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THE CHEMOTHERAPY AND PHARMACOLOGY OF THE POLYMYXINS

BY

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The polymyxins are a group of polypeptide antibiotics produced by many strains of a widely distributed soil organism, *Bacillus polymyxa.* Benedict and Langlykke (1947) were the first to report the antibacterial properties of cultures of a strain of this bacillus. Ainsworth, Brown, and Brownlee (1947) isolated an antibiotic from an organism identified as *Bacillus aerosporus,* which was later found to be synonymous with *B. polymyxa.* This antibiotic was then called " Aerosporin," but is now known as polymyxin A. Simultaneously Stansly, Shepherd, and White (1947) described an antibiotic produced by another strain of *B. polymyxa* which they named " polymyxin," but which is now known as polymyxin D.

The chemotherapeutic properties and pharmacology of polymyxin A were described by Brownlee and Bushby (1948), and the similarities and points of difference between polymyxins A and D and a third antibiotic, polymyxin B isolated in our laboratories, were debated in a symposium at the New York Academy of Sciences in May, 1948 (Brownlee, Bushby, and Short, 1949 ; White, Alverson, Baker, and Jackson, 1949).

Swift (1948) drew attention to the effect of polymyxin A in early cases of pertussis, and Long, Schoenbach, Bliss, Chandler, and Bryer (1949) found polymyxin D to be an effective therapeutic agent in infections in man by *Pseudomonas aeruginosa, Klebsiella pneumoniae, Haemophilus pertussis, Haemophilus influenzae,* and *Escherichia coli.* However, the purest available samples of both these antibiotics, when administered parenterally in man and animals, caused severe proteinuria. The lesions in the kidneys of animals were restricted to the first convoluted tubules, and Brownlee and Short (1948) showed that they could be minimized in dogs and rats by the simultaneous administration of DL-methionine or S-methylcysteine. Efforts were made to eliminate the toxic factor of polymyxin A by further purification. These failed, but the examination of a large series of freshly isolated strains and the search for mutants resulted in the selection of organisms yielding additional antibiotics with similar chemotherapeutic actions, but differing in chemical constitution and pharmacological properties. It was at this stage that agreement was reached that polymyxin should be the generic name of all these antibiotics (Brownlee and Stansly, 1949). The new antibiotics isolated in our laboratories were named polymyxins C and E.

The identification of these various substances as separate entities depended chiefly upon chemical investigations by a number of workers. In addition to a

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fatty acid present in all the polymyxins, which was shown by Wilkinson (1949) to be *d*-6-methyloctan-1-oic acid, the amino-acids present in their hydrolysates were as follows: polymyxin A contained D-leucine, L-threonine, and L-$\alpha\gamma$-diaminobutyric acid (Jones, 1948a and b; Catch and Jones, 1948; Catch, Jones, and Wilkinson, 1948); polymyxin B contained all these amino-acids and in addition D-phenylalanine (Jones, 1949; Catch, Jones, and Wilkinson, 1949); polymyxin C differed from polymyxin B in not containing leucine (Jones, 1949); polymyxin D contained the same amino-acids as polymyxin A with D-serine in addition (Jones, 1949; Bell, Bone, English, Fellows, Howard, Rogers, Shepherd, Winterbottom, Dornbush, Kuschner, and SubbaRow, 1949), and polymyxin E contained the same amino-acids as polymyxin A, but the intact antibiotics could be readily separated by paper chromatography (Jones, 1948c).

In vitro *antibacterial spectra*

The minimal concentrations of polymyxins A, B, C, D, and E, which inhibit the growth of large inocula of genera of bacteria observed by streaking 18-hour broth cultures on 2 per cent blood-agar containing twofold increasing concentrations of the antibiotics, are not significantly different.

The polymyxins may be estimated by a dilution method, or by a plate diffusion test by comparison with a reference standard of the identical polymyxin. Both methods are in use and are equally accurate. The diffusion method has been described by Benedict and Stodola (1948), who used *Brucella bronchiseptica* as a test organism, and by Stansly (1949) and Thorp and Stewart (1949). The activities of samples of polymyxin A were originally compared with a standard sample given a value in arbitrary units per mg. The purest samples of polymyxin A had approximately 10,000 of these units per mg. The samples of polymyxin D used in these investigations were about 50 per cent pure, and when assayed against the polymyxin A standard contained approximately 5,000 units per mg. With polymyxins B and E separate reference standards were set up by comparison with the reference standard of A, and the purest samples of these antibiotics were also found to have a value of approximately 10,000 units per mg. Polymyxin C was never isolated in large quantities, and has been assayed only against the polymyxin A standard. In the investigations reported here the value of the unit can be regarded as the same for all these antibiotics.

A more accurate method of comparison, not applicable to slow-growing organisms, is that of adding large inocula to nutrient broth containing concentrations of polymyxin, which are varied by two-third or four-fifth increments, and observing growth after incubation for four to seven hours. By this means a clean bactericidal end-point is achieved, and the confusion, caused by small numbers of survivors multiplying after 24 hours, is avoided. A comparison of the activity of polymyxin A, B, and E against suitable strains is made in Table I, in which the intrinsic efficiencies are expressed in terms of polymyxin A. It is apparent that, within the limits of experimental error, the intrinsic activities of the three antibiotics against these three organisms tested are identical.

That the efficiency of the polymyxins is related to the concentration of organisms may be shown by observing the antibacterial effect of graded doses of the polymyxins upon inocula of *Salmonella typhosa* of varying size. With tests made in nutrient

TABLE I

COMPARISON OF THE ANTIBACTERIAL ACTIVITY OF POLYMYXINS A, B, AND E AGAINST LARGE INOCULA OF QUICK-GROWING GENERA

The activity (*A*) of B and E is expressed in terms of A. The standard deviation (S.D.) and the number of observations (*n*) are given

Polymyxin	*Escherichia coli*			*Salmonella typhosa*			*Shigella paradysenteriae*		
	A	S.D.	*n*	*A*	S.D.	*n*	*A*	S.D.	*n*
A	(100%)			(100%)			(100%)		
B	100%	27	6	93%	38	3	96%	22	4
E	100%	19	6	103%	16	6	104%	21	4

broth* polymyxins A and C are most, and polymyxin D is least, affected by the size of the inoculum, whilst B and E are intermediate.

