no breakdown products of isoniazid, such as isonicotinic acid, were detected.

Hydrolysis of isonicotinoylglycine. To demonstrate the production of isonicotinic acid and glycine by hydrolysis of the metabolite thought to be isonicotinoylglycine, it was necessary to purify the urine. Urine (200 ml.) was adjusted to pH 2.5 with HCl and extracted continuously with ether for 16 hr. The residual aqueous solution was neutralized with NaOH, concentrated *in vacuo* and filtered from the precipitated solids. The final volume was 10 ml. One ml. of this solution was applied to paper as a thin streak and chromatographed with *n*-propanol:water. The band containing the isonicotinoylglycine was cut out and eluted with three successive 20 ml. of distilled water. The combined water extracts were concentrated *in vacuo* to 1 ml. which was applied to paper as a streak and subjected to ionophoresis in phosphate buffer pH 6.5. The band containing the isonicotinoylglycine was again cut out, eluted, concentrated, chromatographed and eluted as before. Part of the final eluate was chromatographed and the remainder was hydrolysed by heating at 100° for 3 hr. in 5*N*-HCl in a sealed tube. The hydrolysate was neutralized with NaOH and chromatographed. In the hydrolysed eluate isonicotinoylglycine was no longer detectable, but substances giving the same *R_F* values and colour reactions as isonicotinic acid and glycine were present. Glycine was detected by spraying with ninhydrin (0.5%, w/v, in *n*-butanol).

DISCUSSION

Elmendorf, Cawthon, Muschenheim & McDermott (1952) have reported that the 24 hr. urinary ex-

cretion of isoniazid after ingestion of a single dose (3 mg./kg.) by six human subjects varied from 47.8 to 70.7% of the dose. In five of the six cases reported here no isoniazid was detected on paper chromatograms, indicating that less than 20% of the total ingested dose of isoniazid had been excreted unchanged in 24 hr. In the one case where isoniazid excretion was detected it was calculated that about 20% of the dose had been excreted unchanged in 24 hr.

SUMMARY

1. Methods are described for detecting and identifying some tertiary pyridine compounds, in particular pyridine-4-carboxy derivatives.

2. The probable identities of two metabolites excreted in urine by human subjects treated with isoniazid (pyridine-4-carboxyhydrazide) have been established as isonicotinoylglycine and isonicotinic acid. Large amounts of unchanged isoniazid were not excreted.

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REFERENCES

- Boxer, G. E. & Rickards, J. C. (1951). *Arch. Biochem.* **30**, 372.
 Bray, H. G., Thorpe, W. V. & White, K. (1950). *Biochem. J.* **46**, 271.
 Cuthbertson, W. F. J. & Ireland, D. M. (1952). *Biochem. J.* **52**, xxxiv.
 Dalglish, C. E. (1952). *Biochem. J.* **52**, 3.
 Elmendorf, D. F., Cawthon, W. D., Muschenheim, C. & McDermott, W. (1952). *Amer. Rev. Tuberc.* **65**, 429.
 Epstein, J. (1947). *Analyt. Chem.* **19**, 272.
 Fox, S. W. & Field, H. (1943). *J. biol. Chem.* **147**, 651.
 Huebner, C. F. (1951). *Nature, Lond.*, **167**, 119.
 Kelly, J. M., Poet, R. B. & Chesner, L. M. (1952). *Amer. chem. Soc. Abstr.* 122nd Meeting. Sept. p. 3C.
 Knorr, L. (1883). *Ber. dtsch. chem. Ges.* **16**, 2597.
 Kodicek, E. & Reddi, K. K. (1951). *Nature, Lond.*, **168**, 475.
 König, W. (1904). *J. prakt. Chem.* **69**, 105.
 Kulberg, L. M. & Cherkasov, A. I. (1951). *J. anal. Chem. Moscow*, **6**, 364. Cited in *Brit. Abstracts C.* (1952) p. 149.
 Latner, A. L. (1952). *Biochem. J.* **51**, xii.
 Scott, P. G. W. (1952). *J. Pharm., Lond.*, **4**, 681.

The Action of Some Water-soluble Poly- α -amino Acids on Bacteria

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It has been shown that different bacteria and fungi secrete antibacterial polypeptides (for review, see Synge, 1949). The work of Bloom, Watson, Cromartie & Freed (1947) and Bloom & Blake (1948) showed that cells of higher animals, as well, may excrete antibacterial substances of a peptide nature. These facts encouraged us to study the biological properties of water soluble poly- α -amino acids (for review see Katchalski, 1951).

Poly-DL-alanine (Astbury, Dalglish, Darmon & Sutherland, 1948) was chosen as representative of the neutral polyamino acids. It is the only known water-soluble member of this group. The synthetic poly-L-aspartic acid (Berger & Katchalski, 1951) and the natural poly-D-glutamic acid (Hanby & Rydon, 1946) were selected as representatives of the acidic polyamino acids. Poly-L-lysine and poly-DL-lysine (Katchalski, Grossfeld & Frankel, 1948) as

well as poly-DL-ornithine and poly-DL-arginine (Katchalski & Spitnik, 1951) were selected as representatives of the basic polyamino acids.

In the following study the effect of the various polyamino acids on *Escherichia coli* and *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) is described, and an attempt is made to elucidate the mode of interaction between the bacterial cell and the polyamino acids.

Some preliminary notes on our work have appeared (Katchalski, Bichowski-Slomnitzki & Volcani, 1951, 1952). Burger & Stahmann (1952) have reported independent observations on the action of lysine peptides on bacteria. Their findings were in accord with our own data on the inhibition of bacterial growth and on agglutination.

