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Specificities of Haemagglutinating Antibodies Evoked by Members of the Cephalosporin C Family and Benzylpenicillin

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1. Antisera have been produced in rabbits to benzylpenicillin and four members of the cephalosporin C family and to conjugates of these substances with bovine γ-globulin. 2. Deacetoxycephalosporin C reacted less readily and deacetylcephalosporin C lactone more readily with bovine γ-globulin than did benzylpenicillin, cephalosporin C or deacetylcephalosporin C. 3. Antisera to free or conjugated benzylpenicillin agglutinated red cells sensitized with a variety of penicillins, but only reacted to a significant extent with cells sensitized with the cephalosporins tested when the latter contained an *N*-phenylacetyl or chemically related side chain. 4. Antisera to members of the cephalosporin C family agglutinated cells sensitized with these cephalosporins or with penicillin N, but did not react with cephalosporins whose side chains were chemically unrelated to α-aminoadipic acid. 5. Members of the cephalosporin C family and products of hydrolysis of cephalosporin C behaved as hapten inhibitors of antisera to cephalosporin C, but 7-aminocephalosporanic acid was relatively ineffective. 6. These findings are discussed in relation to differences in the chemical properties of penicillins and cephalosporins.

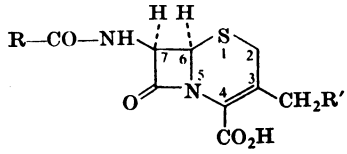
Aminolysis of the β-lactam ring of the cephalosporins (I, R′=O–CO–CH_3) with simple amino compounds in aqueous solution is accompanied by the expulsion of the acetoxy group as acetate, the formation of a double bond in the Δ^4-position and the appearance of an exocyclic methylene group (Hamilton-Miller, Newton & Abraham, 1970*a*; Hamilton-Miller, Richards & Abraham, 1970*b*). Similarly, the opening of the β-lactam ring of compounds in which the acetoxy group has been replaced by a pyridinium ion is accompanied by removal of the latter as pyridine. In contrast, aminolysis of deacetylcephalosporins (I, R′=OH) and deacetoxycephalosporins (I, R′=H) can occur without immediate changes in the dihydrothiazine ring. However, these different compounds all decompose to give, eventually, penaldates and penamaldates derived from the side chain and the carbon atoms of the β-lactam ring, the rate of decomposition increasing as the concentration of the compound is raised and the pH of the solution is lowered.

The initial course of aminolysis of a cephalosporin with polylysine and poly(lysine+serine) appeared to be similar to that with simple amino compounds, but the product obtained with poly(lysine+serine) was more stable than that with polylysine and with neither polymer was evidence obtained from ultraviolet-absorption spectra for the final formation of a penaldate or penamaldate (Hamilton-Miller *et al.* 1970*a*).

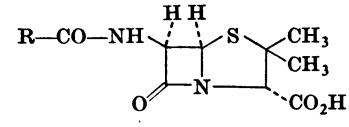
These findings raised the question whether the determinant groups of antigens formed by reaction of cephalosporins with the amino groups of proteins would differ significantly from the groups formed by deacetyl and deacetoxycephalosporins and from the penicilloyl determinants derived from the penicillins (II) in properties other than those dependent mainly on the nature of their *N*-acyl side chains (R–CO in I and II). This paper describes the results of experiments designed to determine the extent of cross-reactions between groups resulting from the reaction of certain cephalosporins and penicillins with rabbit red blood cells and antisera obtained from rabbits immunized with other cephalosporins, benzylpenicillin or conjugates of these compounds with bovine γ-globulin.

METHODS

Penicillins, cephalosporins and their derivatives. Phenoxymethylpenicillin (penicillin V, II, R=C_6H_5–O–CH_2) was obtained from The Distillers Co. (Biochemicals) Ltd., Liverpool, U.K. and was converted into its potassium salt by treatment with potassium 2-ethylhexanoate in butan-1-ol. Methicillin (II, R=2,6-dimethoxyphenyl), ampicillin (II, R=C_6H_5–CH(NH_2)), carbenicillin (II, R=C_6H_5–CH(CO_2H)) and 6-aminopenicillanic acid were

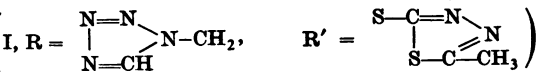


(I)