Polymyxin A was shown to be bactericidal and never merely bacteriostatic (Brownlee and Bushby, 1948). The organisms do not have to be actively multiplying in order to be susceptible and they are affected both in water and in broth. It was also shown that the presence of serum modified slightly the effective lethal concentrations of polymyxin A and reduced the bactericidal rate. Polymyxins B and E behave in exactly the same way. When a constant inoculum is used the number of organisms killed by the antibiotics depends on the concentration.

In vivo *antibacterial activity*

Polymyxin A has been previously shown (Brownlee and Bushby, 1948) to be more effective weight for weight than streptomycin in protecting groups of mice against experimental infections caused by *S. typhosa, Esch. coli, H. pertussis, Br. bronchiseptica,* and to be equally effective in infections caused by *H. influenzae* and *Ps. aeruginosa.* The present comparisons with polymyxins A, B, C, D, and E are restricted to chemotherapeutic experiments with *S. typhosa* and *H. pertussis* in mice.

S. typhosa.—Mice were infected intraperitoneally with 0.5 ml. of a suspension of Rawling's strain, containing 10,000 average lethal doses, in 5 per cent mucin. This caused such an overwhelming infection that the untreated mice were either ill or dead within six hours, and all were dead within 18 hours.

H. pertussis.—The infecting agent was the mouse-passaged strain of Kendrick, Eldering, Dixon, and Misner (1947). A broth suspension containing 10^7 organisms per ml. was washed from a 48-hour culture on Bordet-Gengou medium. Of this a dose of 0.05 ml. containing about 10,000 average lethal doses, injected intracerebrally, caused death within four to nine days.

In both experiments the drugs were administered immediately after infection and again six hours later on the first day; during the subsequent days injections were given at 9 a.m. and 5 p.m. Typical protocols illustrating the protection by five polymyxins for *S. typhosa* and *H. pertussis* are shown in Table II. Against *S. typhosa* all the polymyxins appear to be equally active chemotherapeutically, but against *H. pertussis* polymyxin D is only about half as active as the other forms.

* An aqueous extract of horse muscle, to which sodium chloride was added together with papain digest of horse muscle and adjusted to *p*H 7.4. Nutrical agar was made by the addition of 1.2% New Zealand agar.

TABLE II

COMPARATIVE EFFICIENCY OF FIVE POLYMYXINS IN PROTECTING MICE AGAINST *S. typhosa* AND *H. pertussis*

Organism and conditions	Dose in units	Percentage survival with polymyxins				
		A	B	C	D	E
S. typhosa						
Groups of ten mice dosed	1,000	100	90	100	100	100
twice daily for four days*	500	80	90	100	100	100
	250	80	40	90	80	30
H. pertussis						
Groups of 8–10 mice dosed	1,000	—	—	—	100	—
twice daily for three days†	500	100	100	100	37	80
	250	70	90	90	—	60

* Mice observed for seven days. All control mice died on the first day.
† Mice observed for 14 days. All control mice died on the eighth day.

Absorption.—In these studies we have confined our attention to polymyxins A, B, and E. Polymyxin D was not available in sufficient amount, and polymyxin C was early observed to possess high renal toxicity and therefore was of no further interest.

For the determination of blood levels the serial dilution test of Brownlee and Bushby (1948) was employed, 1 per cent glucose phenol red broth containing 25 per cent (v/v) human or horse serum being used and either *Shigella paradysenteriae* or *Esch. coli* as test organism.

Since the minimal inhibitory concentration of polymyxin varies with the amount of serum, a uniform final serum concentration is aimed at by making the first dilution of the test serum in broth containing no serum. Minimal concentrations of polymyxin may be detected by keeping the inoculum of the test organism small; a suitable inoculum is that which corresponds to a final dilution of 10^{-6} of a 24-hour broth culture. So that a high concentration of the test serum shall not inhibit the small inoculum by reason of its antibacterial properties, the test serum is first heated for 20 min. at 56° C. to inactivate complement. Read after 18 hours' incubation at 37° C., this test detects approximately 2 units per ml. of polymyxin with *Shig. paradysenteriae* or 4 units per ml. with *Esch. coli*. Because the end-point varies with the size of the inoculum, serum containing a known amount of polymyxin must be assayed with each set of assays. It is convenient to add penicillin 0.5 I.U./ml. to the medium in order to inhibit Gram-positive contaminants.

Oral administration.—The polymyxins (A, B, and E) are not readily absorbed from the alimentary tract except in the newborn. Rabbits given 100,000 u. per kg., twice daily, dogs given 500,000 u. per kg., twice daily, and man given 1 mega unit every four hours, show no detectable antibiotic in the blood. Very large oral doses (300,000 units) are, however, lethal to mice in which the LD50 is approximately 10 mega units per kg. and the blood concentration at death about 150 u. per ml.

The alimentary tract of the newborn is more permeable. Thus seven guinea-pigs, less than 24 hours old, given 100,000 units of polymyxin B orally had

blood concentrations of 8 to 64 units per ml. two hours later, whereas the blood of control adult guinea-pigs given 1 mega unit contained no detectable antibiotic. In bovines the antibiotic in doses of 5 or 10 mega units is absorbed, provided it is given within the first forty-eight hours of life. Animals less than twelve hours old, after a single dose of polymyxin E, have detectable blood concentrations for ten to twenty hours. In general, animals which did not absorb polymyxin also failed to absorb lamb dysentery antiserum (*Clostridium welchii* type B antiserum) given simultaneously by mouth in a dose of 70,000 units. (For these titrations we are indebted to our colleagues, Mr. A. Thomson, M.R.C.V.S., and Miss Helen E. Ross, B.Sc.)

