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ANTIBACTERIAL ACTIVITY OF SOME DERIVATIVES OF 7-AMINOCEPHALOSPORANIC ACID AGAINST *STAPHYLOCOCCUS AUREUS* AND SYNERGISM BETWEEN THESE AND OTHER ANTIBIOTICS

BY

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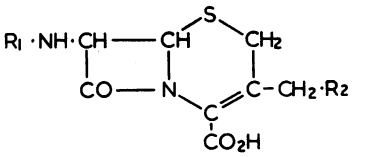
The *N*-phenylacetyl derivative of 7-aminocephalosporanic acid (cephaloram) had roughly the same activity as benzylpenicillin against a number of Gram-positive organisms and about one-eighth of the activity of benzylpenicillin against penicillin-sensitive strains of *Staphylococcus aureus*. This derivative and the *N*-α-phenoxypropionyl derivative of 7-aminocephalosporanic acid were 4 to 8 and 4 to 16 times as active as methicillin against penicillinase- and nonpenicillinase-producing staphylococcal strains, respectively. Neither the presence of horse serum nor changes in inoculum size appreciably affected the activities of any of the derivatives of 7-aminocephalosporanic acid which were tested. After forty-eight subcultures in the presence of antibiotic the increase in minimum inhibitory concentration against the staphylococcus was about four-times as great for cephaloram as for cephalosporin C. The resistant penicillinase-producing strains remained stable after six subcultures in antibiotic-free medium, and all the strains retained coagulase activity. Some degree of cross-resistance was found between the derivatives of 7-aminocephalosporanic acid and those of 6-aminopenicillanic acid. Synergism was observed *in vitro* between certain derivatives of 7-aminocephalosporanic acid and 6-aminopenicillanic acid when they were tested together or with fusidic acid or cephalosporin P_1 against a weak penicillinase-producing strain of *Staphylococcus aureus*. Cephalosporin C and cephalosporin C (pyridine), each in combination with benzylpenicillin, showed a significant degree of synergism in protection experiments in mice infected with a strong penicillinase-producing strain of *Staphylococcus aureus*.

Some of the biological properties of cephalosporin C and cephalosporin C_A (pyridine) were described by Jago & Heatley (1961) and by Crompton, Jago, Crawford, Newton & Abraham (1962). The results are given here of further studies on the antibacterial activity of these and related substances, mainly against penicillinase-producing strains of *Staphylococcus aureus*. The substances concerned are *N*-acyl derivatives of 7-aminocephalosporanic acid, which has the structure (Table 1) where $R_1=H$ and $R_2=CH_3.CO.O$ (Abraham & Newton, 1961; Loder, Newton & Abraham, 1961); the structures of these substances are summarized in Table 1. Two derivatives of 6-aminopenicillanic acid, benzylpenicillin (6-phenylacetamidopenicillanic acid) and methicillin [6-(2,6-dimethoxybenzamido)penicillanic

TABLE 1

DERIVATIVES OF 7-AMINOCEPHALOSPORANIC ACID

(R_1=H; R_2=CH_3.CO.O)



Name	Side-chains R_1	Side-chains R_2	Abbreviated name
Cephalosporin C	H_3N^+	CH_3.CO.O	Ceph C
Cephalosporin C_A (pyridine)*		$C_5H_5N^+$	Ceph C_A
Desacetylcephalosporin C†		HO	Ceph C_D
Desacetylcephalosporin C lactone	^-O_2C	Lactone	Ceph C_C
Cephaloram (7-phenylacetamido-cephalosporanic acid)	$C_6H_5.CH_2.CO$	CH_3.CO.O	—
7-α-Phenoxypropionamido-cephalosporanic acid	$CH_3.CH(O.C_6H_5).CO$	CH_3.CO.O	POP–7–ACA
7-(2,6-Dimethoxybenzamido)-cephalosporanic acid	$C_6H_5(O.CH_3)_2.CO$	CH_3.CO.O	DMB–7–ACA

* Hale, Newton & Abraham (1961); † Jeffrey, Abraham & Newton (1961).

acid], were used for comparison with the derivatives of 7-aminocephalosporanic acid.

The effects of various combinations of derivatives of 7-aminocephalosporanic acid and of 6-aminopenicillanic acid with each other and also with the sodium salts of cephalosporin P_1 and fusidic acid (Fucidin) were studied on a strain of *Staph. aureus*, D3R, which is a weak producer of penicillinase (Crawford & Abraham, (1957). Cephalosporin P_1 (Ritchie, Smith & Florey, 1951; Burton & Abraham, 1952; Baird, Halsall, Jones & Lowe, 1961) and fusidic acid (Godtfredsen & Vangedal, 1962) are acidic steroid antibiotics with similar structural features.