MATERIALS AND METHODS

Poly- α -amino acids. Poly-L-lysine and poly-DL-lysine hydrochlorides with an average degree of polymerization $n=30$ to 35, were prepared according to Katchalski *et al.* (1948). Poly-DL-ornithine hydrochloride ($n=27$) and poly-DL-arginine sulphate ($n=27$) by the method of Katchalski & Spitnik (1951), and poly-L-aspartic acid ($n=70$) according to Berger & Katchalski (1951). Poly-D-glutamic acid derived from *Bacillus anthracis* was kindly supplied by Dr H. N. Rydon (cf. Hanby & Rydon, 1946). Poly-DL-alanine was prepared according to Astbury *et al.* (1948).

Lysine peptides. Di-L-lysine dihydrochloride was prepared according to Erlanger & Brand (1951), tri-L-lysine trihydrochloride according to Brand, Erlanger, Polatnick, Sachs & Kirschenbaum (1951) and tetra-L-lysine tetrahydrochloride according to Waley & Watson (1953).

The above polyamino acids and peptides were used in aqueous solutions adjusted to pH 7.0. The acidic polyamino acids were dissolved as sodium salts.

Organisms. *Escherichia coli* and *Staphylococcus aureus* were obtained from the Department of Bacteriology, Hebrew University Hadassah Medical School, Jerusalem.

Media. The synthetic medium used in the growth experiments with *Esch. coli* contained $(\text{NH}_4)_2\text{HPO}_4$, 0.5 g.; KH_2PO_4 , 0.035 g.; NaCl, 0.2 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g., and glucose, 0.3 g. in 100 ml. distilled water. pH adjusted to 7.4.

The semi-synthetic medium for the growth of *Staph. aureus* contained: acid casein hydrolysate vitamin free (Nutritional Biochemicals Inc.), 3 ml.; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.25 g.; KH_2PO_4 , 0.035 g.; NaCl, 0.15 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g.; vitamin mixture solution (Henderson & Snell, 1948), 1 ml.; traces of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and MnSO_4 , and glucose, 0.3 g. in 100 ml. distilled water, pH adjusted to 7.4.

Bacterial suspensions. The number of viable cells was determined by plating. The turbidity of the suspensions was measured with the Klett-Summerson photoelectric colorimeter using a red filter no. 64 (640 m μ). A viable cell count of 1.2×10^9 cells was found to correspond to 0.031 mg. nitrogen (Kjeldahl).

In the growth experiments, 24 hr. old cultures on Difco nutrient agar were harvested and resuspended in saline to 10^7 viable cells/ml. Each test tube was inoculated with 0.1 ml. In respiration and viability experiments, 20 hr. old cultures on nutrient agar containing 0.2% glucose were

harvested, washed twice with saline, and resuspended in 0.066 M-phosphate buffer of pH 7.4 to 4×10^8 viable cells/ml. In electrophoresis, adsorption and agglutination experiments, 20 hr. old cultures grown on nutrient agar were used. The organisms were washed twice with the salt or buffer solution used in the final experiment, and resuspended in the same salt or buffer solution to the required concentration.

Growth tests. 18 $\times$ 150 mm. rimless Pyrex test tubes containing 2.5 ml. of a double-strength synthetic or semi-synthetic medium were capped and autoclaved for 15 min. at 15 lb. The various polyamino acids were added aseptically as aqueous solutions, previously sterilized by autoclaving. The volume was adjusted to 4.9 ml., and each tube inoculated with 10^6 organisms in 0.1 ml. saline. Growth was recorded turbidimetrically after incubation at 37° for 24 hr. Each test was conducted in duplicate.

Respiration experiments. The conventional Warburg manometric technique was employed. Each vessel contained 2 ml. fluid consisting of: 0.5 ml. 0.066 M-phosphate buffer pH 7.4; 0.5 ml. poly-L-lysine solution; 0.5 ml. 0.166 M-glucose, and 0.5 ml. bacterial suspension. The centre well contained a folded strip of filter-paper moistened with 0.2 ml. of 10% KOH. In experiments where the immediate effect of poly-L-lysine on the respiration was measured, the bacteria were put into the side arm and were tipped after equilibration. When the effect of pre-incubation of bacteria with polylysine on their respiration was investigated, the glucose was kept in the side arm and tipped after 1, 3 and 6 hr. respectively. The flasks were shaken at a rate of 90 oscillations/min. at a constant bath temperature of 37°. Air atmosphere was used and readings taken at 10 min. intervals.

Electrophoresis experiments. These were performed at 22° in the apparatus of Northrop & Kunitz (1925). The position of the stationary layer was determined according to Abramson, Moyer & Gorin (1942). A Spencer phase-contrast turret condenser, a $\times 20$ dark medium objective and $\times 25$ ocular provided the optical system (Hartman, Bateman & Laufer, 1952). To 30 ml. of a bacterial suspension of 2×10^8 viable cells/ml. prepared in various concentrations of NaCl or phosphate buffers different amounts of poly-L-lysine in 0.3 ml. were added. The mixture was left at room temperature for 5–10 min. and then introduced into the cataphoretic cell. The excursion time of twenty individuals was measured, at the stationary layer, over a distance of 50–100 μ . with the current passing first in one and then in the reverse direction. The electrophoretic mobility (u) in μ /sec./volt/cm. was calculated from the average velocity of the bacteria (v), current strength (I), the cross-section of the rectangular chamber (g) and the specific resistance (r) of the bacterial suspension by the formula (cf. Abramson *et al.* 1942):

$$u = vg/Ir.$$

Adsorption experiments. Bacterial suspensions containing a known number of organisms were mixed with equal volumes of poly-L-lysine solutions of known concentration. The mixtures were shaken for 5–10 min. at room temperature and the bacteria centrifuged off in a Servall high-speed centrifuge for 5 min. at 8500 g. Preliminary experiments proved that the adsorption reached an equilibrium within this time interval. The amount of poly-L-lysine in the supernatant was determined as follows: to 5 ml. samples of the clear supernatant in a Pyrex test tube conc. HCl was added, to a final concentration of 10%. Larger samples, up to

25 ml., were taken for hydrolysis when the adsorption experiments were carried out with low polylysine concentrations (1–20 $\mu\text{g./ml.}$). The tubes were sealed and autoclaved for 6 hr. at 15 lb. The hydrolysates were evaporated to dryness in a vacuum desiccator over H_2SO_4 and KOH, and the amount of L-lysine determined by a microbiological bioassay procedure, using a L-lysine-requiring mutant of *Esch. coli* grown in the minimum medium described by Davis & Mingioli (1950). From the data obtained the amount of poly-L-lysine adsorbed was calculated.

