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The Formation of Metabolites from Cephalosporin Compounds

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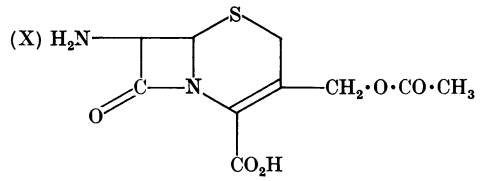
The discovery of cephalosporin and the elucidation of its structure has led to the preparation of a new series of compounds with antibacterial activity. The nucleus of the cephalosporins consists of a dihydrothiazine ring fused to a β-lactam ring (Abraham & Newton, 1961).



Cephalosporin C has an α-aminoadipoyl group in the 7-position (X) and is the only member of this series of compounds that has so far been found in natural products. The cephalosporins differ from the penicillins in that no analogues are produced when precursors such as phenylacetic acid are incorporated in the fermentation medium. The α-aminoadipoyl group can, however, be removed from cephalosporin C by hydrolysis (Loder, Newton & Abraham, 1961) or by treatment with nitrosyl chloride (Morin, Jackson, Flynn & Roeske, 1962), and the 7-amino group can then be substituted if desired. The first cephalosporin analogue to be prepared in this way was 7-phenylacetamidocephalosporanic acid (sodium salt) (called 'phenylacetyl cephalosporin').

In laboratory studies, which are not reported in detail below, we have shown that phenylacetyl cephalosporin has an antibacterial spectrum similar to that of benzylpenicillin, with the added advantages of a greatly increased stability to acid and to penicillinase. Against experimental *Staphylococcus aureus* infections in mice, phenylacetyl cephalosporin gave irregular results in which the protection obtained was not parallel with the dose given. Although it has a low toxicity for mice, some animals receiving low doses were protected, whereas some having larger doses died.

To explain these irregular results, in which no clear-cut end points were obtained, a variable metabolic decomposition of the substance to a compound with decreased antibacterial activity was postulated, and the work reported below describes the investigation of the fate of phenylacetyl cephalosporin and other cephalosporins in the mammalian body.

EXPERIMENTAL

Chromatography and bio-autographs. A suitable solution in 30 μl. quantities was applied to Whatman no. 1 papers, buffered with 0·05M-sodium phosphate at pH 6·0. Undiluted serum and urine samples were applied to the paper in the same volume but, where it was expected that the antibiotic concentration would be low, several (up to five) applications were made on the same spot, the paper being dried in a stream of warm air between applications. The solvents used were: (*a*) butan-1-ol–ethanol–water (4:1:5, by vol.); (*b*) propan-1-ol–water (7:3, v/v). The chromatograms (descending) were allowed to run for 18 hr. After drying, the papers were placed on nutrient–agar plates seeded, for phenylacetyl cephalosporin, with 1% of a 24 hr. broth culture, diluted 1:100, of *S. aureus* (NCTC 7447). For cephalosporin C or *N*-dinitrophenyl cephalosporin C, 1% of a 1:500 dilution of a spore suspension of *Bacillus subtilis* (ATCC 6633) was used. The papers were removed after 15 min., and the plates were incubated at

Table 1. *Comparative R_F values of some cephalosporin compounds and their metabolites*

Compound	Solvent system	R_F value
Cephalosporin C	Propan-1-ol–water (7:3, v/v)	0·20
Cephalosporin C metabolite	Propan-1-ol–water (7:3, v/v)	0·12
Cephalosporin C lactone	Propan-1-ol–water (7:3, v/v)	0·30
Phenylacetyl cephalosporin	Butan-1-ol–ethanol–water (4:1:5, by vol.)	0·67
Phenylacetyl cephalosporin metabolite	Butan-1-ol–ethanol–water (4:1:5, by vol.)	0·52
Phenylacetyl cephalosporin lactone	Butan-1-ol–ethanol–water (4:1:5, by vol.)	0·83
N-Dinitrophenyl cephalosporin C	Butan-1-ol–ethanol–water (4:1:5, by vol.)	0·30
N-Dinitrophenyl cephalosporin C metabolite	Butan-1-ol–ethanol–water (4:1:5, by vol.)	0·18
N-Dinitrophenyl cephalosporin C lactone	Butan-1-ol–ethanol–water (4:1:5, by vol.)	0·63

37° for 18 hr., when the presence of the antibiotics was indicated by clear zones of inhibition.

Bioassay. Phenylacetyl cephalosporin was determined by cup–plate assay with *S. aureus* in tryptose–agar, seeded with 1% of a 1:100 dilution of a 24 hr. broth culture. Cephalosporin C and *N*-dinitrophenyl cephalosporin C were determined by cup–plate assay with *B. subtilis*. When serum samples were assayed, the standards were prepared in horse serum.