METHODS

Antibiotics. With the exception of cephalosporin P_1 all the antibiotics were used as sodium salts, which dissolved freely in distilled water. Cephalosporin P_1 was dissolved in 0.1 M-disodium phosphate buffer.

Minimum inhibitory concentrations. Two-fold serial dilutions of antibiotics, or mixtures of antibiotics, in 0.5 ml. volumes of Oxoid broth, were inoculated with one drop (0.02 ml.) of a 24 hr broth-culture of the test organism diluted 1 in 40 (and referred to as a "large inoculum") unless otherwise stated. With strains of *Staph. aureus* a large inoculum contained about 3×10^7 organisms/ml. The results were read after incubation at 37° C for 1 day and, in some experiments, also for 3 and 6 days.

Enzyme induction and manometric determinations of enzymic activity. The induction of penicillinase and cephalosporin C-ase was carried out with staphylococcal cultures as described by Crompton *et al.* (1962), methicillin (1 μg/ml.) being used as the inducer. After induction, the culture was centrifuged and the cells resuspended in about one-tenth of the original volume of supernatant fluid. The initial rates of hydrolysis of cephalosporin C (30 mg of the sodium salt in a Warburg vessel) and benzylpenicillin (3 mg of the sodium salt in a Warburg vessel) in the presence of the cell suspensions were then compared.

RESULTS

Antibacterial activity of single antibiotics in vitro

Activity of cephaloram, cephalosporin C and benzylpenicillin against various organisms

The activity of cephaloram (7-phenylacetamidocephalosporanic acid) against a variety of bacteria is given in Table 2. Comparable results for cephalosporin C and benzylpenicillin are quoted from Jago & Heatley (1961).

TABLE 2

ACTIVITY OF CEPHALORAM, CEPHALOSPORIN C AND BENZYLPENICILLIN AGAINST DIFFERENT BACTERIA *IN VITRO*

Minimum inhibitory concentrations (μg/ml.) were determined after incubation for 1 day at 37° C as described in Methods. Medium B=Oxoid broth, SB=10% serum broth. Results for cephalosporin C and benzylpenicillin are from Jago & Heatley (1961)

Organism	Medium	Inoculum culture diluted 1 in	Minimum inhibitory concentration of		
			Cephaloram	Cephalosporin C	Benzylpenicillin
Staph. aureus (H)	B	40	0·125	62·5	0·015
Str. pyogenes CN10	SB	40	0·2	31	<0·1
B. anthracis (avirulent)	B	4,000	0·2	31	<0·1
C. diphtheriae gravis	B	4,000	0·1	15·6	0·2
N. gonorrhoeae NCTC 8676	SB	Undiluted	0·24	7·8	0·2
Salm. typhi (avirulent)	B	4,000	7·8	15·6	4·8
Bact. friedlanderi	B	4,000	31	31	19
Sh. shigae	B	4,000	3·9	125	7·8
E. coli, type 2	B	4,000	31	125	15·6
Prot. vulgaris	B	4,000	31	62·5	7·8
Br. melitensis	SB	40	0·1	15·6	—
Br. abortus	SB	40	0·24	31	>6
H. influenzae (incubated 2 days)	Fildes	Undiluted	1	31	6
Ps. pyocyanea	B	4,000	>125	>1,000	>8,000
V. cholerae	B	4,000	3·9	0·8	6

Cephaloram had the same order of activity as benzylpenicillin and about 150-times the activity of cephalosporin C against a number of Gram-positive organisms. Excluding *Vibrio cholerae*, against which cephalosporin C is particularly active, cephaloram was from 2- to 32-times as active as cephalosporin C against the Gram-negative organisms tested. The activity of cephaloram was decreased from 2- to 4-fold when the minimum inhibitory concentration was read after incubation for 6 days instead of for 1 day.

Activity against different strains of Staphylococcus aureus

Table 3 gives the minimum inhibitory concentration of the antibiotics tested against three penicillinase-producing strains of *Staph. aureus* and three strains which do not produce penicillinase. Of the 7-aminocephalosporanic acid derivatives, cephaloram and 7-α-phenoxypropionamidocephalosporanic acid showed the greatest activity, being about 8- and 4-times respectively as active as methicillin. Desacetylcephalosporin C and desacetylcephalosporin C lactone showed a much lower activity. With the exception of benzylpenicillin, the antibiotics were only slightly (2- to 4-times) more active against the nonpenicillinase-producing strains than against the penicillinase producers.