Only a small proportion of an oral dose can be recovered from the faeces in biologically active form. For example, Table III gives the concentrations of polymyxins A, B, and E detected in the faeces of rabbits given 100,000 units per kg. twice daily by mouth. The diet consisted of bran, sugar-beet pulp, and cabbage. The undried faeces were weighed, suspended in phosphate buffer of *p*H 4 in a concentration of 1 g. per 10 ml., adjusted to *p*H 4 if necessary, and heated to 100° C. for 20 min. This treatment kills the vegetative forms, but does not inactivate the antibiotic. The concentration of polymyxin was assayed in the clear supernatant after centrifugation.

TABLE III

THE EFFECT OF THE ORAL ADMINISTRATION OF POLYMYXINS A, B, AND E ON THE BACTERIAL FLORA IN THE FAECES OF RABBITS

	Polymyxin A			Polymyxin B			Polymyxin E		
	Polymyxin concn. u./g.	No. bacteria in millions per g.		Polymyxin concn. u./g.	No. bacteria in millions per g.		Polymyxin concn. u /g.	No. bacteria in millions per g.	
		Total	Polymyxin insensitive		Total	Polymyxin insensitive		Total	Polymyxin insensitive
Before treatment ..	<10	2.4	0.8	<10	0.24	0.08	<10	0.5	0.1
1st day ..	<10	0.16	0.03	<10	0.05	0.03	<10	0.08	0.01
2nd day ..	320	0.06	0.08	320	0.04	0.035	640	0.04	<0.001
3rd day ..	640	0.06	0.03	1,280	0.08	0.06	1,280	0.004	<0.001

In Table III the effects on the aerobic bacterial flora of the faeces are also shown. Viable counts were made of the total aerobes and of those insensitive to 100 units per ml. of polymyxin. The three polymyxins greatly diminish the total number of viable organisms, as well as that of organisms " insensitive " to polymyxin. By the third day of treatment no *Esch. coli* could be grown from 0.01 g. of faeces.

Similarly in adult man 1 mega unit of polymyxin B or E administered four-hourly eliminates *Esch. coli* from the faeces, as shown by directly plating one loopful on to nutrient agar. A four-hourly dose of 20,000 units/kg. in infants has the same effect.

Parenteral administration

Estimates of polymyxins A, B, and E in blood cells and plasma from rabbits, dogs, and man, given the antibiotics parenterally an hour earlier, showed that the polymyxins remained in the plasma and did not enter the cells.

After a single subcutaneous or intramuscular injection the polymyxins readily enter the blood stream, and are lost at about the same rate as other antibiotics such as penicillin or streptomycin. In Table IV are set out the blood levels in nine

TABLE IV

BLOOD LEVELS OF POLYMYXINS A, B, AND E IN RABBITS AND DOGS AFTER INTRAMUSCULAR ADMINISTRATION

RABBITS

	Time in hours	Concentrations in units per ml. for doses					
		40,000 u./kg.		20,000 u./kg.		10,000 u./kg.	
Polymyxin A ..	0.5	76	76	24	20	5	10
	1.0	19	38	24	20	5	10
	2.0	19	38	12	10	2.5	10
	3.0	—	19	12	—	2.5	5
	4.0	—	—	6	5	<2.5	<2.4
	5.0	5	5	3	2.5	—	—
	6.0	<2.5	—	<1.5	<2.5	—	—
Polymyxin B ..	0.5	19	26	24	10	10	13
	1.0	19	20	24	10	10	13
	2.0	5	10	12	5	2.5	9
	3.0	5	10	6	5	<2.5	6.5
	4.0	2.5	—	6	5	—	6.5
	5.0	—	5	3	2.5	—	1.5
	6.0	<2.5	<2.5	<1.5	<2.5	—	1.5
Polymyxin E ..	0.5	40	70	12	10	5	13
	1.0	20	35	12	5	5	13
	2.0	20	35	12	5	2.5	3
	3.0	5	10	6	5	2.5	1.5
	4.0	—	10	3	2.5	<2.5	1.5
	5.0	5	—	1.5	2.5	—	1.5
	6.0	<2.5	5	<1.5	<2.5	—	<1.5

DOGS

Time in hours	Concentrations in units per ml. for											
	Polymyxin A 10,000 u./kg.			Polymyxin B 10,000 u./kg.				Polymyxin E 10,000 u./kg.				
0.5	32	16	—	3	5	8	5	—	—	—	5	
1.0	16	16	10	8	8	4	5	2.5	5	5	2.5	
2.0	16	8	—	4	2	4	5	—	5	3	2.5	
3.0	4	8	5	2	2	2	—	2.5	3	3	<2.5	
4.0	4	2	<2.5	<2	<2	<2	2.5	<2.5	3	<2	—	
5.0	<2	<2	—	—	—	—	2.5	—	—	—	—	

pairs of rabbits for six hours after the intramuscular injection of various doses of polymyxins A, B, and E. The rabbits show a good deal of individual variation, but measurable blood levels persist for from four to six hours. When the polymyxins are given intravenously in a dose of 10,000 units disappearance from the blood stream is much more rapid. With polymyxin A the levels in one rabbit were, at 5 min., 30 min., and one hour, 20, 5, and 2.5 units, and no antibiotic could be detected after two hours. With polymyxins B and E disappearance was a little less rapid, detectable levels being observed at the third, but not at the fourth, hour. Some results in dogs after a single intramuscular dose of 10,000 units per kg. are also set out in the Table. The blood levels tended to be higher after polymyxin A than after polymyxins B and E. Generally detectable blood levels could not be found after the fourth hour.

It is of some interest to note the effect on the blood level of intramuscular administration repeated four-hourly in comparison with that of single doses in man. For example, in an adult one hour after the first dose of 250,000 units of polymyxin E the blood level was less than 2.4 u./ml. One hour after the second dose on the third day of treatment the blood level was 2.4 u./ml., and on the fifth day it had reached 4.8 u./ml. In another adult treated similarly the level one hour after the first dose was 1.2 u./ml., and after the second dose on the fourth day it was 9.6 u./ml. After repeated dosage in man levels as high as 26 u./ml. have been observed.