Agglutination experiments. 1 ml. bacterial suspension, containing 2.4×10^8 organisms in 0.001 M-NaCl or 0.001 M-phosphate buffer pH 7.0, was mixed with 1 ml. of polylysine solution of specified concentration, in the corresponding medium, and the mixture incubated at 37°. The agglutination was recorded after 0.5, 1, 2 and 24 hr.

RESULTS

Effect on bacterial growth. Tests in the presence of varying amounts of different polyamino acids showed that poly-DL-alanine, poly-D-glutamic acid and poly-L-aspartic acid up to concentrations of 500 $\mu\text{g./ml.}$ do not inhibit the growth of *Esch. coli* and *Staph. aureus*. The basic polyamino acids, on the other hand, caused growth inhibition even at low concentration. The minimum concentrations required to inhibit growth are given in Table 1. The data concerning the lysine peptides show that the antibacterial activity increases with molecular weight. L-Lysine did not inhibit growth even at concentration of 10 mg./ml.

In order to clarify whether the growth-inhibiting effect of poly-L-lysine is bactericidal or bacteriostatic, another set of experiments was carried out. *Esch. coli* and *Staph. aureus* were treated with poly-L-lysine under conditions similar to those given in Table 1. The test tubes in which complete growth inhibition occurred after 24 hr., were centrifuged, the supernatants discarded, and 5 ml. of a fresh sterile medium added. The tubes were then incubated for an additional 24 hr. at 37°. The action of polylysine was judged to be bacteriostatic where growth appeared after the above treatment. Poly-

L-lysine hydrochloride was found to be bacteriostatic to *Esch. coli* within the range of 2.5–75 $\mu\text{g./ml.}$ and to *Staph. aureus* within the range of 1.0–75 $\mu\text{g./ml.}$ Poly-DL-lysine hydrochloride acted as a bacteriostatic agent on both organisms at the concentration range 5–75 $\mu\text{g./ml.}$ The above data show that optically active and racemic lysine polymers act as bacteriostatic agents in a rather wide range of concentrations. At concentrations higher than 75 $\mu\text{g./ml.}$ both polymers showed bactericidal activity.

Reversal of poly-L-lysine growth inhibition by poly-L-aspartic and poly-D-glutamic acids. The data presented in Table 2 show that growth of *Esch. coli* in a mixture of poly-L-aspartic acid and poly-L-lysine occurred only when the molar concentrations of the carboxyl groups of the acidic polymer approximately equalled or exceeded the molar concentration of the ϵ -amino groups of the basic polymer. A similar antagonistic behaviour was found between poly-L-lysine and poly-D-glutamic acid. L-Aspartic and L-glutamic acids as well as L-lysine, up to concentrations of 1000 $\mu\text{g./ml.}$ failed to reverse the growth inhibition of poly-lysine.

Table 1. Minimum concentrations of lysine peptides and of basic poly- α -amino acids required to inhibit growth of *Esch. coli* and *Staph. aureus*

(Test tubes containing different amounts of polyamino acids and peptides (0–500 $\mu\text{g./ml.}$) in 5 ml. media, were inoculated with 10^6 organisms and incubated for 24 hr. at 37°. Minimum concentrations in $\mu\text{g./ml.}$ at which no increase in turbidity occurred are recorded below.)

Peptides	<i>Esch. coli</i>	<i>Staph. aureus</i>
Di-lysine hydrochloride	450	.
Tri-L-lysine hydrochloride	150	.
Tetra-L-lysine hydrochloride	75	.
Poly-L-lysine hydrochloride	2.5	0.5 to 1.0
Poly-DL-lysine hydrochloride	5	3
Poly-DL-ornithine hydrochloride	10	5
Poly-DL-arginine sulphate	10	5

Table 2. Reversal of the growth-inhibiting action of poly-L-lysine on *Esch. coli* by poly-L-aspartic acid

(To test tubes containing different amounts of poly-L-lysine in 4.7 ml. medium, inoculated with 10^6 organisms, varying amounts of poly-L-aspartic acid, in 0.3 ml. water, were added. Incubation at 37° for 24 hr. The final concentrations of polylysine hydrochloride and polyaspartic acid are given in μmoles of lysine hydrochloride and aspartic acid residues per ml. 1 μmole lysine hydrochloride residue = 164.7 $\mu\text{g.}$; 1 μmole aspartic acid residue = 115 $\mu\text{g.}$; –, no growth; +, moderate; ++, strong growth.)

Polylysine hydrochloride (μmoles lysine residue/ml.)	Poly-L-aspartic acid (μmoles aspartic acid residue/ml.)					
	0.0	0.01	0.025	0.05	0.10	0.20
0	++	++	++	++	++	++
0.025	–	–	+	++	++	++
0.050	–	–	–	++	++	++
0.10	–	–	–	–	++	++
0.20	–	–	–	–	–	++

Fig. 1. Effect of poly-L-lysine in various concentrations on the O_2 uptake of *Staph. aureus* in the presence of 0.04M-glucose. A, control respiration; B, 50 $\mu\text{g./ml.}$; C, 100 $\mu\text{g./ml.}$; D, 500 $\mu\text{g./ml.}$; E, 1000 $\mu\text{g./ml.}$ poly-L-lysine hydrochloride.

