

support to this assumption. For papain digest we have assumed that the active fragments are the same as those produced by reduction of peptic digest³ and that the proportion of radioactivity absorbable is the same as that in the whole (before dialysis) peptic digest of the 7S preparation.

Comparisons have been made of the 7S antibody (separated by DEAE-cellulose chromatography) and its derivatives from three rabbits, all the derivatives from one serum being investigated simultaneously. In each case, the curves for 7S antibody and peptic digest have been almost identical. The curves for univalent fragments, however derived, have been very close together and parallel. The concentrations of A_1 cells necessary to absorb half the antibody from 7S or 5S preparations from each of the three rabbits were, respectively, 0.16, 0.03 and 0.02 per cent; the cell concentrations required to absorb half the univalent antibody derived from these preparations were, respectively, 19, 10.5 and 6 per cent. That is to say $\frac{K \text{ bivalent } Ab (7S \text{ or } 5S)}{K \text{ univalent } Ab \text{ fragments}}$ was 119, 350 and 300 respectively for the three rabbit sera.

The greatly diminished binding constant of univalent antibody fragments could be explained if the process of splitting the molecule had distorted the antibody specific group; this seems unlikely since, at least for a small hapten, K remains unaltered after splitting the antibody molecule⁵; moreover, if the binding constant of antibody sites were drastically diminished it seems improbable that a comparatively small excess of univalent fragments would inhibit precipitation in the ovalbumin-anti-ovalbumin system². Labelling with iodine does not appear harmful since labelled anti-ovalbumin fragments which we have prepared inhibit precipitation in the same proportion as described by Porter. It is possible that reduction in

the size *per se* of the antibody would reduce K by eliminating some non-specific binding of the antibody molecule, but we find no difference in K between the 7S and the bivalent 5S fragment derived therefrom.

We consider that the great diminution in binding constant which accompanies the reduction of antibody valency from 2 to 1 gives support to our hypothesis that rabbit immune 7S anti- A antibody mostly combines with the red cell by both its specific groups. The possibility of this type of antigen-antibody attachment does not appear to have received consideration and is even excluded from his calculations by Goldberg⁶, although it is not necessary to postulate that the bivalent attachment of antibody on a red cell is to a single antigen molecule. However, Almeida, Cinader and Howatson⁷ have shown electron micrographs which may be interpreted as bivalent attachment of a single antibody molecule to a wart virus particle.

A final resolution of this point may be possible by the examination, which we are about to attempt, of univalent fragments recombined to the bivalent 5S form⁸, where if our hypothesis is correct, recombined pure antibody should regain its 5S binding constant, whereas 5S recombinants made up of one antibody and one non-antibody 3-5S fragment would have the binding constant of a univalent fragment.

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QUINACILLIN: A COMPARISON WITH OTHER PENICILLINASE-RESISTANT PENICILLINS

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QUINACILLIN, 3-carboxy-2-quinoxalinylnicillin, a recently described semi-synthetic penicillin, was found¹ to have 'unusual properties', namely, antibacterial activity highest against *Staphylococcus aureus*, enhancement of antibacterial activity and rate of diffusion in agar with decrease in pH of the medium, and poor inducing ability for staphylococcal penicillinase. In view of these findings, we have examined the interactions of quinacillin with various bacteria and their penicillinases, and are able to confirm and extend the description of this penicillin as possessing "unusual properties".

Quinacillin as a substrate for penicillinase. (a) *Gram-positive bacteria.* The rates of hydrolysis of quinacillin at various concentrations in 0.025 M sodium phosphate buffer pH 7.4 were measured using the hydroxylamine method². With staphylococcal penicillinase made, as previously described³, from *Staph. aureus* E3, the Michaelis constant (K_m) for quinacillin was 61 mM (28 mg/ml.) with a V_{max} 8 per cent that of benzylpenicillin; at an initial concentration of 4.3 mM (2 mg/ml.) quinacillin was not appreciably hydrolysed by this preparation. Richards *et al.*¹, who used the iodometric method of assay at pH 6.5, found that the K_m value for staphylococcal penicillinase (Parker strain) was 5 mg/ml. and the V_{max} was 5 per cent that of benzylpenicillin. They also found no appreciable hydrolysis of quinacillin at lower concentrations.