High levels have been noted in children from 6 to 12 years of age, in whom a single dose of 500,000 units has been introduced at operation into the peritoneum for prophylaxis. Half an hour after this treatment blood levels ranging from 6 to 33 units per ml. have been observed. We are indebted to Mr. C. L. Cookson, of Farnborough Hospital, for the opportunity of making these observations.

It is important to note that after the administration of polymyxin E these high levels have not been found to be associated with any toxic signs or symptoms.

After a single intramuscular injection only small fractions of the antibiotic are excreted in the urine in a biologically active form. In dogs after a single dose of 10,000 units of polymyxin B per kg. the percentage excreted in the urine in 24 hours was 0.3 ± 0.08 per cent (9 observations), and after a corresponding dose of polymyxin E it was 0.8 ± 0.6 per cent (36 observations). After a similar dose of polymyxin A 2.4 ± 2.1 per cent (9 observations) was excreted. As will be seen later, this difference is related to the nephrotoxic action of polymyxin A.

The small quantity of polymyxin excreted in the urine led to the examination of other possible routes of excretion. Brownlee and Bushby (1948) were unable to detect any excretion of polymyxin A in the bile. This was shown to be true also of polymyxins B and E. For example, a dog of 8 kg. was anaesthetized with sodium pentobarbital, the common bile duct was cannulated, and the cystic duct clamped. After a dose of 20,000 units per kg. intramuscularly blood concentrations reached 39 units per ml. at 0.5 hr. and 26 units per ml. at 2.5 hr. After 2.5 hr. the dose was repeated intravenously, giving blood concentrations of 64 units per ml. 5 min. after injection, and 20 units per ml. two hours later, but no polymyxin was detected at any time in the bile.

Polymyxin A, B, or E could not be detected in the cerebrospinal fluid of rabbits at intervals of one hour, two hours, and four hours after the subcutaneous injections of 10,000 units per kg.

Intracisternal administration

Polymyxins A, B, and E, injected under light ether anaesthesia into the cisterna magna of rabbits weighing about 3 kg., generally gave detectable levels in the cerebrospinal fluid for at least six hours. The animals were again lightly anaesthetized for the withdrawal of test samples. Typical protocols are given in Table V.

TABLE V

UNITS OF POLYMYXIN FOUND IN THE CEREBROSPINAL FLUID AFTER INTRACISTERNAL INJECTION OF 1,000 UNITS IN RABBITS

Polymyxin	C.S.F. concentration in units per ml.					
	1 hr.	2 hr.	3 hr.	4 hr.	6 hr.	24 hr.
A	38	—	10	2.4	<2.4	—
	—	38	—	4.8	4.8	—
	64	—	—	8.0	8.0	1
B	76	—	4.8	4.8	2.4	<2.4
	—	9.6	—	2.4	<1.2	—
E	76	—	10	10	4.8	2.4

Toxicity

Acute toxicity determinations

Mice.—The acute toxicity to intravenous and intraperitoneal injection of polymyxins A, B, C, D, and E was studied in groups of six mice weighing 18–20 g. The toxic signs are similar except for the slower recovery of animals given near-lethal amounts of polymyxin D. Toxic doses cause immediate signs of pallor of the ears and pads, muscular incoordination, and respiratory distress. Occasional strychnine-like convulsions are seen, followed by complete flaccidity of skeletal muscle with dyspnoea interrupted by gasping respiration. Death from asphyxia occurs in 3 or 4 min. In animals which recover from barely sub-lethal doses, the curare-like effect on muscle is more evident, and gives way to a phase of vasodilatation and ultimate recovery. All the polymyxins have acute toxicities of the same order. By the intravenous route the LD50 ranges from 60,000 to 90,000 units per kg,, whilst by the intraperitoneal route the range is from 140,000 to 280,000 units per kg.

Rabbits.—Acute toxicity observations in the rabbit have been limited to polymyxin E, whose LD50, intravenously, is similar to that for mice, being approximately 70,000 units per kg. The toxic signs are also similar.

Dogs.—No attempt has been made to determine the LD50 in dogs. Injected subcutaneously with 60,000 units per kg., or intravenously with 30,000 units per kg. of polymyxin E, dogs show no toxic signs. On the other hand, 30,000 units per kg. of polymyxin B intravenously in two animals caused some immediate distress, but the pulse and respiration remained normal and recovery was rapid.

Antidiuretic effect

Polymyxins A, B, C, D, and E all show an antidiuretic effect in rats. Estimates of the delayed urinary excretion after administration of 50 ml./kg. of water show

that the effect is similar with all the polymyxins and is absolute for four to six hours with a single subcutaneous injection of 100,000 units per kg. With lower doses the effect is less complete ; with a dose of 50,000 units per kg. the time for maximal excretion rate is 180–330 min., compared with 80 min. for a control group of rats given saline. This effect is about twice that seen with 0.12 units per kg. of posterior pituitary.

The time for the maximal excretion rate to be reached after a single subcutaneous injection of 250 mg. per kg. of streptomycin is 120–180 min.

Renal toxicity

Observations in rats.—A careful comparison of the nephrotoxic properties of polymyxins A, B, C, D, and E has been made by estimating the total protein excreted in the urine by groups of four Wistar rats during 72 hours after the subcutaneous injection of a single dose of 10,000 units per 100 g. body weight. In Table VI the

TABLE VI

THE EXCRETION OF PROTEIN IN THE URINE OF RATS

Single subcutaneous injections of 10,000 units polymyxins A, B, C, D, and E per 100 g.

Polymyxin	Mean weight protein excreted (mg./100 g.) in groups of four rats		
	Test	Standard polymyxin A	Control
A	34.0 40.1 46.5 29.8	37.0 52.0 52.0 52.0	7.9 9.1 9.1 9.1
B	13.6 3.2 9.6 9.2	37.0 45.0 27.8 27.8	7.9 12.7 7.5 7.5
C	29.0 40.3 57.9	27.8 57.7 57.7	7.5 11.5 11.5
D	43.5 49.0	51.5 36.5	7.8 6.0
E	7.3 7.4 3.2 7.1	37.0 37.0 28.1 28.1	7.9 7.9 2.8 2.8

mean weight of protein excreted per 100 g. body weight for each group of four rats is given in comparison with that after the administration of a corresponding dose of standard polymyxin A in other groups of four rats and with the excretion in control groups of rats receiving a saline injection only. The normal rat in captivity regularly appears to excrete some protein in the urine, and the polymyxins A, C, and D caused a striking increase in the degree of proteinuria, whereas polymyxins B and E caused no appreciable increase in the protein excreted.