Fig. 2. Oxygen uptake of *Esch. coli* in the presence of 0.04M-glucose after various periods of pre-incubation with 500 $\mu\text{g./ml.}$ of poly-L-lysine hydrochloride. A, respiration of cells previously incubated without poly-L-lysine for 0, 3 or 6 hr. at 37°; B, poly-L-lysine added immediately; C, 3 hr. pre-incubation with poly-L-lysine; D, 6 hr. pre-incubation with poly-L-lysine.

Fig. 3. Viable counts of *Esch. coli* incubated with 500 $\mu\text{g./ml.}$ poly-L-lysine hydrochloride for 0, 3 and 6 hr. at 37° (full line). Number of cells after addition of glucose, determined at 15 min. intervals (broken line).

Fig. 4. Viable counts of *Staph. aureus* incubated with 1000 $\mu\text{g./ml.}$ poly-L-lysine hydrochloride for 0, 3 and 6 hr. at 37° (full line). Number of cells after addition of glucose determined at 15-min. interval (broken line).

Action of poly-L-lysine on the oxygen consumption and viability. Poly-L-lysine caused a marked inhibition of respiration of *Esch. coli* and *Staph. aureus*. A representative experiment for *Staph. aureus* is given in Fig. 1. Increasing poly-L-lysine concentrations caused a steady decrease in oxygen consumption. A similar effect was observed with *Esch. coli*.

The effect of pre-incubation of *Esch. coli* with polylysine (500 $\mu\text{g./ml.}$) on the oxygen consumption is given in Fig. 2. The inhibition of oxygen consumption increased with pre-incubation time. A similar effect was found for *Staph. aureus*.

In order to examine the relation between oxygen uptake and the number of viable cells, counts were carried out on nutrient agar medium using cell suspensions of *Esch. coli* and *Staph. aureus* treated exactly as in the respiration experiments. The results are summarized in Figs. 3 and 4. Fig. 3 shows

that during incubation of *Esch. coli* with polylysine a steady decrease in the number of viable cells occurred; the rate of decrease was markedly enhanced on addition of glucose. In the case of *Staph. aureus* (Fig. 4) almost no decrease in viable cells was observed upon incubation. The addition of glucose, at various time intervals caused, also in this case, a marked decrease in the number of viable cells.

A comparison of the rates of respiration with the number of viable cells of *Esch. coli* under similar conditions of pre-incubation indicated that the percentage decrease in the number of viable cells very markedly exceeds the percentage decrease in the rate of oxygen uptake. For *Staph. aureus* the decrease appeared to parallel the depletion in the number of viable cells.

The influence of polyamino acids on the electrophoretic mobilities of *Esch. coli* and *Staph. aureus*. In a study of the influence of the various polyamino acids on the electrophoretic mobility of *Esch. coli* and *Staph. aureus* in 0.001 M-phosphate buffer pH 7.0 or 0.001 M-sodium chloride, it was found that poly-DL-alanine up to a concentration of 100 $\mu\text{g./ml.}$ had practically no effect on either organism. Poly-L-aspartic and poly-D-glutamic acids caused a slight increase in the mobility under similar conditions. The basic polyamino acids, on the other hand, caused, even at low concentrations, a significant change in the electrophoretic mobility. The interaction with poly-L-lysine was studied in greater detail.

Figs. 5 and 6 give the effect of various starting concentrations of polylysine on the electrophoretic mobility of *Esch. coli* and *Staph. aureus*. With increasing polylysine concentrations the mobility drops to zero and finally reverses sign. The mobility of *Esch. coli* (Fig. 5) drops to zero, in the presence of 0.001 M-sodium chloride at a polylysine concentration of 5.0 $\mu\text{g./ml.}$ and in the presence of 0.001 M-phosphate buffer at a polylysine concentration of 7.5 $\mu\text{g./ml.}$ The mobility of *Staph. aureus* (Fig. 6) drops to zero, under similar conditions, at polylysine concentrations of 1.25 and 4.2 $\mu\text{g./ml.}$ respectively. A further increase in polylysine concentration causes a gradual increase in the electrophoretic mobility in the reverse direction. At a very large excess of the peptide the electrophoretic velocity was found to be comparable in magnitude but opposite in sign to that of the control.

The electrophoretic mobilities were measured 5–10 min. after admixture of the peptide solution with the micro-organisms. No additional change was observed for 0.5–1 hr. As *Esch. coli* and *Staph. aureus* are negatively charged in the media used, it could be assumed that the electrostatic attraction between the positively charged polylysine and the negatively charged bacteria plays a significant role in determining the extent of interaction between the cell and the peptide. It seemed, therefore, desirable

Fig. 5. Electrophoretic mobility of *Esch. coli* in the presence of poly-L-lysine. ●—●, 0.001 M-phosphate buffer, pH 7.0; ○—○, 0.001 M-NaCl.

Fig. 6. Electrophoretic mobility of *Staph. aureus* in the presence of poly-L-lysine. ●—●, 0.001 M-phosphate buffer, pH 7.0; ○—○, 0.001 M-NaCl.

to check the influence of the ionic strength of the medium on the above interaction.

