition of acidic polyaspartic acid to the clotting mixture is probably the result of the formation of a biologically inactive complex due to combination between the oppositely charged polyamino acids.

Summary. 1. The synthetic basic poly-α-

TABLE III. Neutralization of the Clot Accelerating Action of Polylysine by Polyaspartic Acid Added at Various Time Intervals to the Fibrinogen-Polylysine-Thrombin Mixture.

Time* interval after which polyaspartic acid or buffer was added	Clotting times* of mixtures†			
	Containing polylysine +		Without polylysine +	
	Polyaspartic acid	Buffer sol.	Polyaspartic acid	Buffer sol.
0	42	22	58	40
5	41.6	21.8		
10	41.0	21.4		
15	39.5	21.0		
20	37.8	20.8		

* Seconds after addition of thrombin.

† Containing (in order of addition):
.2 ml purified fibrinogen sol. 7.5 mg/ml,
.1 " polylysine 600 μg/ml or .1 ml buffer sol.,
.2 " thrombin sol. 3 units/ml,
.1 " polyaspartic acid 1200 μg/ml or .1 ml buffer sol. (added at various time intervals).

amino acids, polylysine, polyornithine and polyarginine, accelerate conversion of fibrinogen to fibrin by thrombin; the acidic poly-α-amino acids, polyaspartic, polyglutamic and polycysteic acids, and also heparin, retard this reaction. 2. The acidic polyamino acids neutralize the clot accelerating activity of the basic polyamino acids. 3. The addition of polylysine makes possible the detection of concentrations of fibrinogen too low to be clotted by thrombin alone.

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1. de Vriees, A., Schwager, A., and Katchalski, E., *Biochem. J.*, 1951, v49, 10.

2. Ginsburg, I., de Vries, A., and Katchalski, E., *Science,* 1952, v116, 15.

3. Katchalski, E., Berger, A., and Neumann, Ch., *Nature,* 1954, v173, 978.

4. Stein, Y., de Vries, A., Wislicki, L., and Katchalski, E., *Metabolism,* 1954, v3, 213.

5. Katchalski, E., Bichowski-Slomnitzki, L., and Volcani, B., *Biochem. J.*, 1953, v55, 671.

6. Stahman, M. A., Graf, L. M., Patterson, E. L., Walker, J. C., and Watson, D. W., *J. Biol. Chem.,* 1951, v189, 45.

7. Laki, K., in *Blood Clotting and Allied Problems,* Josiah Macy, Jr. Foundation, New York, N. Y., 1951, v4, 223.

8. Ware, A. G., Guest, M. M., and Seegers, W. H., *Arch. Biochem.,* 1947, v13, 231.

9. Seegers, W. H., McClaughry, R. I., and Andrews, E. B., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 714.

10. Katchalski, E., Grossfeld, I., and Frankel, M., *J. Am. Chem. Soc.,* 1948, v70, 2094.

11. Katchalski, E., and Spitnik, P., *ibid.,* 1951, v73, 3992.

12. Berger, A., and Katchalski, E., *ibid.,* 1951, v73, 4084.

13. Hanby, E. E., Waley, F. G., and Watson, J., *J. Chem. Soc.,* 1950, vIV, 3239.

14. Katchalski, E., and Berger, A., *Bull. Res. Counc., Israel,* 1951, v2, 314.

15. Astbury, W. T., Dalgliesh, C. E., Darmon, S. E., and Sutherland, G. B. B. M., *Nature,* 1948, v162, 569.

16. Seegers, W. H., Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Science,* 1942, v96, 300.

17. Gerendas, M., *Nature,* 1946, v157, 837.

18. Klein, P. D., and Seegers, W. H., *Blood,* 1950, v5, 742.

19. Biezunski, N., Shafrir, E., de Vries, A., and Katchalski, E., 1954, in preparation.

20. Unpublished observations.

21. Ferguson, W. H., *Am. J. Physiol.,* 1940, v130, 759.

22. Lorand, L., and Middlebrook, W. R., *Bioch. Bioph. Acta,* 1952, v9, 581.

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Inhibition of Degradation of Insulin-I^{131} *in vivo*.* (21287)

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The processes of degradation and of inactivation of insulin by tissues *in vitro* can be inhibited by a number of substances(1,2). Both *in vitro* and *in vivo* the system in which degradation is accomplished does not distinguish between insulin and I^{131}-labeled insulin(1,3). It would seem to be of great importance to determine if the degradation of insulin-I^{131} can be inhibited *in vivo,* because such inhibition might effect potentiation of insulin action and have therapeutic implications. Accordingly, the effects of the following compounds on the degradation of insulin-I^{131} *in vivo* were studied.

(1) *Zinc.* There has been a great deal of study of the chemical and biological interaction between insulin and zinc, and in practice, zinc has been used as a factor in various insulin preparations. It is of interest then, that zinc has been shown to be a potent inhibitor of insulin degradation(1) and inactivation(2) *in vitro,* and accordingly the

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