Observations in rabbits.—Rabbits given 30,000 units per kg. of polymyxin B or E by subcutaneous injection daily for five days show no significant increase in proteinuria (Table VII). Those given corresponding doses of polymyxin A showed a peak excretion of protein at 24–48 hr. Microscopical examination of the urine from the rabbits which received polymyxin B showed no casts and only an occasional renal cell, but after polymyxin A the urine contained both renal cells and casts at the 24 and 48 hr. periods, and renal cells at 48 and 72 hr.

TABLE VII

THE EXCRETION OF PROTEIN IN THE URINE OF RABBITS

Polymyxin 30,000 u /kg. s.c. daily for five days	Weight of protein in mg. excreted in urine in 24 hr. period					
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
A	9	40	185	78	—	—
	0	0	310	260	26	38
B	18	15	0	13	—	—
	0	0	0	12	5	0
	0	0	18	3	8	63
E	4	4	2	—	3	1
	8	8	5	16	9	3
	14	9	3	2	8	1
Control	20	8	0	30	—	—
	10	0	0	0	3	—
	2	2	1	17	2	10

The kidneys of rabbits given polymyxin A, examined histologically, showed congestion and stripping of the epithelium of many tubules with disintegration of the cells of Henle's loops, whereas the kidneys of those given polymyxin B and E were either normal or showed only slight cloudy swelling of some secretory tubules.

Observations in dogs.—In dogs the concentration of protein in the urine was determined daily before and during five days after treatment with polymyxin A, B, and E. Each antibiotic was given subcutaneously in a dose of 10,000 units per kg. four times daily for three days. The results are set out in Table VIII. It will be noted that the urine was in all cases free from protein before treatment with polymyxin and that polymyxin A caused a substantial proteinuria, particularly on the second and third days, which continued, though diminished in degree, for two days after the cessation of treatment. Treatment with polymyxin B caused only a relatively slight degree of proteinuria, whereas after polymyxin E the proteinuria, though detectable, was barely significant.

Microscopical examination of the urine after polymyxin A showed many renal cells and occasional casts, whereas after polymyxin B and E either no renal cells, or at most only occasional ones, were seen.

The kidneys of dogs treated with polymyxin A, when examined histologically, showed severe acute changes ; the tubular epithelium was seen in all stages of disintegration, and many granular hyaline and cellular casts were found in the tubules.

TABLE VIII

A COMPARISON OF THE NEPHROTOXIC ACTION OF POLYMYXINS A, B, AND E IN DOGS

The doses were 10,000 units kg. given four times daily for three days

Polymyxin	Concentration of protein in urine (g./l.)					
	0 day	1 day	2 days	3 days	4 days	5 days
A	0	0.1	2.6	4.4	0.8	—
	0	0.1	3.7	1.0	0.5	0.25
	0	0.8	2.5	1.4	—	—
	0	0.3	1.8	2.6	1.0	1.0
	0	0.1	1.4	1.6	0.5	0.7
B	0	0.3	0.3	0.06	0.08	0.08
	0	0.15	0.14	0.12	—	—
	0	0.10	0.10	0.12	—	—
	0	0.12	0.14	0.20	0.23	0.08
	0	0.00	0.05	0.02	0.80	0.40
	0	0.60	0.50	0.10	0.40	0.30
E	0	0	0.04	0.03	0.04	0.08
	0	0	0.04	0.01	0.02	0.08
	0	0	0.04	0.02	0.06	0.00
	0	0	0.02	0.00	0.04	0.00

The kidneys of dogs treated with polymyxins B and E showed at most only slight hyaline droplet degeneration of convoluted tubular epithelium. As will be seen later from the results of chronic toxicity tests with polymyxin E, even with this antibiotic whose nephrotoxic action is minimal, there was some evidence of tubular damage in dogs treated for prolonged periods with large daily doses.

Excretion of the polymyxins in the urine

There is a relation between the excretion of protein caused by the nephrotoxic action of polymyxin A in dogs and the leakage of the antibiotic into the urine (Brownlee, Bushby, and Short, 1949).

In Table IX are shown the concentrations of the antibiotics in units per ml. in plasma and urine, and the protein excreted in grammes per litre during and after treatment for three days with 10,000 units per kg. of polymyxins A and E four times daily. After polymyxin E the levels in the plasma are not so high and those in the urine are much lower than after polymyxin A. Despite the small amounts of antibiotics excreted they appear to be efficient in overcoming urinary infection by organisms susceptible to their action.

Vascular effects

The early samples of polymyxins B and E contained histamine-like substances which were removed by further purification. When this activity had been eliminated, even the purest samples, when injected intravenously in a dose of 10,000 u./kg. in dogs anaesthetized with sodium pentobarbital, sometimes had a vasodepressant action. The effect when most pronounced was a fall of 40 mm. Hg, requiring some 20 min. to recover. Repeated doses in sensitive animals caused diminishing responses. The response was not abolished by full atropinization, nor was it related

TABLE IX

NEPHROTOXIC ACTION AND RENAL EXCRETION OF POLYMYXINS A AND E IN DOGS

Dosage: 10,000 units per kg. four times daily for three days. P = units per ml. plasma. Uu = units per ml. urine. Up = g. protein per l. urine.