Tissue homogenates. Tissue was taken from freshly killed animals and stored at −20° until required, when 5 g. was homogenized in 50 ml. of phosphate buffer, pH 7 (mixture of sodium and potassium salts), for 30 sec. in a Waring Blendor. Equal volumes of homogenate and antibiotic solution in phosphate buffer were mixed, the concentration of antibiotic being so chosen that a dilution of at least 1:10 in phosphate buffer was required for assay. Control tests with diluted (1:10) tissue homogenate showed that this procedure diluted out any tissue components that might interfere with the assay, and also almost completely stopped any further reaction between the tissue and the antibiotics.

Isolation of the metabolites from liver homogenates. A solution (5 mg./ml.) of phenylacetyl cephalosporin was prepared in a homogenate of 5 g. of rabbit liver in 95 ml. of 0·9% sodium chloride. After incubation at 37° for 4 hr., the 'metabolite' was isolated as follows: 2 vol. of acetone was added, and the precipitate of liver centrifuged off and discarded. The acetone was removed from the supernatant solution under vacuum and the remaining aqueous solution was Seitz-filtered to remove any remaining particles. The pH was adjusted to 3·3–3·5 and extracted five times with equal volumes of ethyl acetate. The pooled ethyl acetate extract was then extracted three times with one-fifteenth volumes of sodium hydrogen carbonate solution maintained at pH 7·0. The pooled aqueous extract was treated with I.R.C. 50 ion-exchange resin (H^+ form) to remove excess of bicarbonate at pH 6–6·5. The solution was freeze-dried to give a solid that was found later to contain approx. 70% of the pure metabolite. The solid (1700 mg.) was extracted with methanol (70 ml.) and the insoluble material removed by centrifuging. The methanol was then slowly removed under vacuum until the metabolite started to crystallize out. The crystals were collected after they had been allowed to form completely in the refrigerator (4°) overnight.

RESULTS AND DISCUSSION

Decomposition in vivo. Samples of serum and urine (collected over a period of 6 hr.) from mice that had received subcutaneous doses of 25 mg. of phenylacetyl cephalosporin/kg. were examined for microbiological activity. The antibiotic was shown to be present in both serum and urine, but the recovery of activity from the urine was much lower than expected (about 20%). Further, chromatography followed by bio-autography revealed the presence of another microbiologically active substance both in serum and urine, although the material given had been pure and homogeneous.

Cephalosporin C and its *N*-dinitrophenyl derivative were then injected subcutaneously into mice; each compound gave rise in the urine to another microbiologically active substance besides the compound administered. With cephalosporin C the second compound was only present in small quantities. The new material always had an R_F value less than that of the parent compound (Table 1), and the product from *N*-dinitrophenyl cephalosporin C retained the strong yellow colour characteristic of 2,4-dinitrophenol.

Decomposition in vitro. In an attempt to determine the site of production of these microbiologically active metabolites, homogenates of mouse liver, kidney, lung, skeletal muscle, spleen and brain were incubated at 37° with the cephalosporins. Chromatography and bio-autographs of samples taken up to 4 hr. showed that the metabolites were rapidly produced by liver, kidney and spleen. Lung and skeletal muscle only produced the metabolites slowly, and brain gave little.

Studies were extended to other mammalian species, and homogenates of kidney and liver from mouse, rat, guinea pig, rabbit, monkey and man were incubated with the three cephalosporins separately. Liver and kidney from all species produced the metabolites from phenylacetyl cephalosporin and *N*-dinitrophenyl cephalosporin C, as shown both by decrease in microbiological activity in the homogenate–antibiotic mixtures and by the production of another microbiologically active compound seen in the bio-autographs. With cephalosporin C, however, the rate at which the metabolite was produced varied from species to species. Mouse, rat, guinea-pig and rabbit tissues rapidly produced the metabolite, whereas tissue from monkey and man decreased the activity of

the preparation much more slowly, and chromatography showed that no metabolite at all was produced (Table 2).

Microbiological activity of the metabolites compared with that of the parent compounds. Comparison of microbiological activity by tube-dilution tests showed that in general the metabolites, isolated from incubated antibiotic–liver homogenate mixtures, have about one-fifth of the activity of the parent compounds (Table 3). This would explain the diminished microbiological activity in the tissue homogenates and also largely account for the poor recovery (about 20 %) from the urine of the experimental animals. Cephalosporin C is decomposed only slightly *in vivo* in the mouse and not at all in man, and is recovered to a much greater extent from these species than is phenylacetyl cephalosporin.