The influence of various concentrations of phosphate buffer on the mobility of *Esch. coli* in the presence of polylysine is shown in Fig. 7. Since the initial mobility of the bacteria, u_0 , drops with the increase in buffer concentration, we used the term $\left(\frac{u_0 - u}{u_0}\right) \times 100$ to express the effect of polylysine on the electrophoretic mobility. u designates the mobility measured in the presence of polylysine. Its value has the sign of u_0 as long as the bacteria travel to the anode, the sign is reversed when the organisms start to travel toward the cathode. In accord with expectation, it was found that high buffer concentrations depress the effect of polylysine on the mobility. At buffer concentrations higher than 0.033 M, polylysine, even at a concentration of 100 $\mu\text{g./ml.}$, is incapable of lowering the electrophoretic mobility to zero; in 0.001 M-phosphate buffer a concentration of polylysine of 10 $\mu\text{g./ml.}$ is already sufficient to reverse the direction of the electrophoretic mobility of *Esch. coli*. Similar results were obtained with *Staph. aureus*.

Fig. 7. Effect of various concentrations of phosphate buffer, pH 7.0, on the electrophoretic mobility of *Esch. coli* in the presence of poly-L-lysine. Concentrations of phosphate buffer: $\circ$ — $\circ$, 0.001 M; $\bullet$ — $\bullet$, 0.0083 M; $\square$ — $\square$, 0.017 M; $\triangle$ — $\triangle$, 0.033 M; $\times$ — $\times$, 0.066 M. u_0 , initial mobility in the absence of polylysine. u , mobility in the presence of polylysine. The initial electrophoretic mobilities in the absence of polylysine were: 0.001 M-phosphate buffer, 3.0; 0.0083 M, 1.7; 0.017 M, 1.5; 0.033 M, 1.15 and 0.066 M, 0.85 $\mu\text{./sec./volt/cm.}$

Fig. 8. Restoration of electrophoretic mobility of *Esch. coli* treated with poly-L-lysine in 0.001 M-phosphate buffer, pH 7.0, by poly-L-aspartic acid (PAA).

Fig. 9. Restoration of electrophoretic mobility of *Staph. aureus* treated with poly-L-lysine in 0.001 M-phosphate buffer, pH 7.0, by poly-L-aspartic acid (PAA).

The effect of polylysine on the electrophoretic mobility was found to be dependent also on the concentration of sodium chloride. However, in this case, higher salt concentrations than 0.033 M were required to diminish markedly the effect of polylysine on the electrophoretic mobility of both organisms.

The electrophoretic mobility of cells, brought to zero by polylysine, could be restored to its original value by the addition of poly-L-aspartic acid in equivalent or larger quantities. Representative experiments are given in Figs. 8 and 9. It is pertinent to remark that the mobility of *Staph. aureus* in 0.001 M-phosphate buffer pH 7 was decreased also by the addition of protamine sulphate or tyrothricin. The mobility dropped to zero at a concentration of 7.5 $\mu\text{g./ml.}$ of the former substance and at 60 $\mu\text{g./ml.}$ of the latter. It could be restored to its original value in both cases, by the addition of 2.7 $\mu\text{g./ml.}$ of poly-L-aspartic acid.

Fig. 10. Uptake of poly-L-lysine by *Esch. coli*, $\circ$ — $\circ$, and *Staph. aureus*, $\bullet$ — $\bullet$. Adsorption experiments were carried out with 2×10^8 cells/ml. in 0.001 M-phosphate buffer, pH 7.0. Temp. = 20°.

Adsorption experiments. The adsorption of poly-L-lysine hydrochloride by 2×10^8 viable cells of *Esch. coli* and *Staph. aureus* in the presence of increasing polylysine concentrations in 0.001 M-phosphate buffer pH 7.0 is given in Fig. 10. In both cases a typical adsorption curve is obtained. Saturation occurred at an adsorption of 6.5 $\mu\text{g.}$ polylysine hydrochloride by *Esch. coli* and 4.2 $\mu\text{g.}$ polylysine by *Staph. aureus*. The corresponding adsorption curves in the presence of 0.001 M-sodium chloride were found to be similar to those in phosphate buffer.

Poly-L-lysine uptake at different initial concentrations and different numbers of cells per ml. of *Esch. coli* and *Staph. aureus* in distilled water was also investigated. In the presence of an excess of polylysine the amount adsorbed was found to be approximately proportional to the number of cells. Under these conditions each cell of *Esch. coli* and

Staph. aureus adsorbed on the average $7.0 \mu\text{g.}/2 \times 10^8$ and $4.5 \mu\text{g.}/2 \times 10^8$ polylysine hydrochloride respectively. These findings are in accord with the above data.

A representative experiment on the effect of pH and concentration of buffer on the adsorption of polylysine by *Esch. coli* is given in Fig. 11. At a very low buffer concentration the adsorption was found to be independent of pH in the range pH 5 to 8. The increase in buffer concentration in all the pH values investigated leads to a marked decrease in the adsorption.

Agglutination of bacteria by poly-L-lysine. Table 3 shows that polylysine can cause agglutination of *Esch. coli* and *Staph. aureus*. Observations were made after incubation for 0.5, 1.0, 20 and 24 hr. The agglutination of *Esch. coli* came to completion in 0.001 M-sodium chloride and 0.001 M-phosphate buffer pH 7 within 1 hr., that of *Staph. aureus* under similar conditions was rather slow.

The concentration range at which agglutination occurred is dependent on the organism as well as on the medium used. With an excess of polylysine resuspension of the agglutinated bacteria occurred.

Fig. 11. Effect of pH and concentration of phosphate buffer on the adsorption of poly-L-lysine by *Esch. coli*. ●—●, pH 5.2; △—△, pH 6.5; ■—■, pH 7.2; ▽—▽, pH 8.0. Initial concentration of poly-L-lysine hydrochloride, $50 \mu\text{g.}/\text{ml.}$ throughout. Number of cells, $3 \times 10^8/\text{ml.}$ Temp. = 20° .

These findings are in accord with the electrophoretic experiments, where it has been shown that by increasing gradually the concentration of polylysine the electrophoretic mobility of the bacteria decreases, drops to zero and finally increases in the reverse direction.