Polymyxin	Polymyxin u./ml. Protein g./l.	Day of treatment			Day after treatment		
		1	2	3	1	2	3
A	P	8	8	32	—	—	—
	Uu	7	106	213	106	53	26
	Up	0	0.1	3.7	0.7	0.5	0.25
	P	4	16	8	4	—	—
	Uu	3	56	106	426	—	—
	Up	0	0.75	2.5	1.4	—	—
	P	24	3	6	—	—	—
	Uu	3	3	213	106	2.6	7.0
	Up	0	0.25	1.8	2.6	1.0	0.8
E	P	2.4	—	—	—	—	—
	Uu	1	2	2	<1	1	<1
	Up	0	0.04	0.04	0.3	0.1	0.5
	P	4.8	—	—	—	—	—
	Uu	4	8	4	2	2	2
	Up	0	0.1	0.04	0.08	0.1	0.2
	P	2.4	—	—	—	—	—
	Uu	4	8	2	1	<1	1
	Up	0	0.03	0.01	0.04	0	0
	P	2.4	—	—	—	—	—
	Uu	16	8	4	<1	<1	<1
	Up	0	0.04	0.02	0.06	0	0

to sensitivity to injected histamine. The polymyxins in similar dosage had variable but less pronounced action in both cats and rabbits.

Apparently this vasodepressant activity was not an obvious side-effect in man, because in the treatment of typhoid carriers in 1948 Dr. C. Knight McDonald gave hourly injections of 2,000 u./kg. intravenously without producing any symptoms suggestive of a fall in blood pressure.

Effects on temperature

A rise of temperature is seen in animals after the injection of polymyxin B (Brownlee, Bushby, and Short, 1949). This has been observed both in rabbits and in dogs. Rabbits were given three injections of 10,000 units per kg. subcutaneously of polymyxin A, B, or E on successive days, and the mean temperatures of groups of six animals were recorded several times daily. Only after polymyxin B was there any significant deviation from the normal. Mean afternoon temperatures on the second and third days were raised to $103.6 \pm 0.8°$ F., the mean normal temperature being $102.0 \pm 1.0°$ F.

A similar temperature effect, with some attendant malaise, was seen in dogs given polymyxin B, but not in those given polymyxins A or E, in doses of 10,000 u./kg., three times daily for three successive days. The afternoon temperature on the second and third days was $103.2 \pm 0.2°$ F., while the control dogs gave a figure of $100.9 \pm 0.4°$ F. The range of normal temperatures in the dog is given by Hobday (1947) as 100–102° F. The examination of more recent batches of polymyxin B in comparison with polymyxin E has not shown any significant difference in their pyrexial effects. It would appear that these effects are not constant features in the reaction to polymyxin B.

Local reactions

The effects at the site of subcutaneous and intramuscular injections were studied in groups of rats given three injections on successive days of 20,000 units of polymyxin A, B, or E, into the same area of approximately one inch square of shaved skin. Twenty-four hours after the last injection the animals were killed and the area of injection examined. Macroscopically the sites showed no reaction or only a mild inflammation with polymyxins A and E, whereas that with polymyxin B was severe and often oedematous.

The histological evidence was confirmatory. Reactions caused by polymyxins A and E were mainly of a limited nature and involved damage to muscle, such as vacuolation, though occasionally there was a fibrinous exudate with polymorphonuclear leucocyte infiltration. In contrast polymyxin B caused severe destruction of muscle and connective tissue usually with necrosis. There was diffuse polymorphonuclear leucocyte infiltration, much fibrinous exudate, and severe oedema. In the course of chronic toxicity tests in dogs the daily subcutaneous injection for 21 days of 20,000 units per kg. of polymyxin E either caused no reaction at the site of injection or at most slight inflammatory oedema.

In vitro *action on blood-cells*

A concentration of 1,000 units per ml. of polymyxin A or E had no toxic effect on human leucocytes after four hours ; in a concentration of 10,000 units per ml. the cells were alive but sluggish, but control cells in serum buffer solution were also less active after four hours. A concentration of 100,000 units per ml. killed leucocytes in three hours. In parallel observations 1,000 units per ml. equivalent of streptomycin base killed leucocytes in 30 min., and 10,000 units per ml. of penicillin-sodium amorphous killed them in two hours. The method was that of Dr. C. G. Paine (Abraham *et al.*, 1941).

The polymyxins are not haemolytic ; with 1,250 units per ml. of A, B, or E there was no haemolysis of a 10 per cent suspension of human red cells in physiological saline after 24 hours at 20° C.

Chronic toxicity tests of polymyxin E

Observations in dogs.—Two dogs received 20,000 units per kg. subcutaneously thrice daily for 21 days (except Saturdays when only two injections and Sundays when no injections were given), and two dogs were similarly treated with doses of 10,000 units per kg. thrice daily. One of the dogs on the larger dosage (No. 33) was in poor condition at the beginning of the test and remained so throughout. The others exhibited no toxic signs.

Blood counts were made at four-day intervals and no changes were observed in the haemoglobin, red cell, reticulocyte, and total and differential leucocyte counts. Blood-sugar and urine estimations were made on alternate days; there were no changes in the blood-sugar and only Dog No. 33, noted above, showed a significant increase in blood-urea which before treatment on two days was 49 and 77 mg. per 100 ml., and from the 10th to the 22nd day varied between 113 and 254 mg. This dog also exhibited a significant proteinuria which, during the first week, ranged from 0.03 to 0.2 g. per litre, and during the remainder of the experiment varied between 0.8 and 2.0 g. per litre. The second animal on the larger dose (No. 34) exhibited a slight degree of proteinuria throughout, on only five occasions exceeding 0.18 g. per litre (0.2, 0.6, 0.3, 0.5, and 0.75), for the most part of the order of 0.03 g. per litre. The urines of the two dogs on the lower dosage (Nos. 35 and 36) were free of protein for much of the experiment. When present, except on one occasion when the level reached 0.8 g. per litre, the usual level was less than 0.02 g. per litre. This degree of proteinuria can hardly be regarded as significant, since untreated dogs exhibit proteinuria of this order from time to time. Their blood-urea levels showed no significant changes.

The urea clearance (Table X) in the two dogs on the larger dose showed a definite fall on the 21st day, but there was no significant change in the clearance figures in the two dogs on the smaller dosage.