(II)

gifts from Beecham Research Laboratories Ltd., Betchworth, Surrey, U.K. Oxacillin (II, R=5-methyl-3-phenyl-4-isoxazolyl) was provided by the Bristol Laboratories, Syracuse, New York, U.S.A. and quinacillin (II, R=3-carboxyquinoxalin-2-yl) by Boots Pure Drug Co., Nottingham, U.K. Cephaloridine (I, R=thien-2-ylmethyl, $R'=C_5H_5\overset{+}{N}$) and the tosylate of 7-aminocephalosporanic acid were supplied by Glaxo Laboratories Ltd., and cephalothin (I, R=thien-2-ylmethyl; R′= O–CO–CH_3) by Eli Lilly and Co., Indianapolis, U.S.A. 7-Aminocephalosporanic acid was prepared from an aqueous solution of the tosylate by isoelectric precipitation. Cefazolin



was a gift of the Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan. Penicillin N (II, R=^-O_2C–CH($\overset{+}{N}H_3$)–$[CH_2]_3$) was a barium salt prepared by Abraham, Newton & Hale (1954) and was about 50% pure. Cephalosporin C (I, R=^-O_2C–CH($\overset{+}{N}H_3$)–$[CH_2]_3$) and other cephalosporins and penicillins were as described by Hamilton-Miller *et al.* (1970*a*). *N*-Phenylacetylglycine was obtained from British Drug Houses Ltd. The α-amide of benzylpenicilloate monohydrate was made as described by Mozingo & Folkers (1949). The α-propylamide of benzylpenicilloate was made as described by Levine & Levytska (1969) and had E_M 24250 by the penamaldyl assay (Parker, de Weck, Kern & Eisen, 1962). The δ-amide of DL-α-aminoadipic acid was as described by Abraham & Newton (1954). A solution containing the 3,10-dehydrodeacetoxy-Δ^4-cephalosporoamide of cephalosporin C (with $\lambda_{max.}$ 230nm) was made by dissolving cephalosporin C (178mg/ml) in dilute aq. NH_3 soln. (sp.gr.0.88 diluted 1:6) and diluting the mixture 1000-fold after 12min at room temperature; its concentration was estimated from the value of *E* at 230nm on the assumption that E_M=12480 (Hamilton-Miller *et al.* 1970*a*). A solution containing the penaldate formed after reaction of cephalosporin C with NH_3 was prepared as described above except that the undiluted solution was left for 6 days at room temperature (Hamilton-Miller *et al.* 1970*a*). The solution was adjusted to pH3 with 12M-HCl and then readjusted to pH9 with 10M-NaOH. The concentration of the penaldate was determined by measurement of *E* at 270nm, a value of 15000 being assumed for E_M (Brown, 1949).

Buffer solutions. The solution described in the text as phosphate-buffered saline was prepared by dilution (1 in 10) of 22.5mM-sodium phosphate buffer, pH7.5, with 0.85% (w/v) NaCl (saline). The veronal buffer used (0.1M, pH8.5) was prepared as described by Dawson, Elliott, Elliot & Jones (1959).

Bovine γ-globulin. This was Cohn fraction II from the Sigma Chemical Co., London, U.K. Its mol.wt. was assumed to be 160000.

Immunization. Two groups of three rabbits each were used for raising antibodies against each antibiotic; one group was immunized with the intact antibiotic, the other with a conjugate of the antibiotic with BGG.* All animals had been bled before immunization. Animals were given four 'primary' injections at intervals of 7 days, each of which consisted of 100mg of antibiotic or 10mg of conjugate. The antibiotic or conjugate was dissolved in 1ml of saline and emulsified with an equal volume of Freund's complete adjuvant; injections were made subcutaneously into the belly in volumes of about 0.15ml. At approximately weekly intervals after the fourth 'primary' injection animals were bled from the marginal ear vein; the blood was allowed to stand for 3h at 37°C, and clot retraction allowed to occur overnight at 4°C. Serum samples obtained in this way were stored at −20°C, after labelling with the number of the animal and the day of bleeding, the day of the first injection being counted as 0. Between 105 and 130 days after the first injection animals were given a 'booster' injection, again of 100mg of intact antibiotic or 10mg of conjugate, in Freund's adjuvant. About 10 days after the booster injections they were bled out by cardiac puncture. With two exceptions (pools 5 and 8) the experiments described were done with pools consisting of equal volumes of final sera from the three animals in a group mixed together. Pools 5 and 8 were each from two animals.

Preparation of conjugates of cephalosporins and penicillin with bovine γ-globulin. To a solution of BGG in water (100mg/ml; 625μM) was added a 100-fold molar excess of the appropriate penicillin or cephalosporin. The solution was adjusted to about pH10.5–11 with 10M-NaOH, and the reaction mixture was left at room temperature for 24h, after which it was dialysed against at least three changes each of 300vol. of deionized water at 4°C and then freeze-dried. Addition of too much alkali to the reaction mixtures caused them to solidify on overnight incubation.