TABLE 3

ACTIVITY OF ANTIBIOTICS AGAINST SIX STRAINS OF *STAPHYLOCOCCUS AUREUS*

Minimum inhibitory concentrations (μg/ml.) were determined as described in Methods. A large inoculum was used and readings were taken after 24 hr. Strains R1 (phage type 75/77/+), RD 21 (phage type 29/6/7/47/54/75/+) and RD 23 (phage type 54/+) were isolated in the Radcliffe Infirmary, Oxford, in 1958 (R1) and 1960 (RD 21 and RD 23). Strain B7 (kindly provided by Dr Mary Barber) was isolated in the Hammersmith Hospital in 1959. Strain G2 (mouse virulent) was provided by Glaxo Laboratories. Strain H is the Oxford staphylococcus (NCTC6571). See Table 1 for explanation of abbreviations

Strain of *Staph. aureus*		Minimum inhibitory concentration (μg/ml.) of							
		Ceph C	Ceph C_A	Cephaloram	Ceph C_C	Ceph C_D	POP-7-ACA	Methicillin	Benzylpenicillin
Penicillinase-producing	R1	125	15·6	0·5	1,000	500	1	3·9	500
	B7	250	15·6	0·5	1,000	1,000	1	3·9	500
	G2	125	7·8	0·5	1,000	1,000	0·5	3·9	500
Nonpenicillinase-producing	H	62·5	3·9	0·125	500	250	0·25	2	0·015
	RD 21	125	3·9	0·25	1,000	500	0·5	1	0·03
	RD 23	62.5	3·9	0·25	1,000	250	0·25	1	0·03

TABLE 4

DEVELOPMENT OF RESISTANCE OF SIX STRAINS OF *STAPHYLOCOCCUS AUREUS* TO CEPHALOSPORIN C *IN VITRO*

The organisms were subcultured daily (using 0·02 ml. of inoculum per transfer) in increasing concentrations of antibiotic. The numbers in parentheses after values for minimum inhibitory concentrations (μg/ml.) represent each value as a multiple of the minimum inhibitory concentration for the corresponding parent culture

Strain of *Staph. aureus*		Minimum inhibitory concentration (μg/ml.) after transfer				
		0	12	24	36	48
Penicillinase-producing	R1	125	500 (4)	4,000 (32)	4,000 (32)	4,000 (32)
	B7	250	500 (2)	2,000 (8)	2,000 (8)	4,000 (16)
	G2	250	500 (2)	2,000 (8)	2,000 (8)	4,000 (16)
Nonpenicillinase-producing	H	125	500 (4)	1,000 (8)	2,000 (16)	2,000 (16)
	RD 21	125	500 (4)	1,000 (8)	2,000 (16)	2,000 (16)
	RD 23	125	500 (4)	2,000 (16)	2,000 (16)	2,000 (16)

With a 500-fold decrease in the inoculum size of the six strains of *Staph. aureus* there was no decrease of more than 2- to 4-fold in minimum inhibitory concentration, except with benzylpenicillin and the penicillinase-producing strains.

With strain R1, the presence of horse serum (50% v/v) in the medium did not alter significantly the minimum inhibitory concentration values of any of the antibiotics.

Development of resistance in vitro. The six strains of *Staph. aureus* were subcultured 48-times in increasing concentrations of cephalosporin C and cephaloram in broth. Tables 4 and 5 indicate that, with most of the strains used, resistance (when expressed in terms of the original minimum inhibitory concentration) increased more rapidly, and became about 4-times greater, with cephaloram than with cephalosporin C after forty-eight transfers. However, after forty-eight transfers the minimum inhibitory concentration of cephaloram was still 2- to 16-times smaller than the original value for cephalosporin C and 64- to 256-times smaller than the minimum inhibitory concentration of cephalosporin C after forty-eight transfers.

TABLE 5

DEVELOPMENT OF RESISTANCE OF STRAINS OF *STAPH. AUREUS* TO CEPHALORAM *IN VITRO*

The procedure used was that described in Table 4. The numbers in parentheses represent each minimum inhibitory concentration (μg/ml.) as a multiple of the value for the corresponding parent culture

Strain of *Staph. aureus*		Minimum inhibitory concentration (μg/ml.) after transfer				
		0	12	24	36	48
Penicillinase-producing	R1	0·5	8 (16)	16 (32)	32 (64)	64 (128)
	B7	1	8 (8)	16(16)	32 (32)	64 (64)
	G2	0·5	8 (16)	8 (16)	16 (32)	16 (32)
Nonpenicillinase-producing	H	0·125	2 (16)	8 (64)	16 (128)	32 (256)
	RD 21	0·25	2 (8)	16 (64)	16 (64)	16 (64)
	RD 23	0·25	2 (8)	2 (8)	32 (128)	32 (128)

Cultures of strains R1, B7, RD21 and RD23, which had acquired resistance to cephalosporin C and cephaloram respectively after forty-eight transfers in the presence of these antibiotics, were subcultured 6-times in antibiotic-free broth. The penicillinase-producing strains retained their resistance whereas the nonpenicillinase-producing strains were unstable, retaining only about one-eighth of their acquired resistance. All the cultures retained coagulase activity and were of the same phage type as the parent strains.