Penicillinase preparations⁴ from *B. cereus* and *B. licheniformis* were found to hydrolyse quinacillin (initial concentration 4.3 mM) at 11 and 13 per cent, respectively,

of the rates at which they hydrolysed benzylpenicillin. Both these preparations had K_m values for quinacillin of about 54 mM.

(b) *Gram-negative bacteria.* Cell-free preparations made from *Pr. morgani* G (ref. 5), *Pr. rettgeri* 410, *K. aerogenes* 366 (ref. 6) and *E. coli* 214T (ref. 7) did not appreciably hydrolyse quinacillin (initial concentration 4.3 mM). A cell-free extract of *A. cloacae* 53 (ref. 6) hydrolysed quinacillin (4.3 mM) at about 7 per cent of the rate at which it destroyed benzylpenicillin (5.4 mM) and intact cells of this organism hydrolysed quinacillin at about one-seventh of this rate (Table 1). Disruption of this strain has been shown⁶ to increase its hydrolytic activity towards benzylpenicillin 25 times. Thus it seems that quinacillin can pass the substrate accessibility barrier in this strain more readily than benzylpenicillin.

Quinacillin as an antibacterial agent. Minimum inhibitory concentrations of quinacillin were determined, by means of the tube test, for six strains of *Staph. aureus*, E3, 13137, 6637, 5974 (all of which produce penicillinase, the last three being resistant to methicillin and cloxacillin⁸), E3T and Oxford (benzylpenicillin-sensitive, penicillinase-negative strains). Two inoculum sizes were used, about 10^6 and about 10^2 viable units per tube. The results

Table 1. DESTRUCTION OF 2 PENICILLINS BY *A. cloacae* 53 (rates expressed as $\mu\text{g}/\text{min}/10^9$ cells/ml.)

| | Intact cells | Disrupted cells | Ratio (to nearest whole number) of penicillinase activity of disrupted to intact cells (= 'permeability factor') |
|------------------|--------------|-----------------|--|
| Quinacillin | 3 | 20 | 7 |
| Benzylpenicillin | 12 | 303 | 25 |

Table 2. ANTIBACTERIAL ACTIVITY OF QUINACILLIN AGAINST SIX STRAINS OF *Staph. aureus*
Minimum inhibitory concentrations ($\mu\text{g/ml.}$)

| Strain of <i>Staph. aureus</i> | Methicillin | | Cloxacillin | | Quinacillin | |
|--------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| | 10 ⁶ | 10 ⁷ | 10 ⁶ | 10 ⁷ | 10 ⁶ | 10 ⁷ |
| Penicillin Inoculum | | | | | | |
| E3 | 7.5 | 3.7 | 5.0 | 1.0 | 6.3 | 3.1 |
| 13137 | 1,000.0 | 125.0 | 500.0 | 5.0 | 50.0 | 12.5 |
| 6637 | > 1,000.0 | 25.0 | 1,000.0 | 5.0 | 400.0 | 6.3 |
| 5974 | 500.0 | 100.0 | 1,000.0 | 5.0 | 25.0 | 6.3 |
| E3T | 3.2 | 1.6 | 0.8 | 0.4 | 6.3 | 6.3 |
| Oxford | 1.6 | 0.8 | 0.4 | 0.2 | 6.3 | 1.6 |

Table 3. MINIMUM INHIBITORY CONCENTRATIONS OF QUINACILLIN, TESTED AGAINST GRAM-NEGATIVE BACTERIA BY TUBE DILUTION METHOD

| Inoculum size approximately 10 ⁶ organisms | Minimum inhibitory concentrations ($\mu\text{g/ml.}$) | |
|---|---|-----------------|
| | 10 ⁶ | 10 ⁷ |
| <i>K. aerogenes</i> 83, 370, 415, 418 | > 4,000 | > 8,000 |
| <i>K. aerogenes</i> 1, 43, 366, 373, 407, 414 | > 4,000 | > 4,000 |
| <i>K. ozaenae</i> 61 | > 4,000 | > 4,000 |
| <i>E. coli</i> 214T | > 2,000 | > 2,000 |
| <i>Pr. morgani</i> G | 680 | > 2,000 |
| 185 | > 2,000 | 500 |
| <i>Pr. rettgeri</i> 410 | 500 | 4,000 |
| <i>Achromobacter</i> 510 | > 4,000 | > 20,000 |
| <i>A. cloacae</i> 53 | > 20,000 | > 20,000 |

Table 4. INDUCING ABILITIES OF FOUR COMPOUNDS FOR PENICILLINASE OF *Staph. aureus* E3

| Compound | Concentration $\mu\text{g/ml.}$ | | Ratio of induced to basal penicillinase activity at maximum |
|---------------------------|---------------------------------|--------------------------------|---|
| | to give maximal induction | to give half maximal induction | |
| Quinacillin | 12.5 | 2.07 | 17.8 |
| 6-Aminopenicillanic acid* | 10.0 | 3.0 | 16.3 |
| Methicillin* | 3.0 | 0.27 | 36.0 |
| Cloxacillin† | 0.1 | 0.013 | 71.3 |

* Ref. 3. † ref. 15.