Sensitization of red cells. Blood was collected into an equal volume of modified Alsever's solution [glucose, 2.05% (w/v); NaCl, 0.42% (w/v); trisodium citrate dihydrate, 0.8% (w/v)] from the marginal ear vein of rabbits and the cells were sensitized essentially by the method of de Weck (1964). A solution (0.5ml) of the antibiotic in saline (50mg/ml) was added to 0.25ml of veronal buffer and then 0.5ml of the rabbit blood–Alsever's solution mixture was added. The pH of the mixture was adjusted when necessary to between 8.2 and 8.6, and the mixture (containing antibiotic in a final concentration of 20mg/ml) was incubated at 37°C for 2h with gentle agitation at intervals to prevent sedimentation of the erythrocytes. The cells were then

* Abbreviation: BGG, bovine γ-globulin.

spun down (2000*g* for 10min), washed three times in phosphate-buffered saline and resuspended in 10ml of saline. For use in the haemagglutination assay the cell suspension was adjusted to give a value of *E* at 500nm of between 7 and 3.5; a suspension with *E* 5 was found by direct counting to contain an average of 7×10^7 erythrocytes/ml.

Cells could not be sensitized in this way with deacetoxycephalosporin C, and neither tanned rabbit cells (Feinberg, Davison & Flick, 1956) nor 'HQF' sheep cells (Fulthorpe, Parke, Tovey & Monkton, 1963) proved satisfactory for use with this compound. The $CrCl_3$ treatment described by Gold & Fudenberg (1967) was likewise not suitable for this purpose, but the following modification of it gave fairly satisfactory results: a suspension of packed twice-washed rabbit red cells was mixed with 0.2ml of a 60mg/ml solution of cephalosporin C or deacetoxycephalosporin C in water and 0.2ml of a 0.15% (w/v) solution of $CrCl_3,6H_2O$ in saline. This mixture, which had pH about 4.5, was incubated for 2h at 37°C, and the cells were then washed and resuspended as were cells sensitized conventionally. Cells treated in this way sometimes agglutinated spontaneously, but usually they gave satisfactory results in haemagglutination assays.

Reaction of radioactive benzylpenicillin with red cells. For these experiments potassium 6-([G-^{3}H]phenylacetamido)penicillanate (0.78mCi/μmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and potassium 6-([1-^{14}C]phenylacetamido)penicillanate (59 nCi/μmol) was that made by Sabath, Jago & Abraham (1965). The former compound was diluted 250–750-fold with unlabelled benzylpenicillin before use and the latter was used undiluted.

The reaction mixtures were essentially the same as those used when red cells were sensitized with unlabelled compounds: 1vol. of blood, 1vol. of benzylpenicillin in saline (usually at 50mg/ml) and 0.5vol. of veronal buffer were incubated together and samples were taken at intervals. The cells were washed at least four times with a total of more than 100vol. of phosphate-buffered saline, and suitable volumes (usually 50μl) of the final suspension were taken for measurements of radioactivity and of *E* at 500nm. When [^{14}C]benzylpenicillin was used, samples were counted at infinite thinness in a Nuclear–Chicago gas-flow counter. In experiments with [^{3}H]benzylpenicillin each sample was dissolved in a 1.0M solution of Hyamine hydroxide in methanol (Nuclear Enterprises Ltd., Edinburgh, U.K.). A suitable amount of each resulting solution was added to 3.5ml of the water-immiscible scintillation mixture of Bruno & Christian (1961) and the sample counted in a Nuclear–Chicago Unilux IIA scintillation counter.

Red cell 'ghosts' were prepared for determination of their radioactivity by addition to the cell suspension of 19vol. of a solution obtained by mixing 0.155M-NaH_2PO_4 and 0.13M-Na_2HPO_4 to give pH7.0 and dilution of the resulting suspension with 14.5vol. of water (Dodge, Mitchell & Hanahan, 1963). The 'ghosts' were sedimented by centrifugation at 20000*g* for 20min and washed three times at the centrifuge with water.

In these experiments parallel runs were carried out in the absence of isotope, to provide suitable quenching controls; samples from the 'cold' runs were prepared in the same way as those from the 'hot' runs, and were counted in the presence of a known amount of standard [1,2-^{3}H]*n*-hexadecane.