TABLE X

UREA CLEARANCE OF DOGS TREATED WITH POLYMYXIN E

Days	Volume of blood cleared of urea per minute = UV/B*			
	20,000 u. per kg. 3 times daily for 21 days		10,000 u. per kg. 3 times daily for 21 days	
	dog 33	dog 34	dog 35	dog 36
−2	13.1	19.2	12.6	14.4
9	8.8	19.6	12.4	18.8
16	2.43	16.7	9.4	16.9
21	1.52	5.2	14.3	16.5

* U = mg. urea per 100 ml. urine; V = ml. urine excreted per minute; B = mg. urea per 100 ml. blood.

The dogs were killed on the 22nd day and no macroscopic abnormalities were present. All the organs were submitted to Dr. David Trevan for histological study, but only the kidneys were abnormal.

Dog No. 33 had a chronic interstitial nephritis, and while it is probable that the repeated doses of polymyxin caused some additional damage this was impossible to assess. The hyaline degeneration of the convoluted tubular epithelium and the changes in the first part of the convoluted tubules in Dog 34, and the lesser changes in the tubular epithelium in dogs on smaller dosage, were probably attributable to the toxic action of the antibiotic. These changes are transitory and in other experiments, when a few days were allowed to elapse after the cessation of treatment, were no longer to be observed after polymyxin E. It is noteworthy that Dog. No. 36 also exhibited some acute and subacute interstitial nephritis which was unlikely to be causally related to the administration of polymyxin.

Observations in rabbits.—Four rabbits were given 30,000 units per kg. in a single dose subcutaneously daily for the same period as the dogs described above.

The animals remained well, with healthy appetites, and exhibited no abnormal changes in temperature. Blood examination every four days showed no changes in haemoglobin, red cell, reticulocyte, and total and differential leucocyte counts.

All the rabbits had proteinuria of some degree before the beginning of the experiment, 0.02, 0.14, 0.14, and 0.5 g. per litre, and all exhibited throughout proteinuria of some degree. In R.1029 the level was always 0.1 or less than 0.1 g. per litre on all but three occasions, when it reached 0.4, 0.12, and 0.95. In R.1030 the level never exceeded its highest control value, 0.14 g., and was usually less than half that. In R.1031, apart from the fourth day when the value reached 1.0 g. per litre, and during a period from the tenth to the sixteenth day when it was regularly 0.5–0.55 g. per litre, the values did not reach the highest control level. In R.1000 the level was less than its highest control level throughout, generally less than 0.05 g. per litre.

The microscopical appearances in the kidneys of the first three animals after they had been killed on the 23rd day did not reveal any significant damage attributable to polymyxin except possibly R.1030, which showed some swelling and fine granulations in the convoluted tubular epithelium.

Discussion

This paper is chiefly concerned with the pharmacology and chemotherapy of polymyxin E in comparison with polymyxins A and B. In its bactericidal activity polymyxin E does not differ significantly from polymyxins A and B. *In vitro* it has the same wide spectrum for gram-negative organisms and quantitatively equivalent activity. In animal experiments it has the same striking chemotherapeutic activity against *H. pertussis* and *S. typhosa* as polymyxin B, though both these antibiotics may be a little less active than polymyxin A.

Polypeptide antibiotics in general exhibit certain specific damaging effects in the body. The most serious toxicity of this kind exhibited by the polymyxins is their effect upon the epithelium of the convoluted tubules of the kidneys. With polymyxins A and D this is severe though reversible and readily demonstrable in rats, rabbits, dogs, and in man. With polymyxin B this nephrotoxic effect is much less evident even in rats, rabbits, and dogs in captivity, all of which, in control observations, exhibit intermittent proteinuria of variable degree. In rats after a single large subcutaneous injection of polymyxin B there was no perceptible increase in the protein excreted in the urine. In rabbits after large single daily injections for several days increase in protein excreted was barely evident, particularly in comparison with the effect of polymyxin A, but in dogs, which appear to be more sensitive, after repeated subcutaneous injection there was a definite but slight increase in the protein excreted in the urine.

The nephrotoxic effect of polymyxin E is even less than that of polymyxin B. In rats after a single injection and in rabbits after repeated injections there was no increase in protein excreted, and even in the more sensitive dog there were only just detectable increases after repeated injections. However, during prolonged treatment for 21 days with 20,000 units per kg. thrice daily, small but definite increases were observed.

After treatment with polymyxin E for 21 days the renal damage attributable to the antibiotic consisted of hyaline droplet degeneration of the epithelium of the convoluted tubules with some nuclear changes, both of which were less after the smaller than after the larger doses of polymyxin. It should be noted that in other short-term experiments with dogs in which a few days were allowed to elapse before obtaining the kidneys for section none of these changes could be found ; they must therefore be readily reversible. It should also be emphasized that two out of four of the dogs used for the chronic toxicity test were already suffering from interstitial nephritis, and that, in assessing the significance of these observations in rats, rabbits, and dogs, we are always concerned with the effects upon kidneys which cannot be regarded as comparable with normal human kidneys, and in dogs many may have been damaged by leptospira infection.

In view of the relation which appears to exist between the extent of renal damage caused by polymyxin A and the amount of the antibiotic excreted in the urine, the very low levels of antibiotic in the urine observed after repeated injections of polymyxin E lends support to the view that it has little nephrotoxic activity.

From studies, both of which are yet unpublished, made in conjunction with Dr. P. N. Swift, of Farnborough Hospital, and with Dr. N. M. Coutts, of the Brook Hospital, in infants suffering from pertussis only about one-tenth exhibited proteinuria during treatment with 10,000 units per kg. of polymyxin E at four-hourly intervals for five or more days. These infants presumably had normal kidneys apart from any slight damage attributable to pyrexia or infection. It is therefore evident that the nephrotoxic effects of polymyxin E are minimal.

Whereas polypeptides injected intravenously sometimes cause a fall in blood pressure, this effect with polymyxin E was only irregularly observed in experimental animals.

Polymyxin E by subcutaneous or intramuscular injection does not cause, at the site of injection, tissue injury comparable to that observed after the injection of corresponding doses of polymyxin B, and in man the injection of polymyxin E does not give rise to any more discomfort than an injection of penicillin.