In some additional experiments it was found that an increase in phosphate buffer concentration leads to an increase in the minimum concentration required to cause agglutination. The agglutination experiments under these conditions were again in agreement with the corresponding electrophoretic data.

DISCUSSION

The fact that the neutral and acidic polyamino acids failed to show any effect on the growth of the bacteria, while all the basic polyamino acids tested were strongly inhibitory, indicates that the cationic nature of the basic polypeptides plays an important role in determining their antibacterial activity. The importance of the polypeptide structure in determining the biological activity investigated is stressed by the fact that the basic amino acid monomers do not show any growth-inhibiting action towards the organisms tested. The observation that the antibacterial activity of the lysine peptides is a function of their molecular weight is in accord with the finding of Burger & Stahmann (1951), that the antiviral activity of lysine peptides increases with chain length.

The antibacterial activity of the basic polyamino acids resembles closely those of the natural protamines and histones (Miller, Abrams, Dorfman & Klein, 1942; Weissman & Graf, 1947) as well as those of the basic 'tissue polypeptide' isolated by Bloom *et al.* (1947). Both the synthetic and the natural compounds inhibit growth, cause agglutination and reduce the oxygen uptake of various organisms.

The absorption and electrophoretic experiments probably reflect an early step in the interaction

Table 3. *Agglutination of Esch. coli and Staph. aureus by poly-L-lysine*

(For details, see methods. In all experiments 1.2×10^8 cells/ml. were used. The bacteria were incubated with polylysine for 24 hr. at 37° and agglutination recorded visibly. —, No agglutination; +, moderate agglutination; ++, strong agglutination.)

Poly-L-lysine hydrochloride ($\mu\text{g.}/\text{ml.}$)	<i>Esch. coli</i>		<i>Staph. aureus</i>	
	Phosphate buffer, pH 7.0, 0.001 M	NaCl, 0.001 M	Phosphate buffer, pH 7.0, 0.001 M	NaCl, 0.001 M
0	—	—	—	—
5	—	—	—	—
10	++	++	+	—
20	++	++	+	+
30	++	++	++	++
40	—	++	++	++
50	—	—	++	++
75	—	—	—	—
100	—	—	—	—
200	—	—	—	—

between the polyamino acids and the bacteria. At present it is impossible to determine whether the interaction of the basic polyamino acids with the bacteria is an exclusively surface phenomenon, or whether the peptides may also penetrate into the cell. The forces responsible for the interaction of the synthetic polymer and the complicated systems of the cell are obscure. It may be assumed, however, that the interaction is determined by non-specific electrostatic forces as well as specific adsorption forces of the hydrogen bond and van der Waal's type. Such forces have been demonstrated in the association of macromolecules such as insulin (Doty, Gellert & Rabinovitch, 1952) or tropomyosin (Bailey, 1948), as well as in lyophobic colloids (Verwey & Overbeek, 1948).

The role of the electrostatic forces in the attraction between the negatively charged bacteria and the positively charged polylysine is shown by the following: (a) the lack of activity of neutral and acidic polyamino acids towards bacteria, (b) the change in mobility after adsorption of the basic polyamino acids, (c) the part played by the ionic strength of the environment in determining the extent of adsorption, and (d) the removal of polylysine from the bacteria by the action of the strongly negatively charged polyaspartic acid.

From the classical Debye-Hückel theory (1923) it is well known that an increase in the total ionic strength of the medium leads to a reduction in the electrostatic attraction between ions with opposite charge. If the attraction between polylysine and the bacterial surface is determined partially by electrostatic forces, it could be predicted that an increase in salt concentration would diminish the association between the components. This prediction has been substantiated. The complex between protamines or histones and nucleic acids is of a similar type and it is known to dissociate in the presence of a high salt concentration (Mirsky, 1947).

A neutralization of a basic polypeptide by an acidic polypeptide may be the basis of the antagonistic interaction of anthrax polyglutamic acid and the tissue polypeptide (cf. Bloom *et al.* 1947, 1948). In this connexion it is perhaps pertinent to note that a variety of naturally occurring antibacterial peptides such as tyrocidine and polymyxins, as well as many cationic detergents possess positively charged groups which may facilitate the approach to the negatively charged bacteria. The electrophoretic behaviour of bacteria treated with cationic detergents (Dyar & Ordal, 1946; McQuillen, 1950a) resembles their behaviour in the presence of polylysine.

The presence of specific non-electrostatic adsorption forces is suggested by the fact that bacteria with zero net charge are nevertheless capable of adsorbing polylysine from the medium. In *Esch. coli*

and *Staph. aureus* these forces are of considerable magnitude as they are capable in the experimental conditions given in Figs. 5 and 6 of reversing the charge on the bacteria, and lead to mobilities almost equal in magnitude but opposite in direction to those of the control bacteria. Very little can be said about these non-electrostatic adsorption forces, but it may be expected that they will be determined by the structure of the bacterial surface as well as by the chemical nature of the polymer.

A comparison of the agglutination data with the electrophoretic measurements shows that agglutination occurs in the polylysine concentration region in which the electrophoretic mobility drops to zero. This accords with the behaviour of a lyophobic colloidal suspension, where it has been found that the electrical charge of the colloidal particles prevents their association (Verwey & Overbeek, 1948). At high polylysine concentrations, resuspension of the bacteria occurs because of the positive charge acquired by the organisms.