Determination of free penicillin in sensitized red cells. The cells were sensitized with benzylpenicillin in the usual way, but after washing were resuspended finally in about 1ml of phosphate-buffered saline. The extinction of the suspension at 500nm was measured, and the suspension diluted appropriately with saline or water and then assayed for penicillin by the hole-plate method, *Staphylococcus aureus* (N.C.T.C. 6571) being used as the test organism.

In every assay of this type, the supernatant from the final washing of the red cells was checked for absence of antibacterial activity. When cell suspensions were assayed in saline, lysis occurred on the plates. The resulting values for penicillin did not differ significantly from the corresponding values obtained after prior lysis of the cells by dilution of the suspensions in water.

Haemagglutination assays. These were done with the Microtiter system (Cook Engineering Co., Va., U.S.A.) in V plates containing eight rows each of 12 wells. Doubling dilutions of sera were made in 25μl volumes of saline to give a series of dilutions up to 1 in 1024, the last well in each row being used for control purposes. A sample (25μl) of a suspension of suitably sensitized red cells, standardized to *E* 3.5–7 at 500nm, was added to each well, and the plates were sealed with transparent adhesive tape and incubated for 2h at 37°C before being read. Each row contained serum diluted from 2 to 2048 times. The titre of a serum was defined as the maximum number of times that the serum could be diluted before agglutination failed to occur in the presence of the red cells. Thus the lowest titre measurable was 2.

Usually agglutination was either complete or absent, but where there was any doubt whether it had occurred the plate was held at an angle of about 45° for about 1min and observed from below; under these conditions 'buttons' of red cells that had not agglutinated formed a definite tail as they moved across the bottom of the wells, whereas if agglutination had occurred there was no perceptible movement of the cells.

Inhibition of haemagglutination by haptens. This was also studied in the Microtiter system with V plates. Antiserum was diluted in solutions (each 25μl) containing various concentrations of the inhibitor in saline. After 20min, 25μl of a suspension of sensitized red cells was added to each well. The plates were read after incubation for 2h at 37°C. The antibody titre was plotted against log(hapten concentration) and the line of best fit calculated, any plots giving a regression coefficient of less than 0.85 being rejected. The concentration of hapten that would decrease the titre to 1 was determined from the calculated regression line.

Assay for penicilloyl and α-aminoadipyl groups. The assay for penicilloyl groups was carried out as described by Parker *et al.* (1962), with 200μg/ml of benzylpenicillin–BGG conjugates or 20μg/ml of the α-propylamide of benzylpenicilloic acid. E_M for the mercuric penamaldyl protein was assumed to be 21300.

This assay was found to be inapplicable to cephalosporin conjugates. The amount of conjugation with cephalosporins containing an α-aminoadipyl side chain was estimated by hydrolysis of the conjugate (5–10mg) with 6M-HCl at 105°C for 18h and determination of the

α-aminoadipic acid content of the hydrolysate in an amino acid analyser. The mol.wt. of the conjugate was assumed to be 160000, uncorrected for the degree of substitution.

RESULTS

Conjugation of benzylpenicillin and cephalosporins with bovine γ-globulin. The number of benzylpenicilloyl residues and the number of α-aminoadipyl residues that were associated with 1 mol of BGG after reaction of the latter with benzylpenicillin and four cephalosporins respectively under the conditions used are given in Table 1. The amount of conjugation with deacetoxycephalosporin C was less and that with deacetylcephalosporin C lactone was considerably greater than were the amounts with the remaining substances.

Reaction of penicillins and cephalosporins with red cells. Preliminary experiments showed that [^{14}C]benzylpenicillin was bound to the same extent by red cells from rabbit blood that had been stored at 4°C for 14 days as by cells from freshly drawn blood. Cells which had been washed and resuspended in saline behaved in the same way as cells in a 1:1 mixture of serum and Alsever's solution. Human red cells did not bind noticeably more benzylpenicillin than did rabbit cells.

After rabbit cells had been incubated with concentrations of [^{14}C]benzylpenicillin ranging from 40 to 1.25mg/ml for 1h at 37°C, a linear relationship was observed between log(c.p.m./E_{500nm}) for the suspension of washed cells and log[penicillin] (Fig. 1). The uptake of labelled benzylpenicillin was nearly linear with time for the first hour. After this the rate decreased, but a plateau level was not reached until after more than 9h. An Arrhenius plot of the rates of uptake during the first hour at 15, 25, 36 and 42°C indicated that the apparent energy of activation of the process was 28.6kcal/mol.

