DEGRADATION OF SELECTED CEPHALOSPORINS BY HOMOGENATES OF THE ALIMENTARY TRACT OF RATS

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The increasing use of cephalosporin derivatives in the treatment of bacterial infections has stimulated efforts to synthesise substances based on the 7-aminocephalosporanic acid (7ACA) nucleus that appear in the blood in high concentration when administered orally. In addition to being efficiently absorbed from the gastro-intestinal tract, a good oral antibiotic should be able to withstand degradation in the gut before absorption. Substitutions at the 7- and at the 3-position on the cephalosporin nucleus may influence both absorption from and stability in the gut; with this in mind we chose three cephalosporin derivatives for our studies (fig. 1).

Cephaloridine (CER) is very effective when given by intramuscular injection (Muggleton, O'Callaghan and Stevens, 1964), but is poorly absorbed when given orally to rats (Sullivan and McMahon, 1967) and to man (Muggleton et al., 1964). Cephalexin (CEX), on the other hand, is very well absorbed by mice (Wick, 1967) and by rats and man (Muggleton et al., 1968). The third cephalosporin, 7-(thienyl-2'-acetamido)-3-methylceph-3-em-4-carboxylic acid, or TMC, has the 7-substituent of cephaloridine and the 3-substituent of cephalexin, and in our experience is absorbed to an intermediate extent after oral administration to rats.

We attempted to find out whether the low serum levels obtained in rats after oral administration of cephaloridine were due to poor absorption from the alimentary tract or to destruction of the cephaloridine before it could be absorbed. In this paper, in-vitro degradation of the three cephalosporins by different parts of rat alimentary tract has been examined.

MATERIALS AND METHODS

Bioassay of antibiotics

All three cephalosporins were assayed by a diffusion method on large agar plates. For CER the assay organism was Staphylococcus aureus no. NCTC7447, deep-seeded in a single layer of Difco Tryptose Agar. For CEX and TMC the assay organism was Bacillus subtilis no. ATCC6633 (NCIB8533). The most suitable medium for this organism contained (as percentages w/v) Oxoid Peptone L37 (0.5), Oxoid Lab-Lemco (0.3), sodium citrate (1.0) and Oxoid Agar no. 3 (1.2), at pH 7. 1 per cent. of a 1 in 100 dilution of a concentrated spore suspension in distilled water was inoculated into the melted agar. All plates were incubated at 37°C overnight. Concentrated standards of CER and TMC were prepared in sterile 0.05M phosphate buffer at pH 7; CEX was prepared in 0.05M phosphate buffer at pH 6. Working standards were prepared freshly every day by dilution from the concentrated solutions in the appropriate buffers.

High concentrations of tissue in the samples of homogenate reduced the recovery of all three cephalosporins to a similar extent. Where cephalosporin levels were high, this effect

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could be removed by dilution. Where levels were very low, e.g., in the caecum, the assay was too insensitive to permit sufficient dilution and the results quoted in such cases may have been underestimated by as much as 30 per cent.

**Thin-layer chromatography**

Thin layers were prepared from a mixture of Merck Kieselgel HF 265+366 (3 parts) and Bio-rad Cellex MX (1 part), which combined the running characteristics of the silica and the hardness of the micro-crystalline cellulose. The solvent system was n-butanol/ethanol/0.1M ammonium acetate (ratio 30:5:8) adjusted to pH 5.0 with glacial acetic acid. Components were detected under UV light or after spraying with ninhydrin reagent (Levy and Chung, 1953).

**Enzyme assays in rat intestinal homogenates**

The alimentary tract of two 100-g female Albino Wistar rats was divided into four parts: (1) stomach; (2) first 6 in. (15 cm) of small intestine; (3) rest of small intestine; (4) caecum and rectum. Like portions were combined and homogenised in 20-ml pre-cooled (5°C) 0.2M phosphate buffer, pH 7.0 in an Ultra Turrax homogeniser.