The amount of penicillinase (induced by methicillin) and the ratio of penicillinase to cephalosporin C-ase activity were the same in both the parent R1 culture and the culture derived from it which had acquired a 32-fold resistance to cephalosporin C. A high concentration of cephalosporin C (10 mg/ml.) was required to obtain a measurable rate of hydrolysis by the manometric method. Under the conditions used the measured ratio of penicillinase to cephalosporin C-ase activity was about 180:1.

Cross resistance of strains of Staphylococcus aureus *which had acquired resistance* in vitro *to cephalosporin C and cephaloram.* Cultures derived from strains R1, B7, RD21 and RD23, which had acquired resistance to cephalosporin C or cephaloram after subculture in the presence of one of these substances, were tested in parallel

TABLE 6

CROSS RESISTANCE OF STRAINS OF *STAPHYLOCOCCUS AUREUS* WHICH HAD ACQUIRED RESISTANCE TO CEPHALOSPORIN C AND CEPHALORAM *IN VITRO*

Minimum inhibitory concentrations were determined after incubation for 24 hr, a large inoculum being used. The numbers give the increases in minimum inhibitory concentration as multiples of the original value for the corresponding parent cultures. See Table 1 for explanation of abbreviations. * Small inoculum (approximately 6×10^4 organisms/ml.)

Parent strain of *Staph. aureus*		Resistance acquired to	Increase in minimum inhibitory concentration for						
			Ceph C	Cephalo-ram	Ceph C_A	POP-7-ACA	Methi-cillin	Benzyl-penicillin	Benzyl-penicillin *
Penicillinase-producing	R1	Ceph C	32	128	64	32	16	0	64
		Cephaloram	32	128	64	32	16	0·5	64
	B7	Ceph C	32	128	32	32	16	—	—
		Cephaloram	16	64	16	16	2	—	—
Non-penicillinase-producing	RD 21	Ceph C	16	128	64	128	4	32	—
		Cephaloram	16	64	32	64	16	—	—
	RD 23	Ceph C	32	64	32	64	16	4	—
		Cephaloram	16	64	16	32	8	16	—

for sensitivity to other derivatives of 7-aminocephalosporanic acid and to methicillin and benzylpenicillin. The results are shown in Table 6. Each culture showed some cross-resistance to all the antibiotics tested, although with benzylpenicillin and the penicillinase-producing strain R1 this was only evident with a small inoculum.

Strains which were 16- to 32-times as resistant as the parent to cephalosporin C had increased their resistance from between 32- and 128-fold against the other 7-aminocephalosporanic acid derivatives and from 4- to 16-fold against methicillin. Those which were from 64- to 128-times as resistant to cephaloram had increased their resistance from between 16- to 64-fold against the other 7-aminocephalosporanic acid derivatives and from between 2- to 16-fold against methicillin.

Though resistance increased more readily to cephaloram than to cephalosporin C, the degree of cross-resistance was less with strains which had acquired resistance in the presence of cephaloram than with those which had acquired resistance in the presence of cephalosporin C. Thus the actual inhibitory concentration of cephaloram or cephalosporin C was of the same order with organisms made resistant by the same number of subcultures in the presence of either one of these substances.

TABLE 7

CROSS-RESISTANCE OF METHICILLIN-RESISTANT STRAINS OF *STAPHYLOCOCCUS AUREUS* (PENICILLINASE-PRODUCING)

The minimum inhibitory concentration was determined with a large inoculum, readings being taken after 1 and 3 days. The numbers in parentheses give the value as a multiple of the minimum inhibitory concentration with the parent strains, or, in the case of 13137 and 6637, with strain R1. Strain 13137 (naturally resistant to methicillin) was isolated by Jevons (1961), and 6637 (also naturally resistant) by Stewart & Holt (1963). Strain 18R (phage type 83) is a laboratory-trained methicillin-resistant strain used together with its parent 18 and isolated by Barber (1961). Strain E3/500 is a laboratory-trained methicillin-resistant variant of strain E3, both of phage type 77 and isolated by Knox & Smith (1961). E3/500, unlike its parent, has lost the ability to produce penicillinase. See Table 1 for explanation of abbreviations. * This culture, though resistant to methicillin, is sensitive to benzylpenicillin