Chromatograms of urine, collected over a period of 24 hr., from male adult human volunteers injected intramuscularly with 1 g. of cephalosporin C did not show the presence of any metabolite. When a similar dose of phenylacetyl cephalosporin was given to volunteers, large quantities of metabolite were found, however, and the total recovery (over 24 hr.) was only about 20 % in terms of the activity of the phenylacetyl cephalosporin injected.

Identity of the metabolites. The R_F values of the metabolites were always lower than those of the parent compounds and were also different from

Table 2. *Progressive loss of microbiological activity when cephalosporins are incubated with liver homogenates from mouse and man*

	Microbiological activity (minimum inhibitory concn. in μg./ml.)					
	With mouse liver		With monkey liver		With human liver	
Time (hr.)	Cephalosporin C	Phenylacetyl cephalosporin	Cephalosporin C	Phenylacetyl cephalosporin	Cephalosporin C	Phenylacetyl cephalosporin
0 (starting activity)	1000	250	1000	400	1000	500
½	567	80	590	278	615	440
1	453	38	580	136	680	335
2	418	47	580	100	688	245
4	373	40	540	69	600	150

Table 3. *Comparative microbiological activities of cephalosporin C and phenylacetyl cephalosporin with their metabolites*

Tube-dilution tests, in nutrient broth at pH 7·4, and incubated for 24 hr. at 37°, were used.

		Microbiological activity (minimum inhibitory concn. in μg./ml.)			
Organism	Strain	Cephalosporin C	Cephalosporin C metabolite	Phenylacetyl cephalosporin	Phenylacetyl cephalosporin metabolite
Staphylococcus aureus	NCTC 7447	125	500	0·2	0·8
	11123	62	500	0·2	0·8
	11174	31	250	0·4	0·8
	11152	125	> 500	0·4	1·6
	11146	31	500	0·4	1·6
	16601	31	500	0·2	0·8
	10991	62	250	0·4	1·6
	10996	62	500	0·4	3·2
	11071	62	500	0·4	1·6
	3664	8	> 500	1·6	8
	RS2	31	> 500	25	62
	RS6	62	500	0·8	2
Escherichia coli	1	125	> 500	31	250
	4	500	> 500	16	500
	6	125	> 500	31	500
Aerobacter aerogenes	10	31	> 500	31	250
Proteus sp.	43	125	> 500	31	62
	2	32	> 500	16	62
	14	16	> 500	16	125
	42	16	> 500	16	125
	50	16	> 500	31	250
	51	16	> 500	31	125

Table 4. *Effect of mouse-liver homogenate on the microbiological activities of phenylacetyl cephalosporin and cephalosporin C and their pyridine and thiourea derivatives*

	Microbiological activity (minimum inhibitory concn. in μg./ml.)					
Time (hr.)	Cephalosporin C	Cephalosporin C pyridine derivative	Cephalosporin C thiourea derivative	Phenylacetyl cephalosporin	Phenylacetyl cephalosporin pyridine derivative	Phenylacetyl cephalosporin thiourea derivative
0 (starting activity)	1000	50	500	250	175	25
½	567	52	490	80	186	27
1	453	50	480	38	205	26
4	373	40	340	40	185	23

each other (Table 1), ruling out the possibility that the cephalosporins gave a common metabolite. By analogy with the penicillins, the retention of microbiological activity suggested that the β-lactam ring remained intact. The retention of the characteristic ultraviolet absorption in the region of 260 mμ indicated that the double bond remained and that the dihydrothiazine ring was not changed. As the *N*-dinitrophenyl cephalosporin C metabolite retained its strong yellow colour, the 7-acyl substituent was probably unaffected.

Another possibility was oxidation of the sulphur atom to sulphoxide or sulphone. Hydrogen peroxide oxidation of phenylacetyl cephalosporin, cephalosporin C and *N*-dinitrophenyl cephalosporin C gave compounds of decreased microbiological activity. The R_F values of these compounds were less than those of the parent compounds, but were different from those of the liver metabolites, and the possibility that the metabolites might be the sulphoxides or sulphones of the parent compounds can therefore be discounted.

The remaining possibility was that the metabolites were the hydroxy acids of the parent compounds. Such hydroxy acids would be expected to form the corresponding lactones in acid solution; when the metabolites were incubated at pH 3·5 they gave microbiologically active materials with R_F values greater than those of the parent compound and identical with those of authentic specimens of the corresponding lactones. The properties of cephalosporin C hydroxy acid prepared by citrus-peel enzymes (Jeffery, Abraham & Newton, 1961) corresponded closely to those of the cephalosporin metabolite, whether it was obtained from urine or produced *in vitro* in tissue homogenates. It is therefore concluded that the metabolites of phenylacetyl cephalosporin, cephalosporin C and the *N*-dinitrophenyl cephalosporin C found in the experimental animals are the corresponding hydroxy acids.