(Table 2) show that there is cross-resistance between cloxacillin, methicillin and quinacillin, but it appears that resistant strains are slightly more susceptible to quinacillin than to the other two, at least using large inocula. The 'inoculum size effect' obtained with this series of organisms is probably a reflexion of populations that are not homogeneous, in that they contain small proportions of highly resistant bacteria⁸. In common with other penicillins, quinacillin was extremely poor at inhibiting the growth of *B. cereus*, the minimum inhibitory concentration being 5 mg/ml. for an inoculum of 10⁶ organisms. Quinacillin was relatively inactive as an antibacterial agent against Gram-negative bacteria (Table 3). Richards *et al.*¹ also found that Gram-negative bacteria were resistant to quinacillin.

Quinacillin as an inducing agent for penicillinases. The ability of quinacillin to induce penicillinase production in *Staph. aureus* E3 was determined as described previously³ and the results are shown in Table 4. It can be seen that the concentrations for half maximal induction ('induction constants'¹⁰) with quinacillin, methicillin and cloxacillin were 2.07, 0.27 and 0.013 $\mu\text{g/ml.}$, respectively. Thus the 'induction constant' for quinacillin is some eight times larger than that for methicillin and 160 times larger than that for cloxacillin. In addition, as judged by the concentrations required for maximal induction and by the amount of enzyme production at these concentrations, quinacillin was a less effective inducing agent than either methicillin or cloxacillin. Using the Parker strain of *Staph. aureus*, Richards *et al.*¹ reported a twenty-five-fold difference between the 'induction constants' for methicillin and quinacillin, so, allowing for strain differences, it seems that quinacillin is a much poorer inducing agent than would be expected from its activity against penicillinase-producing staphylococci.

It appeared that the figures obtained here for quinacillin in induction experiments were very similar to those found for 6-aminopenicillanic acid in an earlier series of experiments³. To determine whether quinacillin was broken down to 6-aminopenicillanic acid (which could explain these similarities) during the process of induction, quinacillin was incubated at 37° C with washed cells from an overnight culture of E3 in phosphate-buffered saline at pH 7.4 for 3 h. The cells were centrifuged down, and enough of the supernatant fraction to contain approximately 50 μg quinacillin was applied to Whatman No. 1 paper and chromatographed in *n*-butanol 4:acetic acid 1:water 5, descending overnight; markers of quinacillin,

the penicilloic acid derived from it (made by dissolving quinacillin in N soda, and neutralizing with N hydrochloric acid after 30 min at room temperature) and 6-aminopenicillanic acid were also applied to the chromatogram. When the run had been completed, the paper was dipped in 0.2 per cent ninhydrin in acetone¹¹, heated for 5 min at 100° C and then 'fixed' in alcoholic copper nitrate. Under these conditions, the quinacillin developed a pale-yellow colour, fluorescent in ultra-violet light (this yellow fluorescence could also be observed before ninhydrin treatment). The penicilloic acid developed a red colour which did not fluoresce. The R_F values of these two compounds in the solvent used were 0.82 and 0.65, respectively. No trace of a spot similar to that derived from 6-aminopenicillanic acid (R_F value 0.53—which develops a golden-yellow colour, slightly fluorescent¹¹) could be observed in the incubation mixture. Nor was any evidence of a penicillin acylase obtained when the butyl acetate extraction technique¹² was used, with benzylpenicillin as substrate, with this strain of *Staph. aureus*. Hence, it appears that the resemblance between the values of certain induction parameters of quinacillin and 6-aminopenicillanic acid is a mere coincidence.