Strain of *Staph. aureus*	Minimum inhibitory concentration (μg/ml.) for: Methicillin after day 1	Methicillin after day 3	Ceph C after day 1	Ceph C after day 3	Ceph C_A after day 1	Ceph C_A after day 3	Cephaloram after day 1	Cephaloram after day 3	POP–7–ACA after day 1	POP–7–ACA after day 3	DMB–7–ACA after day 1	DMB–7–ACA after day 3
" Methicillin-sensitive " R1	3·9	7·8	125	250	15·6	31·25	0·5	1	1	1	125	250
Naturally resistant 13137	15·6 (4)	1,000 (128)	125 (0)	8,000 (32)	31·25 (2)	1,000 (32)	2 (4)	16 (16)	—	—	—	—
6637	7·8 (2)	250 (32)	1,000 (8)	8,000 (32)	125 (8)	1,000 (32)	0·62 (0)	5 (5)	4 (4)	16 (16)	250 (2)	>1,000 (>4)
Laboratory-resistant 18R	125 (128)	500 (128)	1,000 (8)	4,000 (16)	250 (16)	1,000 (16)	4 (4)	16 (8)	4 (4)	16 (4)	4 —	—
18 (" methicillin-sensitive " parent)	0·97	3·9	125	250	15·6	62·5	1	2	1	4	—	—
E3/500*	15·6 (2)	250 (16)	250 (2)	4,000 (16)	62·5 (4)	250 (8)	0·5 (0)	4 (4)	—	—	—	—
E3 (" methicillin-sensitive " parent)	7·8	15·6	125	250	15·6	31·25	0·5	1	—	—	—	—

There appears to be no significant difference between the behaviour of the penicillinase- and nonpenicillinase-producing strains, apart from their behaviour to benzylpenicillin.

Cross-resistance of methicillin-resistant strains of Staphylococcus aureus

Four methicillin-resistant strains of *Staph. aureus* (three penicillinase- and one nonpenicillinase-producing) were tested for cross-resistance to cephalosporin C, cephalosporin C_A, cephaloram and, in some experiments, 7-α-phenoxypropionamidocephalosporanic acid and 7-(2,6-dimethoxybenzamido)cephalosporanic acid. The results are given in Table 7.

Both the strains with " natural " resistance to methicillin and those with acquired resistance showed significant cross-resistance with cephalosporin C and its derivatives. Relative to the methicillin-sensitive strains R1 and E3 repectively, the increase in resistance of strains 6637 and E3/500 to methicillin was accompanied by a similar increase in resistance to cephalosporin C and cephalosporin C_A but a smaller increase in resistance to cephaloram. With these strains the minimum inhibitory concentration for methicillin after 3 days was about 50-times that for cephaloram. With strains 13137 and 18R there was a considerably smaller increase in resistance to all the cephalosporins than to methicillin.

Though there was variation between different strains, some cross-resistance between methicillin and cephalosporin C and its derivatives occurred in both directions.

Activity of combinations of antibiotics in vitro *against a strain of* Staphylococcus aureus

Nine antibiotics, comprising fusidic acid, cephalosporin P_1 and derivatives of 7-aminocephalosporanic acid and 6-aminopenicillanic acid, were tested in pairs, in all possible thirty-six combinations, against a strain of *Staph. aureus* which is a weak producer of penicillinase (strain D3R, phage type 6/47+, kindly provided by Dr Mary Barber). The inhibitory concentrations of the antibiotics alone and in various combinations were determined by the serial dilution method after 1, 3 and 6 days incubation at 37° C. Some of the results for 1 and 3 days are summarized in Table 8. This shows the fraction of the inhibitory concentration of each of a number of the antibiotics alone which was required, when combined with one-quarter of the inhibitory concentration of another antibiotic alone, to suppress visible growth. Of the thirty-six combinations, about half showed various degrees of synergism. A number of pairs which contained fusidic acid, cephalosporin P_1, or benzylpenicillin as one component showed strong synergism and the effect was most striking with the combinations of fusidic acid or cephalosporin P_1 with benzylpenicillin.