In most of the experiments carried out under standard conditions (20mg of [^{14}C]benzylpenicillin/ml for 2h at 37°C) about 1% of the added radioactivity was taken up by the red cells. In 11 experiments, the mean value obtained for the binding of benzylpenicillin was 1.88×10^8 molecules/red cell (S.D. 0.69×10^8). This cell-bound radioactivity was not removed by washing with a solution containing unlabelled benzylpenicillin, but most of it was released when the cells were lysed. Thrice-washed 'ghosts' from cells that had reacted with [^{3}H]benzylpenicillin contained about 0.08% of the radioactivity originally associated with the cells, or about 0.0008% of the total antibiotic added. In one experiment the antibacterial activity released by lysis from benzylpenicillin-sensitized cells corresponded to 0.9×10^8 molecules/red cell. Thus a major part of the radioactivity appeared to be associated in the cell with intact benzylpenicillin.

Table 1. *Conjugation of benzylpenicillin and cephalosporins with bovine γ-globulin*

The extent of the reaction of BGG with benzylpenicillin was determined by the penaldyl assay and by the use of ^{14}C- or ^{3}H-labelled benzylpenicillin. The reaction with cephalosporins was estimated from the amount of bound α-aminoadipic acid. For details see the Methods section. (Binding values are calculated on the assumption of a mol.wt. of 160000 for BGG.)

Antibiotic	Residues bound/mol of BGG
Benzylpenicillin	16
	14*, 12†
Cephalosporin C	16
Deacetylcephalosporin C	19
Deacetoxycephalosporin C	7
Deacetylcephalosporin C lactone	30

* [^{14}C]Benzylpenicillin.
† [^{3}H]Benzylpenicillin.

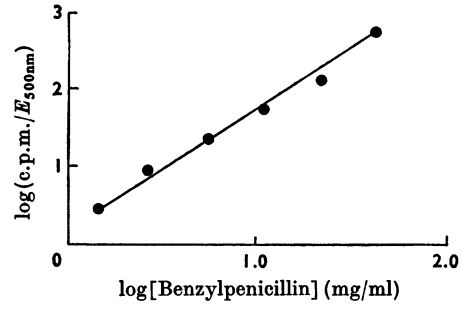


Fig. 1. Uptake of ^{14}C in 1h by rabbit red cells in the presence of different concentrations of [^{14}C]benzylpenicillin.

Antibody titres and haemagglutination. An analysis was made of the results of a series of assays with each serum pool (Table 2) and with sensitized red cells from different rabbits. The average titre observed for all pools was 64 ($\log_2$ titre = 6) and the $\log_2$ titres for individual pools ranged from 4.3 (pool 8) to 7.7 (pool 10). The mean value of V (coefficient of variation) for all 10 pools was 0.16 (169 titrations in all). Thus a serum with value for $\log_2$ titre of 6 would be expected to give a $\log_2$ titre of 6 ± 0.96 on 68% of the occasions on which it was assayed. Since a $\log_2$ titre of 1 represents one twofold dilution the error observed in all the haemagglutination assays amounts to 1 dilution either side of the mean.

Cross-reactivity of antisera to benzylpenicillin. Rabbit red cells were sensitized with various penicillins and cephalosporins and were tested for agglutination by pools 1 and 3, obtained from rabbits immunized with free and conjugated

Table 2. *Titres of antisera from rabbits immunized with benzylpenicillin and various cephalosporins*

Rabbits were immunized with free benzylpenicillin and cephalosporins and with conjugates of these substances with bovine γ-globulin (for details see the Methods section). In each case (with two exceptions) the antisera from three rabbits were pooled. The pooled sera nos. 5 and 8 were from two rabbits. Sera of pools 1 and 3 were assayed against red cells sensitized with benzylpenicillin. All the other sera were assayed against cells sensitized with cephalosporin C.

Immunization with	Antiserum pool no.	No. of assays	Mean $\log_2$ titre
Benzylpenicillin			
Free	1	20	6.5
Conjugated	3	20	7.1
Cephalosporin C			
Free	2	30	6.4
Conjugated	4	28	6.8
Deacetylcephalosporin C			
Free	5	9	6.9
Conjugated	6	10	6.1
Deacetoxycephalosporin C			
Free	7	10	4.9
Conjugated	8	9	4.3
Deacetylcephalosporin C lactone			
Free	9	5	5.8
Conjugated	10	6	7.7

Table 3. *Cross-reactions of various penicillins and cephalosporins to antisera to benzylpenicillin*

Haemagglutination titres were determined as described in the Methods section. When two different values are given they apply to different assays of the same antiserum. The antisera were pools 1 and 3 respectively (Table 2).