From the quantitative adsorption experiments the amount of polylysine adsorbed by each cell can be calculated. The maximum amount of polylysine adsorbed in the presence of 2×10^8 viable cells of *Esch. coli* was found to be $6.5 \mu\text{g}$. If it is assumed that the viable cells represent about 80% of the total bacterial population, it may be calculated that each cell combined on the average with 9.5×10^7 lysine residues. A similar calculation for the adsorption of *Staph. aureus* gives for the maximum amount adsorbed 6.15×10^7 lysine residues per organism. As each of the lysine residues bears one positive charge, it is of interest to compare the total number of negative charges initially present on the surface of each bacterium with the number of lysine residues adsorbed. The number of charges, σ , per unit surface may be calculated from the electrophoretic mobility by the formula (cf. Abramson *et al.* (1942) page 133):

$$\sigma = \sqrt{\frac{NDkT}{2000\pi}} \sqrt{[\Sigma C_i (e^{-\frac{Z_i e \xi}{kT}} - 1) + \Sigma C_j (e^{\frac{Z_j e \xi}{kT}} - 1)]},$$

where C_i and C_j are concentrations of the cations and anions in moles/l., and Z_i and Z_j their valency. In the phosphate buffer used two cations, Na^+ and K^+ , and two anions, H_2PO_4^- and HPO_4^{2-} were present. ξ = the zeta potential, N = Avogadro's number, D = dielectric constant, k = Boltzmann's constant, T = absolute temperature, e = charge of electron, u = electrophoretic mobility.

At 25° , $e\xi/kT$ is equal to $\frac{1}{2}u$, when u is expressed in $\mu/\text{sec}/\text{volt}/\text{cm}$. σ , in electronic charges/square micron is given by

$$\sigma = 369\,000 \sqrt{[\Sigma C_i (e^{-\frac{1}{2}Z_i u}{kT}} - 1) + \Sigma C_j (e^{\frac{1}{2}Z_j u}{kT}} - 1)]}.$$

Table 4. *Number of lysine residues adsorbed and net charge per cell of Esch. coli and Staph. aureus*

(The data below were calculated from the adsorption experiments described in Fig. 10 and the electrophoretic experiments described in Figs. 5 and 6 carried out in 0.001 M-phosphate buffer, pH 7.)

Organism	Initial concentration of poly-L-lysine hydrochloride ($\mu\text{g./ml.}$)	Electrophoretic mobility ($\mu\text{./sec./volt/cm.}$)	Number of lysine residues adsorbed/cell	Net charge/cell (in electrons)
<i>Esch. coli</i>	0.0	-3.0	0.0	(-) 7.62×10^4
	7.5	0.0	8.7×10^7	0.0
	25.0	+2.4	9.5×10^7	(+) 8.50×10^4
<i>Staph. aureus</i>	0.0	-2.75	0.0	(-) 6.90×10^4
	4.2	0.0	4.35×10^7	0.0
	20.0	+1.25	6.15×10^7	(+) 3.69×10^4

As the surface of each bacterium is approximately $3 \mu^2$ (McQuillen, 1950b) the total number of charges per organism is given by 3σ , where σ is expressed in electron charges per square micron.

In Table 4 the net charge of *Esch. coli* and *Staph. aureus* and the amount of lysine residues adsorbed per organism is given. In both cases the number of lysine residues which have been adsorbed when the net charge has been brought to zero considerably exceeds the number of negative charges initially present on the bacterial surfaces. The adsorption of polylysine can therefore hardly be explained as a simple neutralization process. The relatively large adsorption of polylysine without corresponding effect on the bacterial net charge can be explained either by a corresponding adsorption of small counter anions, such as chloride, or by the extrusion of cations such as hydrogen ions from the bacterial cell. No search for either of these mechanisms was made.

It is of interest to note that the data of McQuillen (1950a) concerning the adsorption of cetyltrimethylammonium bromide by Gram-positive bacteria and the effect of adsorbed detergent on the net charge of the organism reveals a similar discrepancy between the decrease in negative charge and the number of cationic detergent molecules adsorbed.

The above discussion dealt with the first stages of the interaction between the basic polyamino acids and the organisms investigated. As shown above, the adsorption of the basic polymers causes a decrease in oxygen consumption and rate of multiplication and finally leads to the death of the cell. It is as yet impossible to present a detailed picture of the biochemical reactions involved in these effects. By analogy with known interactions between substances of high molecular weight, it may be suggested that the following reactions are of possible significance in determining the antibacterial properties of the polymers investigated: (a) the interaction between the surface of the cell with the cationic

polymer may damage the cell surface; (b) a reaction between the nucleic acids of the cell and the synthetic polyamino acids may occur; (c) the basic polymers may interact with certain enzymes and thus block essential physiological processes in the cell.

The bactericidal cationic detergents as well as some basic natural polypeptides such as tyrocidine (Hotchkiss, 1944, 1946) are known to damage the cell surface of bacteria and facilitate the leakage of phosphorus- and nitrogen-containing compounds from the cell into the surrounding medium. In some preliminary experiments it was observed that the amount of phosphorus released immediately after addition of polylysine was negligible. Moreover, electron microscopical observations of bacteria treated with polylysine revealed no changes in the bacterial cell.

The basic polyamino acids form a precipitate with nucleic acids (Burger & Stahmann, 1952) resembling the complex between protamines and histones and nucleic acids. It can be assumed that a similar reversible complex may be formed *in vivo*. A complex between polylysine and nucleic acid may interfere with normal protein synthesis and with other cell processes in which nucleic acids participate.

Data in the literature indicate that specific high molecular weight substances, such as proteins and polypeptides, may reversibly inhibit enzyme activity. As a typical example the inhibition of pepsin by the basic peptide pepsin inhibitor may be quoted (Herriot, 1941). Another example may be found in the inhibition of trypsin by the pancreas and soy-bean inhibitors (Kunitz & Northrop, 1936; Kunitz, 1947). In some of these interactions it has been shown that the electrostatic attraction between the enzyme and the relatively high molecular weight inhibitor plays a significant role in the formation of the inactive complex. It seems, therefore, plausible that polylysine, polyornithine and polyarginine may also reversibly inactivate certain enzymes by an analogous reaction.