In the combinations cephalosporin C and fusidic acid, cephaloram and fusidic acid, fusidic acid and benzylpenicillin, cephalosporin C or cephalosporin C_A with cephalosporin P_1, cephalosporin P_1 with methicillin or benzylpenicillin, cephalosporin C, cephalosporin C_A or 7-(2,6-dimethoxybenzylamido)cephalosporanic acid with benzylpenicillin respectively, synergism was still apparent when only one-eighth or one-sixteenth of the inhibitory concentration of the first antibiotic of the pair

TABLE 8

EFFECT OF COMBINATION OF ANTIBIOTICS UPON THE GROWTH OF A WEAK PENICILLINASE-PRODUCING STRAIN OF *STAPHYLOCOCCUS AUREUS* D3R

Inhibitory concentrations were determined by the serial dilution method with a large inoculum. A quarter of the inhibitory concentration of each antibiotic listed on the vertical sides of the Table is combined with a fraction (recorded as its reciprocal) of the inhibitory concentrations of each antibiotic listed above the columns. Each combination shown inhibits growth. The Table records the results after 1 day and after 3 days (figures in parenthesis) incubation, respectively. The higher the number recorded the greater the degree of synergism. For example, the third figure from the right at the top (32) shows that one-quarter of the inhibitory concentrations of cephalosporin C alone combined with one-thirty-second or with one-eighth of that of fusidic acid alone inhibits growth after 1 or 3 days incubation respectively. Only values above 4 have been taken to reflect a significant degree of synergism. See Table 1 for explanation of abbreviations

Reciprocal of the fractions of the minimum inhibitory concentration after 1 and 3 days' incubation

Antibiotics	Ceph C	Cephaloram	Ceph C_A	POP-7-ACA	DMB-7-ACA	Ceph P_1	Fusidic acid	Benzylpenicillin	Methicillin
Ceph C		4 (4)	8 (4)	0 (0)	0 (2)	32 (8)	32 (8)	32 (32)	8 (2)
Cephaloram	4 (4)		16 (4)	4 (8)	2 (4)	2 (8)	32 (16)	0 (0)	4 (2)
Ceph C_A	8 (4)	16 (4)		16 (4)	4 (8)	8 (16)	4 (16)	16 (64)	2 (4)
POP-7-ACA	0 (0)	4 (8)	8 (4)		8 (2)	8 (4)	2 (2)	2 (2)	2 (2)
DMB-7-ACA	2 (0)	0 (4)	4 (8)	4 (0)		0 (4)	0 (4)	16 (32)	0 (0)
Ceph P_1	16 (4)	2 (8)	16 (8)	4 (4)	0 (4)		8 (4)	64 (512)	8 (32)
Fusidic acid	32 (8)	32 (16)	4 (8)	0 (2)	0 (4)	8 (4)		64 (512)	8 (16)
Benzylpenicillin	16 (16)	0 (0)	16 (32)	0 (2)	16 (32)	32 (64)	32 (64)		0 (0)
Methicillin	8 (0)	4 (2)	2 (4)	2 (2)	2 (0)	4 (16)	8 (16)	0 (0)	

alone was used. In general, the growth of organisms in the tubes containing sub-inhibitory amounts of pairs of antibiotics, though recorded as a " positive " growth, was less than in the control tubes after 1 day's incubation.

Both fusidic acid and cephalosporin P_1 were synergic with cephalosporin C, cephalosporin C_A, methicillin and benzylpenicillin. Fusidic acid was also clearly synergic with cephaloram. For example, mixtures of these substances in the following concentrations inhibited growth after incubation for 24 hr:

Cephaloram (μg/ml.)		Fusidic acid (μg/ml.)
0·50 alone		0·25 alone
0·25	with	0·007
0·125	with	0·007
0·062	with	0·015
0·031	with	0·031

Benzylpenicillin showed synergism with cephalosporin C, cephalosporin C_A and 7-(2,6-dimethoxybenzamido)cephalosporanic acid as well as with cephalosporin P_1 and fucidic acid. Of the remaining synergistic pairs, cephalosporin C_A appeared to be the most frequent constituent.

Cultures of *Staph. aureus* strain D3R were grown in Oxoid broth from a very large inoculum in a series of J tubes rocked at 37° C in the manner described by Crawford & Abraham (1957). The changes in optical density of cultures to which had been added different antibiotics alone and in various combinations confirmed the findings in the serial-dilution tests. For example, cephalosporin P_1 (0.5 μg/ml.) and benzylpenicillin (250 μg/ml.) were synergistic, growth in the tubes containing this mixture being delayed for more than twice as long (more than 30 hr) as in the tubes containing either antibiotic alone in double these concentrations (10 hr). By a similar criterion, there was synergism between cephalosporin C (50 μg/ml.) and

cephalosporin P_1 (0.5 μg/ml.). These two pairs of antibiotics showed no synergistic effect in serial-dilution tests, when the nonpenicillinase-producing strain of *Staph. aureus* RD21 was used. Indeed, in the combination cephalosporin P_1 and benzylpenicillin there appeared to be some antagonism, because 4-times the minimum inhibitory concentration of benzylpenicillin alone had to be combined with one-quarter of the minimum inhibitory concentration of cephalosporin P_1 alone before growth was inhibited. No synergism was observed with the pairs: cephalosporin C and benzylpenicillin, cephalosporin C_A and benzylpenicillin, and cephalosporin C and cephalosporin P_1 when the strong penicillinase-producing strain R1 or G2 was used as an inoculum, though these combinations were synergistic with strain D3R.