Red cells sensitized with	Titres of antisera to benzylpenicillin: Free	Conjugated
Benzylpenicillin	128	512
Penicillin V	64	256
Carbenicillin	32	256
Quinacillin	16	256
Oxacillin	16	32
Methicillin	8	128
Ampicillin	16	64
Penicillin N	8	16
Cephaloram	32, 64	32, 64
Cephalothin	16	64, 128
Cephaloridine	32	8
Cephaloglycin	<2	<2
Cefazolin	<2	<2
Cephalosporin C	2	2

benzylpenicillin respectively. Certain penicillins (phenethicillin, propicillin and cloxacillin) were not used, as they caused extensive lysis of red cells. The results are given in Table 3. All the penicillins tested cross-reacted with anti-benzylpenicillin sera although with some substances, such as methicillin and penicillin N, the sera showed considerably lower titres than with benzylpenicillin itself. Of the cephalosporins tested, cephalosporin C (with the same side chain as penicillin N) showed only a minimal cross-reaction whereas cephaloglycin (with the same side chain as ampicillin) and cefazolin did not cross-react to a measurable extent. However, cephaloram, whose phenylacetyl side chain is identical with that of benzylpenicillin, and cephalothin and cephaloridine, with the closely related thienylacetyl side chain, showed strong cross-reactions.

Cross-reactivity of antisera to members of the cephalosporin C family. The results obtained with red cells sensitized by various cephalosporins and by penicillin N respectively against sera of pools 2, 4, 5, 6, 7, 8, 9 and 10 (Table 2) are shown in Table 4. Only members of the cephalosporin C family and penicillin N, having in common an α-aminoadipyl side chain, cross-reacted with all of these antisera. Cells that had reacted with deacetylcephalosporin C lactone gave higher titres than did cephalosporin C-sensitized cells with the same antiserum. With one exception (*n*-butylcephalosporin) cephalosporins whose side chains were other than α-aminoadipyl showed minimal or undetectable cross-reaction in all cases. However, two of the eight pooled sera (5 and 8) showed titres with cells sensitized with butylcephalosporin that were similar to those with cells sensitized with penicillin N. When the two separate components of pools 5 and 8 were tested against cells sensitized with butylcephalosporin all four sera showed similar haemagglutinating activity.

Cells that had reacted with cephalosporin C in the presence of chromium trichloride (see the Methods section) gave the same end-point with the serum of pool 4 as did cells sensitized with cephalosporin C in the conventional manner. Cells treated with benzylpenicillin in the presence of chromium trichloride lysed spontaneously.

Inhibition of haemagglutination by haptens. Table 5 shows the behaviour of benzylpenicillin and two derivatives of benzylpenicilloic acid as inhibitors of the haemagglutination of benzylpenicillin-sensitized red cells by antisera to benzylpenicillin. The penicilloamides were much more effective inhibitors than was benzylpenicillin itself. 6-Aminopenicillanic acid and *N*-phenylacetylglycine were even less effective inhibitors than benzylpenicillin in a test with one pooled antiserum.

Table 6 shows the ability of several members of the cephalosporin C family and of other compounds to inhibit the haemagglutination of cephalosporin C-sensitized red cells by antisera to cephalosporin C. Cephalosporin C itself and other members of the cephalosporin C family appeared to be as effective

Table 4. *Cross-reactions of various penicillins and cephalosporins to antisera to members of the cephalosporin C family*

Haemagglutination titres were determined as described in the Methods section. When two different values are given they apply to different assays of the same antiserum.

Red cells sensitized with	Titres of antisera: To cephalosporin C, Free	To cephalosporin C, Conjugated	To deacetylcephalosporin C, Free	To deacetylcephalosporin C, Conjugated	To deacetoxycephalosporin C, Free	To deacetoxycephalosporin C, Conjugated	To deacetylcephalosporin C lactone, Free	To deacetylcephalosporin C lactone, Conjugated
Cephalosporin C	128	64, 128	256	64	32	16	256	128
Deacetylcephalosporin C	128	128	256	256	32	16	128	1024
Deacetoxycephalosporin C	128	128						
Deacetylcephalosporin C lactone	256	1024	1024	512	64	64	128	1024
Penicillin N	32	32, 128	16	16	8	4, 16	32	64
n-Butylcephalosporin	<2	<2	32	<2	<2	16	<2	<2
Cephaloram	2	2	<2	<2	<2	<2	<2	<2
Cephalothin	<2	2	2	<2	<2	<2	<2	<2
Cephaloridine	2		<2	<2	<2		<2	<2
Cefazolin	<2	<2	<2	<2	<2	<2	<2	<2

Table 5. *Hapten inhibition of antisera to benzylpenicillin*

Determinations were made as described in the Methods section with red cells sensitized with benzylpenicillin. The concentration of haptens required to decrease the titre of antiserum to 1 were obtained by extrapolation (for details see the text). Pools 1 and 3 are those described in Table 2.