Equal volumes of each homogenate were incubated at 37°C with a solution of the cephalosporin in the phosphate buffer; samples were taken at intervals, cleared by centrifugation and examined.

(a) **β-Lactamase assay.** Degradation of the β-lactam ring was followed by the reduction in UV absorption of the cephalosporin solution at 255 nm (O'Callaghan, Muggleton and Ross, 1968) and the appearance of pyridine peaks after breakdown of CER. The hydroxylamine method of Knox and Smith (1962) as modified for cephalosporins by Hamilton-Miller, Smith and Knox (1965) was not compatible with the intestinal homogenates even when a more dilute buffer was used; reasonable standard curves were obtained at room temperature in homogenates but, after incubation at 37°C, wide variation in results occurred.

(b) **Acylase assay.** Solutions of the cephalosporins in rat intestinal homogenates were incubated at 37°C for 18 hr and examined by thin-layer chromatography for 7-substituent liberated by acylase. The technique used could detect \( \geq 1 \) per cent. liberation of thienylacetic acid or phenylglycine.

Bio-autography on Difco Micro Assay Culture Agar plates seeded with *Staphylococcus aureus* NCTC7447 was used in conjunction with a phenylacetyl chloride spray (Sabath, Jago and Abraham, 1965) to detect any 7-amino-3-methylceph-3-em-4 carboxylic acid liberated by acylase from CEX or TMC.
(e) Trypsin and chymotrypsin were assayed by the method of Schwert and Takenaka (1955).

Action of proteolytic enzymes on cephalosporins

Solutions of all three cephalosporins (5 mg per ml) were incubated with each proteolytic enzyme at its optimum pH overnight at 37°C. The following control solutions were also incubated overnight at 37°C: (a) enzyme + bovine plasma albumin (BPA), to check enzyme activity; (b) enzyme alone, to check for low molecular weight impurities; (c) the cephalosporin alone, to check for any chemical decomposition at the pH and temperature of the test; and (d) BPA alone.

The solutions were examined for any reaction by thin-layer chromatography, UV assay and bioassay.

In-vitro degradation of cephalosporins by rat intestinal homogenates

Homogenates of parts of rat alimentary tract were prepared as described above. Twelve rats (six groups of two) were used in each experiment. Samples of each homogenate (3 ml) were incubated at 37°C with the appropriate cephalosporin (100 μg per ml) and then centrifuged at 2000g for 15 min. The supernatants were assayed microbiologically for residual cephalosporin. In further tests the alimentary tract from 50-g, 100-g, and 200-g rats of either sex were used.

RESULTS

In-vitro degradation of cephalosporins by rat intestinal homogenates

Initial experiments showed that the fourth portion of rat intestine, consisting of caecum and rectum and referred to as the caecal portion, was most active and that comparatively little in-vitro degradation occurred elsewhere in the alimentary tract during incubation for 1 hr at 37°C. Incubation time was extended to 4 hr and typical results are shown in the table. Some degradation occurred in the lower portion of the small intestine, but most occurred in the caecal portion and appeared to depend on the structure of the cephalosporin. After 2 hr CER had been completely destroyed in the caecal portion, but was only slightly degraded in the other portions; even after 4 hr at least 65 per cent. of the CER remained in the first three portions; TMC was slightly degraded in the first three portions and about 75 per cent. degraded in the fourth portion after 4 hr; CEX, when compared with a control solution in phosphate buffer, was largely undegraded in the first three portions and even after 4 hours’ incubation 57 per cent. remained in the homogenate of the fourth portion.

Degradation of the cephalosporins by rat intestinal homogenates after 1, 2 and 4 hr at 37°C is summarised in fig. 2.