Quinacillin, benzylpenicillin, methicillin, cloxacillin and 6-aminopenicillanic acid were tested for inducing ability towards the penicillinase of *B. cereus*. Various concentrations of each inducing agent were dissolved in broth, 2 ml. of a 6-h 37° C culture of *B. cereus* 569 added to each 100 ml., and bottles were incubated overnight at 37° C. Portions were centrifuged (3,000g for 15 min), after mM *p*-chloromercuribenzoate had been added to prevent further induction, and the turbidity measured at 700 m μ . The supernatant fraction was adjusted to pH 7.4 with N sodium hydroxide and the rate of hydrolysis of benzylpenicillin estimated by the hydroxylamine method. Results were expressed in terms of the amount of increase in penicillinase activity as compared with a control culture grown in broth alone; Table 5 shows the results obtained. It is clear that quinacillin, although a better inducing agent than benzylpenicillin (probably due to its greater stability to this penicillinase), is not so efficient at the higher concentration as methicillin, cloxacillin or 6-aminopenicillanic acid.

Quinacillin did not cause any increased enzyme production in five strains of *K. aerogenes*—43, 370, 373, 407 and 414—nor in *K. ozaenae* 61; this is in common with the non-inducibility of these penicillinases by all other compounds tested⁶. When *Pr. morgani* 185 (ref. 5) and *Pr. rettgeri* 410 were grown overnight at 37° C in the presence of half the minimum inhibitory concentration of quinacillin, penicillinase was induced. The activities of these cultures after disruption, expressed in terms of μg benzylpenicillin hydrolysed/min/10⁸ cells/ml., were 5.6 and 3.7, respectively. Uninduced preparations of these two organisms hydrolysed less than 0.1 μg benzylpenicillin/min/10⁸ cell/ml.

Quinacillin as an inhibitor of benzylpenicillin hydrolysis by penicillinases. Penicillins which are only slowly hydrolysed by penicillinases have been shown to inhibit benzylpenicillin hydrolysis by *B. cereus*¹³, *B. licheniformis*¹¹, *K. aerogenes*¹⁴, *E. coli*¹⁴ and *Pr. morgani*¹⁴ penicillinase preparations. Mixtures of 1.3 mM (0.5 mg/ml.) benzylpenicillin and mM quinacillin were incubated with enzyme

Table 5. INDUCING ABILITIES OF FIVE COMPOUNDS FOR PENICILLINASE OF *B. cereus* 569

| Compound | Ratio of concentration of inducing agent to minimum inhibitory concentration for that inducing agent | |
|-----------------------------------|--|-----|
| | 1/10 | 1/4 |
| Benzylpenicillin (1,250) | 9 | 7 |
| Quinacillin (5,000) | 31 | 39 |
| Cloxacillin (500) | 21 | 54 |
| 6-Aminopenicillanic acid (10,000) | 66 | 92 |
| Methicillin (313) | 25 | 140 |

Figures in brackets are minimum inhibitory concentrations of drugs for *B. cereus* (large inoculum, in $\mu\text{g/ml.}$).

Figures in body of the Table are enzymatic activity after induction, taking basal level = 1.

preparations from *Staph. aureus* E3, *B. cereus* 569 and *B. licheniformis*, which had been adjusted to hydrolyse 2 µg benzylpenicillin/min^{11,15}. The rates of hydrolysis in the presence of quinacillin, measured by the hydroxylamine method, were compared with those obtained in the absence of quinacillin but no differences were observed. Hence, quinacillin, in contrast to the other poorly hydrolysed penicillins, does not inhibit any of the penicillinase preparations from Gram-positive bacteria examined.

When cell-free penicillinase preparations of *E. coli* 214T and *Pr. morgani* G were tested by the modified spectrophotometric method¹¹, no inhibition of the hydrolysis of benzylpenicillin (2.7 mM) could be detected in the presence of concentrations of quinacillin as high as 260 µM. However, under similar conditions 50 per cent inhibition of the penicillinase activities of *K. aerogenes* 366 and 373 was observed with 70 µM and 161 µM quinacillin, respectively; thus, quinacillin is about as active an inhibitor of the *Klebsiella* penicillinases as oxacillin and BRL 1577 (ref. 14). The fact that quinacillin does not inhibit the *E. coli* and *Proteus* penicillinase preparations is in contrast to the inhibitory action of other penicillins stable to the penicillinases of these bacteria.

Discussion. Richards *et al.*¹ found that quinacillin possessed unusual properties. We have confirmed that this penicillin is a poor inducing agent for staphylococcal penicillinase, and in addition we have shown that it is a relatively inefficient inducer of *B. cereus* penicillinase.