Hapten	Concn. (μM) of hapten required to decrease the titre to 1: Pool 1 antiserum	Pool 3 antiserum
Benzylpenicillin	15400	5600
α-Propylamide of sodium benzylpenicilloate	6.3	2.8
α-Amide of sodium benzylpenicilloate	40	1.3
6-Aminopenicillanic acid		>10000
Phenylacetylglycine		>13000

Table 6. *Hapten inhibition of antisera to cephalosporin C*

Determinations were made as described in the Methods section with red cells sensitized with cephalosporin C.

Hapten	Concn. (μM) of hapten required to decrease titre to 1: Pool 2 antiserum	Pool 4 antiserum
Cephalosporin C	34	39
Deacetylcephalosporin C	67	82
Deacetoxycephalosporin C	36	83
Deacetylcephalosporin C lactone	80	130
7-Aminocephalosporanic acid	49500	78500
DL-α-Aminoadipic acid	7020	8800
δ-Amide of DL-α-aminoadipic acid	2800	4700
Derivative with $\lambda_{max.}$ 230nm from cephalosporin C	48	35
Penaldate with $\lambda_{max.}$ 270nm from cephalosporin C	36	49

as inhibitors as the 3,10-dehydrodeacetoxy-Δ^4-cephalosporoamide. The penaldate formed on decomposition of the latter was apparently equally effective in a similar concentration, but the concentrations of 7-aminocephalosporanic acid, DL-α-aminoadipic acid and the δ-amide of DL-α-aminoadipic acid required to decrease the titre to 1 were 1000, 200 and 80 times as great respectively.

DISCUSSION

The finding that deacetoxycephalosporin C did not sensitize red cells under normal conditions may be correlated with the fact that the β-lactam ring of this substance is considerably more resistant than that of cephalosporin C to aminolysis with hydroxylamine at pH 7.0 and with aqueous ammonia (Hamilton-Miller *et al.* 1970*a*). All the penicillins used in the present work readily formed a hydroxamate under the conditions described by Boxer & Everett (1949). Cephaloram, cephaloridine, cefazolin, cephaloglycin and *n*-butylcephalosporin

appeared to be as labile as cephalosporin C in dilute ammonia. It is thus a reasonable assumption that the behaviour of these substances in haemagglutination assays was not greatly influenced by differences in their ability to react with the red cell membranes. However, antisera to several members of the cephalosporin C family showed higher titres against cells sensitized with deacetylcephalosporin C lactone than against cells sensitized with these cephalosporins themselves (Table 4). This may be partly a reflection of the higher reactivity of the β-lactam ring in the lactone (Abraham & Newton, 1961).

The conjugation of benzylpenicillin with proteins is believed to involve the formation of benzylpenicilloyl groups linked to the ϵ-amino groups of lysine residues (Levine & Ovary, 1961; Schneider & de Weck, 1966). In the present work the number of penicilloyl groups per mol of bovine γ-globulin, as estimated by the penamaldyl assay, was similar to the number of protein-bound groups estimated by use of radioactive benzylpenicillin (Table 1). Since amino acid analysis indicated that the preparation of bovine γ-globulin contained about 100 lysine residues/mol it appeared that about 15% of these residues had reacted under the alkaline conditions used.

The number of mol of cephalosporin C and deacetylcephalosporin C that reacted with bovine γ-globulin (as estimated from the number of α-aminoadipic acid residues bound per mol of protein) was similar to the number of mol of benzylpenicillin which conjugated under the same conditions. The finding that deacetoxycephalosporin C reacted less readily and the observation that a larger number of residues of α-aminoadipic acid were bound to protein after reaction with deacetylcephalosporin C lactone appears to parallel the relative behaviour of these two compounds to red cells. As in the latter case, it may be associated with the lower reactivity of the β-lactam ring of deacetoxycephalosporin C to nucleophilic reagents and with the fact that the β-lactam ring of the lactone is less stable than that of cephalosporin C in neutral or alkaline solution.