Only the caecal portions were used to examine the effect of age and sex on in-vitro degradation. The weight of the caecum varied considerably with age, so all homogenates were diluted to a similar concentration (0.1 g per ml). The results illustrated in fig. 3 show that, once again, CEX was hardly affected after 1 hr and recovery from the various homogenates was very consistent. With CER the larger and older rats caused more degradation than the smaller young rats and the homogenates from females were more active than those from males (degradation for 100-g females was less than in previous tests). A similar pattern emerged for TMC, but the differences were less significant.
Assay for cephalosporin-degrading activities in rat intestinal homogenates

When rat intestinal homogenates were examined for β-lactamase activity, only the caecal portions had detectable activity and β-lactamase-producing bacteria were isolated from these portions.

A bacteriological survey of the caecum of 50-g, 100-g and 200-g rats was carried out. Similar organisms were detected in all three weight groups;

<table>
<thead>
<tr>
<th>Portion of intestine</th>
<th>Cephalosporin</th>
<th>Concentration of antibiotic* (µg per ml) remaining in sample after</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 hr</td>
</tr>
<tr>
<td>Stomach</td>
<td>CER†</td>
<td>98±3</td>
</tr>
<tr>
<td></td>
<td>TMC</td>
<td>73±3</td>
</tr>
<tr>
<td></td>
<td>CEX</td>
<td>97±9</td>
</tr>
<tr>
<td>First part of small intestine</td>
<td>CER</td>
<td>92±6</td>
</tr>
<tr>
<td></td>
<td>TMC</td>
<td>81±6</td>
</tr>
<tr>
<td></td>
<td>CEX</td>
<td>87±5</td>
</tr>
<tr>
<td>Rest of small intestine</td>
<td>CER</td>
<td>87±5</td>
</tr>
<tr>
<td></td>
<td>TMC</td>
<td>63±7</td>
</tr>
<tr>
<td></td>
<td>CEX</td>
<td>82±3</td>
</tr>
<tr>
<td>Caecum and rectum</td>
<td>CER</td>
<td>0 (&lt;1.5)</td>
</tr>
<tr>
<td></td>
<td>TMC</td>
<td>46±4</td>
</tr>
<tr>
<td></td>
<td>CEX</td>
<td>62±2</td>
</tr>
<tr>
<td>None (control of cephalosporin in buffer at 37°C)</td>
<td>CER</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>TMC</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>CEX</td>
<td>96</td>
</tr>
</tbody>
</table>

* Values are means of six results ± standard error of mean.
† See fig. 1.

greater numbers were isolated from the larger, older rats. Several strains of Proteus spp., which are often β-lactamase producers, were isolated from both 100-g and 200-g rats, but not from 50-g rats. Most of the β-lactamase-positive strains of Proteus were isolated from the 200-g rats.

The β-lactamases produced by many Gram-negative organisms are inhibited by some cephalosporins (O'Callaghan et al., 1966, 1968). Two such compounds, the cephalosporins 5/1 (2:6-dimethoxybenzamidocephalosporanic acid) and 291/1 (3-(o-chlorophenyl)-5-methylisoxazole-4-carboxamidocephalosporanic acid) were used to inhibit the action of partially purified β-lactamase from Enterobacter cloacae strain P99 (O'Callaghan et al., 1968) on CER as a control system; they were also used in attempts to inhibit degrading activity of rat intestinal homogenates. Test solutions were incubated at 37°C and samples taken for microbiological assay at 3 ½ and 22 hr. Cephalosporins
5/1 and 291/1 both protected CER from the β-lactamase of strain P99 and, to a less extent, from the degrading activity of the caecal homogenates (fig. 4).

![Graph](image)

**Fig. 2.**—In-vitro degradation of cephalosporins by homogenates of rat alimentary tract at 37°C. Dotted lines indicate progress of cephalosporin degradation in a buffer solution at 37°C. S = Stomach; 1st = first six inches of small intestine; 2nd = rest of small intestine; C = caecum + rectum. CER, TMC, CEX: see fig. 1.