As a substrate for penicillinases derived from various sources, quinacillin shows in general a marked resistance to hydrolysis; its affinity for staphylococcal and *Bacillus* penicillinases was uniformly low, but it was hydrolysed rather more rapidly by the *Bacillus* enzymes. Penicillinase preparations from *K. aerogenes*, *E. coli* and *Pr. morgani* did not detectably destroy the compound, although a strain of *A. cloacae* did hydrolyse quinacillin. An interesting feature of this penicillin is that it does not inhibit penicillinases from *B. cereus* and *B. licheniformis*, which

is in contrast to the action of other poorly hydrolysed penicillins. Similarly, penicillinase preparations from *E. coli* and *Pr. morgani* were not inhibited by quinacillin, and it was found to be only a very weak inhibitor of *K. aerogenes* penicillinases.

The discovery that a penicillin which is a poor inducer of staphylococcal penicillinase can also be an effective antistaphylococcal agent discounts the correlation that has been made by some authors¹⁵⁻¹⁸ between inducing ability and antibacterial activity for penicillinase-producing staphylococci. Until now penicillins which are biologically active against penicillinase-producing *Staph. aureus*, besides being resistant to hydrolysis by this enzyme, have also been found to be very good inducing agents and can markedly inhibit various penicillinases. It is interesting that although quinacillin does possess the first two characteristics it is not a particularly good inducing agent nor an efficient inhibitor of penicillinases.

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A COMPARISON OF EFFECTS OF SOME CHEMOTHERAPEUTIC AGENTS AND THOSE OF X-RAYS ON THE REPRODUCTIVE CAPACITY OF MAMMALIAN CELLS

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WHILE chemotherapeutic agents have been extensively investigated for ability to 'cure' a wide range of transplanted tumours *in vivo*¹ and have less frequently been investigated qualitatively *in vitro* in cultures of mammalian cells², there has been a paucity of quantitative investigation of the effect on cell reproductive capacity of agents known to have clinical usefulness in palliation of human cancer^{3,4}. The technique of clonal cell culture described by Puck *et al.*⁵ by which macroscopically visible colonies can be grown *in vitro* from single mammalian cells has been extensively applied to the examination of effects of ionizing radiation (see Porter⁶ for a representative list of references). It is only recently, however, that this method has been used to investigate the dose-response to a chemotherapeutic agent⁷.

The present communication reports investigations of the dose-response relationship for the reproductive capacity of two strains of mammalian cells exposed *in vitro* to several commonly used anticancer drugs and compares these relationships with that for X-irradiation of the same cells.

The drugs used in these experiments were of the following types: (1) *Nitrogen mustard*. (a) 'HN3' (tri-(2-chloroethyl)-amine, 'Trillekamin', Crookes). (b) Mannitol

mustard (Mannomustine dihydrochloride, 'Degranol', Chinon). (2) *Ethylene imine* ('Thio-TEPA', Lederle). (3) *Epoxide* ('Epodyl', I.C.I., 32865). (4) *Antimetabolite* (5-fluorouracil, Roche). All drugs were diluted with sterile normal saline to desired final concentrations immediately prior to addition to the growth medium.

The mammalian cell lines used were HeLa S-3_{oxf}, an aneuploid derivative of human cervical carcinoma kindly supplied by Dr. C. E. Ford, Medical Research Council, Harwell, and re-cloned in this laboratory, and the mouse strain L_{oxf} fibroblast kindly provided by Dr. J. Paul, Department of Biochemistry, Glasgow, and re-cloned in this laboratory by Dr. A. H. W. Nias. They were grown in Medium 199 (Glaxo) supplemented with 5 µg/ml. amphotericin B ('Fungizone', Squibb), and for HeLa: 10 or 20 per cent freshly drawn human AB serum; for strain L: 2 per cent pooled calf serum (Oxoid) and 0.5 per cent 'Bacto-Peptone' (Difco) for stock growth and 10 per cent pooled calf serum for clonal growth.

The technique of clonal culture was modified only slightly from that of Puck *et al.*⁵; cells were trypsinized ('Bacto-Trypsin', Difco, 0.25 per cent in 0.3 per cent sodium citrate made isotonic with sodium chloride) from a stock bottle containing a nearly confluent sheet of cells. They were re-suspended in the appropriate medium and

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