Antistaphylococcal activity of certain antibiotics alone and in combination in vivo

The weak penicillinase-producing strain of *Staph. aureus* D3R which was used for testing for synergism *in vitro* was not suitable in protection experiments in mice. Therefore a strain, G2, virulent to mice, and a strong producer of penicillinase, was used as a test organism for studying synergism *in vivo*. The following four pairs of antibiotics were tested: (1) cephalosporin C and benzylpenicillin, (2) cephalosporin C_A and benzylpenicillin, (3) cephalosporin C and fusidic acid, and (4) cephaloram and benzylpenicillin. With strain D3R synergism was observed *in vitro* with pairs (1) and (2) and to a slightly lesser degree with (3), but it was not observed with (4). With strain G2 no effect was observed *in vitro* with any of the combinations.

The results of the experiments in mice with pairs (1) and (2) and strain G2 are given in Table 9. They show that there was a significant synergistic effect *in vivo*

TABLE 9

SURVIVAL OF MICE TREATED WITH ANTIBIOTICS ALONE AND IN COMBINATION AFTER INFECTION WITH A STRONG PENICILLINASE-PRODUCING STRAIN OF *STAPHYLOCOCCUS AUREUS*, G2

Albino mice (20 g), in groups of six, were given nine doses of antibiotic in 0·3 ml. of saline subcutaneously over 48 hr, the first dose being given immediately after they had been injected intraperitoneally with 0.5 ml. of a suspension, containing approximately 5×10^8 staphylococci of strain G2. The mixtures of antibiotics contained equal amounts by weight of each component. Groups of thirty mice were used as controls. See Table 1 for explanation of abbreviations

Antibiotic	Days after infection	Survivors after dose per mouse (single antibiotic or mixture) of 4 mg	2 mg	1 mg	0·5 mg	0·25 mg	0
Expt. 1. *Effect of cephalosporin C and benzylpenicillin*							
Ceph C	1	6	6	5	1	2	1
alone	3	6	6	4	1	2	1
Benzylpenicillin	1	3	3	1	2	1	
alone	3	3	3	1	1	1	
Ceph C and	1	6	6	6	6	3	
benzylpenicillin	3	6	6	4	6	2	
Expt. 2. *Effect of cephalosporin C_A and benzylpenicillin*							
Ceph C_A	1		5	4	1	2	3
alone	3		5	4	1	1	2
Benzylpenicillin	1		2	2	1	0	
alone	3		1	2	0	0	
Ceph C_A and	1		6	6	6	5	
benzylpenicillin	3		6	6	6	3	

both with cephalosporin C_A and benzylpenicillin and with cephalosporin C and benzylpenicillin. With cephalosporin C and fusidic acid, the effect was hardly significant and with cephaloram and benzylpenicillin no synergism was observed.

DISCUSSION

Cephaloram showed roughly the same activity as benzylpenicillin against a number of the Gram-positive organisms tested, but about one-eighth of the activity of benzylpenicillin against three penicillin-sensitive strains of *Staph. aureus.*

Against three penicillinase-producing strains of *Staph. aureus,* cephaloram and 7-α-phenoxypropionamidocephalosporanic acid were 4- to 8-times as active *in vitro* as methicillin and about 250-times as active as cephalosporin C; and against the nonpenicillinase-producing Oxford staphylococcus they were 8- to 16-times as active as methicillin and 500-times as active as cephalosporin C. The activities of all four compounds were little affected by 50% (v/v) horse serum or by changes in size of inoculum. On subculture in the presence of cephaloram or cephalosporin C all the staphylococcal strains became more resistant, but after forty-eight subcultures the increase in minimum inhibitory concentration (when considered as a multiple of the original minimum inhibitory concentration) was about 4-times as great for cephaloram as for cephalosporin C or for methicillin, as reported by Barber (1961), who used a similar number of subcultures on a solid antibiotic-containing medium. The resistant strains retained their coagulase activity, and the penicillinase-producing strains (but not the nonpenicillinase-producing strains) were stable for six subcultures in the absence of antibiotic. Experiments with one strain indicated that the increase in resistance was not associated with any increase in the ability to produce a cephalosporinase.