Acylase activity was not detected. A sample of the incubated rat homogenate containing 300 μg of CER was applied to the chromatogram. Total
breakdown of the acyl linkage would have liberated 102 μg of thienylacetic acid and therefore 1 per cent. breakdown (1 μg) could be detected by the thin-layer chromatography system. Phenylglycine was not liberated from CEX in detectable amounts either. Control solutions of thienylacetic acid and phenylglycine were not degraded by the homogenates. No acylase activity was detected by bio-autography.

When supernatants from the homogenates were examined for tryptic and chymotryptic activity, negligible amounts were detected in the stomach portions. The small intestine was divided into two equal parts in this experiment and both enzymes were detected in each part; there was seven times more trypsin and ten times more chymotrypsin in the second half of the intestine than in the first half. Caecal portions were not assayed.

![Graph](Image)

**Fig. 4.**—Inhibition of activity of β-lactamase from *Enterobacter cloacae* strain P99, and of β-lactamase activity of homogenates of rat caecum, on cephaloridine, by cephalosporins 5/1 and 291/1.

In the study of the action of proteolytic enzymes on the three cephalosporins, trypsin and chymotrypsin (pH 7.8) from pancreatic juice and pepsin (pH 3) and rennin (pH 3.7) from gastric juice were used. Papain (pH 6.2) and bromelain (pH 7.8), powerful proteolytic enzymes from plants, were also examined. No reproducible breakdown of the cephalosporins was observed in any of these tests.

**Discussion**

Most previous papers on the metabolism of cephalosporins have described in-vitro and in-vivo deacetylation by tissue esterases (Lee, Herr and Anderson, 1963; O'Callaghan and Muggleton, 1963; Okui, Hattori and Nishida, 1967). Metabolism of the phenylacetic acid 7-side chain of cephaloram to phenylaceturic acid in the gastro-intestinal tract has been reported (Culp, Marshall and McMahon, 1963), and the metabolism of cephalothin, cephaloridine, 7-phenoxyacetylicecephalosporanic acid, cephaloglycin and cephalexin has also
been studied with the aid of $^{14}$C labelling (Sullivan and McMahon, 1967; Sullivan, Billings and McMahon, 1969a and b).

The cephalosporins studied here did not have ester-linked 3-substituents and so were not susceptible to breakdown by tissue esterases. Other possible points of attack were the $\beta$-lactam ring and the acyl linkage by which the 7-substituent is attached to the nucleus. Rat intestinal homogenates were therefore assayed for $\beta$-lactamase and acylase activity.

$\beta$-Lactamase degradation involving hydrolysis of the C–N bond of the $\beta$-lactam ring could be the first step in the production of metabolites such as the thienylacetylglycine previously reported in rat urine after oral dosage with cephalothin and cefaloridine (Sullivan and McMahon). The most likely source of $\beta$-lactamase was the bacterial flora of the gastro-intestinal tract, and $\beta$-lactamase-producing bacteria were isolated from the caecal portions. The $\beta$-lactamase inhibition studies confirmed that some of the degrading activity of the caecum was due to bacterial $\beta$-lactamase. It is possible that the remaining degrading activity could be attributed to $\beta$-lactamases not inhibited by cephalosporin 5/1 or 29/1. The supernatants from the homogenates absorbed UV light and this reduced the sensitivity of the UV assay. Dilution of the supernatants reduced the high blank reading, but also reduced the amount of enzyme in a sample; pyridine liberated from the cephalexin was readily detected. The hydroxylamine assay is normally more specific for residual $\beta$-lactam ring, but incubation in intestinal homogenates gave variable results. $\beta$-Lactamase activity was not detected higher up the rat intestine, but this may have been due to lack of sensitivity of the assay rather than to absence of activity.

The lack of acylase activity in these in-vitro tests may also have been due to lack of sensitivity of the assay, although it was designed to detect $\geq$1 per cent. degradation. Any thienylacetyl or phenylglycyl groups coming off may have reacted further with components of the homogenate in a way not possible in the thienylacetic acid or phenylglycine controls.