Since antisera to members of the cephalosporin C family reacted to a significant extent only (with one exception) with red cells sensitized by the same cephalosporins and by penicillin N, it appeared that the δ-(D-α-aminoadipyl) side chain of all these compounds played a dominant role in the system used. Titres obtained with cells sensitized by penicillin N may be relatively low, since the preparation of the latter was impure and the concentration in which this antibiotic was used for sensitization was thus lower than that of the other compounds. Nevertheless, the fact that some of the antisera showed titres against cells sensitized with *n*-butylcephalosporin is presumably to be accounted for by the presence of an *n*-alkyl chain of four carbon atoms in both compounds.

In contrast, antisera to benzylpenicillin reacted with red cells sensitized by penicillins with quite different side chains, although the titres varied with the nature of the penicillin. However, such antisera reacted with cells sensitized by the cephalosporins used only when the latter contained the phenylacetyl side chain of cephaloram or the closely related thienylacetyl side chain of cephalothin. In this case, also, it appeared that the nature of the cephalosporin side chain was of major importance in the reaction.

The finding that strong hapten inhibition of the anti-cephalosporin C sera was produced by cephalosporin C itself and by a solution of the corresponding penaldate appeared to provide additional evidence that the δ-(D-α-aminoadipyl) side chain played an important part in the reaction with antibody. But since the δ-amide of DL-α-aminoadipic acid was a much less-effective inhibitor, some or all of the additional fragment of the penamaldate may be involved in the reaction.

The behaviour of the rabbit anti-benzylpenicillin sera to red cells sensitized with different penicillins is consistent with results reported earlier in which cells were sensitized with penicillin N (Ley *et al.* 1958), methicillin (de Weck, 1962; Schwartz & Vaughan, 1963) and ampicillin (Girard, 1968), and in which hapten inhibition was studied with methicillin and other penicillins (Van Arsdel & O'Rourke, 1963). These studies led to the conclusion that the nucleus of the penicillin molecule provided a common and significant feature of the determinants derived from different penicillins. The extent to which variations in titre were dependent on the contributions of different side chains to the behaviour of the determinant towards a heterogeneous group of antibodies, or on differences in the facility with which the penicillins concerned reacted with the red cell, remained uncertain. However, two findings with the anti-benzylpenicillin and anti-cephalosporin C sera used in the present experiments indicated that the antibodies were heterogeneous (J. M. T. Hamilton-Miller, unpublished work). There was no significant correlation beween the haemagglutinating titre of an antiserum pool and the amount of precipitable antibody it contained; some free antibody still remained in the supernatant after precipitation under optimum conditions or with an excess of antigen. These findings are consistent with the conclusion of Brandriss, Smith & Steinman (1965) that rabbits make antibodies of varying degrees of specificity to penicillin.

The agglutination of red cells sensitized with cephalothin by antisera to benzylpenicillin confirms

an earlier finding of Batchelor, Dewdney, Weston & Wheeler (1966), who reported that there was a high degree of cross-reaction between benzylpenicillin and cephalothin and cephaloridine, but very little between these cephalosporins and the penicillin nucleus, and stated that this might be due to the presence of immunologically similar side chains in the penicillin and cephalosporin concerned. A similar conclusion was reached by Shibata, Atsumi, Horiuchi & Mashimo (1966), who used the amount of antibody precipitation to analyse the cross-reactivity of benzylpenicillin, cephalothin, cephaloridine and cephaloram.

The results described here point to clear-cut differences between the immunological properties of the penicillins and those of the cephalosporins in the haemagglutination test system. They could be interpreted in terms of the known differences in the chemical behaviour of the two groups of substances when subjected to aminolysis by simple amino compounds in aqueous solution. Thus the finding that the penaldate from cephalosporin C was as active as the immediate product of aminolysis of the latter in causing hapten inhibition of haemagglutination by antisera to cephalosporin C of cephalosporin C-sensitized red cells would be consistent with breakdown to a penaldate of the product first obtained on conjugation of cephalosporin C with protein. Since no significant difference was observed between the behaviour of antisera obtained after immunization with cephalosporin C itself and a cephalosporin C–protein conjugate respectively, it could be supposed that such breakdown occurs *in vivo* as well as *in vitro* and that there is no major determinant corresponding to an entire cephalosporoate type of molecule. On the basis of changes in ultraviolet absorption, which occurred when cephalothin and cephaloridine were coupled with human γ-globulin, Stemberger & Wiedermann (1970) suggested that the dihydrothiazine ring of the cephalosporins disintegrated, leaving a penaldate type of structure linked to protein. However, no direct evidence about the structure of the determinant groups that are formed when cephalosporins react with proteins has so far been obtained. The extent to which fission of a cephalosporoyl group to yield a penaldyl group occurs in protein conjugates and (if it does occur) the fate of the remaining fragment of the molecule requires further investigation.

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