The finding that staphylococci which had acquired resistance *in vitro* to any one of the derivatives of 7-aminocephalosporanic acid tested showed strong cross-resistance to the others was not unexpected in view of the structural relationships of the compounds concerned. Such staphylococci also showed an increased resistance to methicillin, but this was somewhat less than that to the derivatives of 7-aminocephalosporanic acid. Similarly, strains with natural or acquired resistance to methicillin showed a smaller increase in resistance to cephaloram. The results indicate that some degree of cross-resistance is normally to be expected between derivatives of 7-aminocephalosporanic acid and those of 6-aminopenicillanic acid, but that it is likely to vary in extent from one compound and from one strain of *Staph. aureus* to another.

Crawford & Abraham (1957) reported that cephalosporin C showed synergism with benzylpenicillin *in vitro* against the weak penicillinase-producing strain of *Staph. aureus,* D3R. These findings have been confirmed and it has been shown that cephalosporin C_A (pyridine) and 7-(2,6-dimethoxybenzamido)cephalosporanic acid are also synergistic with benzylpenicillin against this organism, though cephaloram is not.

Godtfredsen & Vangedal (1962) and Barber & Waterworth (1962) found that fusidic acid and benzylpenicillin were synergistic *in vitro* against strains of *Staph.*

aureus which were weak producers of penicillinase and that synergism was most evident when a large inoculum was used. The present results show that there is also considerable synergism between cephalosporin P_1 and benzylpenicillin against *Staph. aureus* D3R. In addition there is synergism, though less striking, between either cephalosporin C, cephalosporin C_A or methicillin and both cephalosporin P_1 and fusidic acid. Cephaloram also appears to show a significant degree of synergism with fusidic acid.

The synergism shown by fusidic acid, cephalosporin P_1 and some of the derivatives of 7-aminocephalosporanic acid with benzylpenicillin against *Staph. aureus* D3R is clearly strain-dependent. With fusidic acid and benzylpenicillin the existence of synergism has been correlated with the ability to produce a relatively small amount of penicillinase and it has been suggested that the destruction of benzylpenicillin is delayed by the inhibiton of growth caused by fusidic acid (Barber & Waterworth, 1962). It may be that a small proportion of resistant organisms, which begin to multiply in the presence of a subinhibitory concentration of fusidic acid or cephalosporin P_1, can be killed by a subinhibitory amount of benzylpenicillin before the concentration of the latter is reduced to less than its intrinsic minimum inhibitory concentration by penicillinase. Similarly, the synergism between cephalosporin C and benzylpenicillin might be ascribed to a decreased production of penicillinase by the culture in the presence of subinhibitory concentrations of cephalosporin C. If this were so, however, the failure of cephaloram to show synergism with benzylpenicillin would remain unexplained.

In contrast to its effect with *Staph. aureus* D3R, cephalosporin P_1 appeared able under certain conditions to antagonize the action of benzylpenicillin on a strain of *Staph. aureus* which did not produce penicillinase. A similar finding was reported in 1945 by Chain & Duthie, who showed that the bacteriostatic action of helvolic acid (an antibiotic structurally related to cephalosporin P_1 and fusidic acid) inhibited the bactericidal effect of benzylpenicillin on *Staph. aureus.*

Synergism between cephalosporin P_1 and cephalosporin C was also strain-dependent. But in this and other instances involving mixtures of cephalosporin P_1, or fusidic acid, with derivatives of 7-aminocephalosporanic acid or with methicillin, there is no obvious reason to suppose that penicillinase is involved, since the components of the mixtures are either unaffected by the enzyme or highly resistant to it. Whether synergism in these and other cases is connected with the absence of cross-resistance to the separate components of the mixtures, or whether it has a different biochemical basis, remains to be decided.

Although no indication was obtained of synergism *in vitro* between cephalosporin C and benzylpenicillin, or between cephalosporin C_A and benzylpenicillin, with *Staph. aureus* strain G2 (which is a strong producer of penicillinase), these pairs appeared to show a significant degree of synergism in protection experiments with strain G2 in the mouse. It may be that the apparent difference between the behaviour of strain G2 *in vivo* and *in vitro* is only a quantitative one and that the underlying mechanism of the synergism *in vivo* is similar to that with strain D3R *in vitro.* Cephaloram and benzylpenicillin, which showed no synergism *in vitro* with strain D3R, also showed no synergism *in vivo* with strain G2.

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