SUMMARY

1. The basic poly- α -amino acids, poly-L-lysine, poly-DL-lysine, poly-DL-ornithine and poly-DL-arginine, inhibit the growth of *Escherichia coli* and *Staphylococcus aureus* in synthetic and semi-synthetic media. The neutral poly-DL-alanine and the acidic poly-L-aspartic and poly-D-glutamic acids do not inhibit the growth of both organisms.

2. The growth-inhibitory effect of poly-L-lysine can be reversed by poly-L-aspartic and poly-D-glutamic acids.

3. Poly-L-lysine caused a decrease in oxygen consumption of *Esch. coli* and *Staph. aureus*. The decrease in respiration was compared with the decrease in number of viable cells.

4. The electrophoretic mobilities of *Esch. coli* and *Staph. aureus* in the presence of poly-L-lysine

was investigated. With increasing concentrations of the cationic polymer, the mobility drops to zero and finally reverses sign. The electrophoretic mobility of cells brought to zero with poly-L-lysine could be restored to its original value by the addition of poly-L-aspartic and poly-D-glutamic acids.

5. The amount of poly-L-lysine adsorbed by *Esch. coli* and *Staph. aureus* under various conditions was estimated.

6. Agglutination of *Esch. coli* and *Staph. aureus* occurred at poly-L-lysine concentrations at which the electrophoretic mobilities dropped approximately to zero. An excess of poly-L-lysine caused resuspension.

7. The role of electrostatic and nonelectrostatic forces in the interaction between the basic poly-amino acids and the bacteria was discussed.

REFERENCES

- Abramson, H. A., Moyer, L. S. & Gorin, M. H. (1942). *Electrophoresis of Proteins*. New York: Reinhold Publishing Corporation.
- Astbury, W. T., Dalglish, C. E., Darmon, S. E. & Sutherland, G. B. B. M. (1948). *Nature, Lond.*, **162**, 596.
- Bailey, K. (1948). *Biochem. J.* **43**, 271.
- Berger, A. & Katchalski, E. (1951). *J. Amer. chem. Soc.* **73**, 4084.
- Bloom, W. L. & Blake, F. G. (1948). *J. infect. Dis.* **83**, 116.
- Bloom, W. L., Watson, D. W., Cromartie, W. J. & Freed, M. (1947). *J. infect. Dis.* **80**, 41.
- Brand, E., Erlanger, B. F., Polatnick, J., Sachs, H. & Kirschenbaum, D. (1951). *J. Amer. chem. Soc.* **73**, 4027.
- Burger, W. C. & Stahmann, M. A. (1951). *J. biol. Chem.* **193**, 13.
- Burger, W. C. & Stahmann, M. A. (1952). *Arch. Biochem. Biophys.* **39**, 27.
- Davis, B. D. & Mingioli, E. S. (1950). *J. Bact.* **60**, 17.
- Debye, P. & Hückel, E. (1923). *Phys. Z.* **24**, 185.
- Doty, P., Gellert, M. & Rabinovitch, B. (1952). *J. Amer. chem. Soc.* **74**, 2065.
- Dyar, M. T. & Ordal, E. J. (1946). *J. Bact.* **51**, 149.
- Erlanger, B. F. & Brand, E. (1951). *J. Amer. chem. Soc.* **73**, 4025.
- Hanby, W. E. & Rydon, H. N. (1946). *Biochem. J.* **40**, 297.
- Hartman, R. S., Bateman, J. B. & Lauffer, M. A. (1952). *Arch. Biochem. Biophys.* **39**, 56.
- Henderson, L. M. & Snell, E. E. (1948). *J. biol. Chem.* **172**, 15.
- Herriot, R. M. (1941). *J. gen. Physiol.* **24**, 325.
- Hotchkiss, R. D. (1944). *Advanc. Enzymol.* **4**, 192.
- Hotchkiss, R. D. (1946). *Ann. N.Y. Acad. Sci.* **46**, 479.
- Katchalski, E. (1951). *Advanc. Protein Chem.* **6**, 123.
- Katchalski, E., Bichowski-Slomnitzki, L. & Volcani, B. E. (1951). *Bull. Res. Council, Israel*, **1**, 153.
- Katchalski, E., Bichowski-Slomnitzki, L. & Volcani, B. E. (1952). *Nature, Lond.*, **169**, 1095.
- Katchalski, E., Grossfeld, I. & Frankel, M. (1948). *J. Amer. chem. Soc.* **70**, 2094.
- Katchalski, E. & Spitnik, P. (1951). *J. Amer. chem. Soc.* **73**, 3992.
- Kunitz, M. (1947). *J. gen. Physiol.* **30**, 291, 311.
- Kunitz, M. & Northrop, J. H. (1936). *J. gen. Physiol.* **19**, 991.
- McQuillen, K. (1950a). *Biochim. biophys. Acta*, **5**, 463.
- McQuillen, K. (1950b). *Biochim. biophys. Acta*, **6**, 66.
- Miller, B. F., Abrams, R., Dorfman, A. & Klein, M. (1942). *Science*, **96**, 428.
- Mirsky, A. E. (1947). *Cold. Spr. Harb. Symp. quant. Biol.* **12**, 143.
- Northrop, J. H. & Kunitz, M. (1925). *J. gen. Physiol.* **7**, 729.
- Synge, R. L. M. (1949). *Quart. Rev. chem. Soc., Lond.*, **3**, 245.
- Verwey, E. J. W. & Overbeek, J. Th. G. (1948). *Theory of the Stability of Lyophobic Colloids*. New York, London, Amsterdam: Elsevier Publishing Co. Inc.
- Waley, S. G. & Watson, J. (1953). *J. chem. Soc.* p. 475.
- Weissman, N. & Graf, L. M. (1947). *J. infect. Dis.* **83**, 116.