In our earlier experiments with subcutaneous doses of 10,000 units per kg. thrice daily for three days polymyxin E never caused the pyrexia and malaise regularly observed in dogs with polymyxin B ; recent samples of polmyxin B show only minor pyrexial effects without malaise. Kagan, Krevsky, Milzer, and Locke (1951) comment on the variability in the reports on the toxic reactions of polymyxin B. Referring to the pyrexial reaction, they draw attention to the fact that, of five separate workers, only one noted pyrexial reactions and these were in 62 of 66 patients.

We can offer but little evidence about the fate of the polymyxins in the body. When mixed with serum they at once lose 50 per cent of their activity *in vitro,* and it is significant that even within 5 min. after intravenous injection much lower blood levels of either polymyxin B or E are observed than would be expected. Assuming no immediate loss by excretion or by entry into body cells or into the tissue spaces the actual levels are only about 10 per cent of those expected. These polymyxins do not long persist in active form in the blood stream, especially after a single injection. They are excreted only in trivial amounts in the urine, and they are not excreted in the bile, and we have no evidence of direct excretion into the alimentary tract. In this connexion it will be remembered that when the

polymyxins are added to faeces there is a large immediate loss of activity (Brownlee and Bushby, 1948). The polymyxins do not pass the blood-brain barrier. We have not yet accurate information concerning their molecular size. Though they go through some semipermeable membranes it is unlikely that they pass the glomerular filter in more than minute amounts. Their rapid disappearance in biologically active form from the circulating blood may most probably be accounted for by adsorption to protein. There is, however, no evidence of adsorption or entry into the red blood cells, since, after admixture of polymyxin with blood *in vitro* and subsequent centrifugation, the concentration in plasma is that which would be expected from admixture of the same dose of polymyxin with that volume of plasma or serum. Some support for this hypothesis is afforded by the fact that after repeated injection much higher blood levels are observed, a fact which could readily be attributed to partial saturation of the adsorbing surfaces.

Of the ultimate fate of the polymyxins we know even less. We have not explored their distribution in the organs, and the only evidence which we can offer indicates that in the liver there is no more active polymyxin than would be accounted for by its presence in the blood included within the organ. It is not improbable that the polymyxins, though they are completely resistant to destruction by pepsin, trypsin, or papain *in vitro,* are, like other polypeptides, broken down in the body.

Apart from any question of toxicity all the polymyxins have certain disadvantages as therapeutic agents. They are not absorbed from the alimentary tract and after parenteral administration give relatively low blood levels ; furthermore they do not pass the blood-brain barrier.

Additional disadvantages from which the polymyxins suffer are their toxic side-effects, particularly their nephrotoxicity. Here polymyxins B and E show their superiority over the other members of the group, since these two polymyxins have only slight nephrotoxic action. In children and adults with normal kidneys polymyxins B and E in therapeutic dosage show little evidence of causing renal damage, and even when slight damage occurs it is readily reversible. In experimental animals with already damaged kidneys large and repeated doses are necessary to cause significant renal damage. The remaining side-effects are not of great practical importance, though polymyxin E has some advantage over polymyxin B in causing less tissue injury at the site of injection.

On the other hand, there are certain advantages. They are rapidly bactericidal and even minute amounts excreted in the urinary tract can be efficient sterilizing agents (Pulaski and Rosenberg, 1949 ; Jawetz and Coleman, 1949).

Their non-absorption from the alimentary tract can be advantageous when it is desired to restrict bactericidal action to the lumen of the intestine (Ross, Burke, Rice, Washington, and Stevens, 1950 ; Kagan, Krevsky, Milzer, and Locke, 1951), and for the treatment of meningitis polymyxins B or E can be administered intrathecally (Kagan, 1949 ; Hayes and Yow, 1950 ; Swift and Bushby, 1951).

The polymyxins are particularly suitable for local application even when large raw areas are involved. Jackson, Lowbury, and Topley (1951), after prolonged clinical trial of polymyxin E in burns, found no toxic symptoms, even with the extensive use of 1 per cent cream, and in 0.1 per cent strength the antibiotic was effective both prophylactically and therapeutically in *Ps. aeruginosa* infections.

SUMMARY

1. Polymyxins A, B, C, D, and E all have similar antibacterial spectra without significant quantitative differences.

2. The efficiency of the polymyxins as bactericidal agents depends upon the size of inoculum of rapidly growing genera of bacteria.

3. The chemotherapeutic activity of polymyxins A, B, and E in mice has been compared against *S. typhosa* and *H. pertussis*; polymyxins B and E are slightly less active than polymyxin A.

4. Administered orally, polymyxin E, like polymyxins A and B, is not absorbed from the alimentary tract, except in the newborn animal.

5. These antibiotics all cause a sharp fall in the counts of viable faecal aerobes, even of those insensitive to polymyxin.

6. Administered in single doses by subcutaneous or intramuscular injection in rabbits, dogs, and in man, polymyxins A, B, and E enter the blood stream and variable blood levels persist for from three to sixteen hours. The blood levels are higher with polymyxin A than with polymyxins B and E. With repeated doses higher blood levels can be obtained.

7. After single parenteral injections only a small fraction of the dose is excreted in the urine, and none is detectable in the bile.

8. After intracisternal administration in rabbits polymyxin E can be detected in the cerebrospinal fluid up to twenty-four hours.

9. Polymyxins A, B, and E all have about the same LD50 in mice after intravenous injection. By the intraperitoneal route their toxicities are of the same order.

10. Polymyxins A, B, and E in large doses all exhibit an antidiuretic effect in rats.

11. Polymyxins B and E exhibit far less nephrotoxic action than polymyxin A. In rats, rabbits, and dogs after single and repeated doses of these two polymyxins renal change is minimal, and in this respect polymyxin E, except in prolonged experiments with large doses in dogs, gave but little evidence of injury to the kidneys.

12. Early samples of polymyxins B and E injected intravenously, apart from the presence of a histamine-like substance as an impurity, showed occasional vasodepressant action in experimental animals. No sign of this effect has been observed in man after intravenous injection of polymyxin E.

13. Polymyxin E causes less local reaction at the site of injection than polymyxin B.

14. The results of "chronic toxicity" tests of polymyxin E in dogs and rabbits are recorded.

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