Proteolytic enzymes found in the gastro-intestinal tract were tested for cephalosporin-degrading activity. The cephalosporin nucleus may be dissected into cystine and dehydrogenated valine, and cystine and valine can be incorporated into the nucleus during biosynthesis (Trown, Smith and Abraham, 1963). The $\text{CONH}^-$ group linking the 7-substituent to the nucleus is potentially susceptible to proteolytic attack.

The gastric juice enzymes, pepsin and rennin, have similar specificities (Dixon and Webb, 1964) and have greater activity on proteins than on peptides. They also prefer bonds involving aromatic amino acids, but a free $\alpha$-amino group (as e.g. in cephalaxin) is disadvantageous. Neither enzyme degraded the cephalosporins significantly. Similarly the pancreatic enzymes trypsin and chymotrypsin had no detectable effect. These enzymes were previously tested on cephalothin without demonstrable hydrolysis in vitro (Sullivan and McMahon). Two other proteolytic enzymes, papain and bromelain, both from plant extracts and with similar specificity, gave no reproducible degradation.

The qualitative tests for degrading activity described above showed that
cephalosporins were degraded in the caecum but not higher up the gastro-intestinal tract. Quantitative results obtained by bioassay of cephalosporins incubated with intestinal homogenates confirmed that in-vitro degradation was largely confined to the caecum. All three cephalosporins were recovered virtually intact from homogenates of the first section of small intestine. Recoveries from the rest of the intestine were lower than from the first section. The rest of the intestine contained about twice as much tissue as the shorter first section, and this may have increased breakdown or perhaps interfered with the bioassay to give apparently increased breakdown, although most of the tissue effect could be avoided by dilution of the samples. The homogenates of caecum, however, destroyed most of the CER and much of the TMC in 1 hr although the CEX was still highly resistant to breakdown.

The effect of the sex and weight (or age) of the rats on the rate of destruction of antibiotic could be studied only in the caecal homogenates because little breakdown occurred elsewhere in the gut. Increased breakdown of cephaloridine in the older rats can probably be explained by an increase in the number of bacteria of the caecum with age. The apparent sex difference is not easily explained.

The greater resistance of CEX than of CER to breakdown by homogenates of caecum emphasises the importance of the substituents on the cephalosporin nucleus. Cephalexin is more resistant to breakdown than cephaloridine when incubated for 2 hr at 37°C with several bacterial species, e.g., Enterobacter cloacae and Proteus morgani (Muggleton et al., 1968).

Extensive breakdown of the cephalosporins examined occurred only in the caecum and rectum of the rat and varied considerably with the structure of the cephalosporin. From this it is concluded that rapid degradation in the alimentary tract is not the primary reason for the poor oral absorption of cephaloridine and TMC. In-vivo experiments (O'Callaghan et al., 1970) led to similar conclusions.

**SUMMARY**

In-vitro tests with homogenates of rat intestine showed that cephaloridine, cephalexin and 7-(thienyl-2'-acetamido)-3-methylceph-3-em-4-carboxylic acid were degraded more quickly in the caecum and rectum than in other parts of the alimentary tract; even after 4 hr at 37°C at least 57 per cent. of the low dose of cephalosporin used remained intact in other portions of intestine. The extent of breakdown in the caecum varied with the structure of the cephalosporin. Cephalexin was more stable than cephaloridine to biological degradation.

Different parts of rat intestine were assayed for β-lactamase and acylase activity and the effect of proteolytic enzymes on the cephalosporins was studied. The only degrading activity found was due to β-lactamase-producing bacteria in the caecum.

We wish to thank Miss A. M. Harris who carried out the bacteriological survey of rat caeca and Mr K. V. Chanter who developed the thin-layer chromatography technique for separation of cephalosporins.
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