More cephalosporin analogues have now been made and, when injected into mice, all the acetoxy compounds tested have given rise to other microbiologically active substances. Thus metabolites were found in the urine after the injection of cephalosporins in which the α-aminoadipic acid side chain in the 7-position was replaced by phenylmercaptoacetic acid, dichloroacetic acid, pentanoic acid, vinylacetic acid, β-aminopropionic acid, glutamic acid, propylmercaptoacetic acid or 2-thienylacetic acid. The metabolites had smaller R_F values than those of the materials injected and seem likely to have been the corresponding hydroxy acids. For the 7-(2-thienylacetamido)cephalosporanic acid, the results are in agreement with those of Lee, Herr & Anderson (1962), who found that the corresponding hydroxy acid was formed when this substance was injected.

The identification of the metabolites as hydroxy acids is further confirmed by the failure of either live animals or tissue homogenates to produce a corresponding substance when the acetoxy group is replaced by a nucleophilic substituent, such as thiourea or pyridine. The preparation of such a pyridine compound has been described by Hale, Newton & Abraham (1961). The bonding in such compounds is no longer that of an ester, and it would therefore be expected that these substances would not be attacked by an esterase, either *in vitro* or *in vivo*. In fact, little if any decomposition of the pyridine or thiourea derivatives of phenylacetyl cephalosporin or cephalosporin C was found (Table 4), and chromatography showed that no metabolite was formed.

We must therefore conclude that the activity of a cephalosporin analogue in the body is at least in part dependent on the decomposition it may undergo. Thus a substance such as phenylacetyl cephalosporin may have high activity *in vitro* against a range of pathogenic organisms, but its effectiveness *in vivo* will depend both on the extent to which it is deacetylated and on the antibacterial activity of the deacetyl compound formed. Further, deacetylation of cephalosporins proceeds at different rates in different animals; thus both man

and mouse can deacetylate phenylacetyl cephalosporin, whereas man is unable to deacetylate cephalosporin C.

SUMMARY

1. Phenylacetyl cephalosporin was observed to give rise to a further microbiologically active material in the urine of mice receiving the compound subcutaneously. This also occurred with cephalosporin C and its *N*-dinitrophenyl derivative.

2. Many tissues, notably liver and kidney, are able to produce these compounds *in vitro*.

3. The metabolites have less microbiological activity than their corresponding parent compounds.

4. The metabolites are produced by deacetylation of the parent cephalosporins; different animal species have the ability to deacetylate different cephalosporins to different extents, e.g. man and monkey can deacetylate phenylacetyl cephalosporin but not cephalosporin C.

5. It is deduced that the clinical effectiveness of any cephalosporin will depend, not only on its microbiological activity *in vitro*, but also on the rate at which it is deacetylated in the body and the activity of the resulting deacetyl compound.

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The Fermentation of Glucose by *Chlorella vulgaris*

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The anaerobic metabolism of algae has received little attention. Genevois (1927, 1929) showed that several different species produce acid but little carbon dioxide anaerobically. Gaffron (1939) and Michels (1940) showed that *Chlorella pyrenoidosa* and *Scenedesmus obliquus* form lactic acid from glucose anaerobically. Gaffron & Rubin (1943) found that strains of *Scenedesmus*, but not of *Chlorella*, produce hydrogen after a period of anaerobiosis. Barker (1935) studied, rather thoroughly, the anaerobic metabolism of the colourless alga, *Prototheca zopfii*, and found that lactic acid was formed from glucose anaerobically. He claimed that the conversion was quantitative, i.e. 1 mole of glucose gave 2 moles of lactic acid as in homolactic fermenting bacteria. Damasche (1957) has made a similar claim for *C. pyrenoidosa*, but neither Barker nor Damasche supplies experimental values that support this conclusion.

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Damasche also showed that the acid fermentation of glucose by *C. pyrenoidosa* is replaced, after some days of anaerobosis, by a fermentation in which hydrogen is produced.

On the other hand, there are algae with a typical yeast type of alcoholic fermentation, e.g. *Ochromonas malhamensis* (Reazin, 1956). Weis & Mukerjee (1958) cite no quantitative results but recognize three types of algal fermentation. In the first, typified by *C. pyrenoidosa*, only lactic acid is produced from glucose; in the second, e.g. *C. ellipsoidea*, no acid is produced; and in the third type, represented by *C. vulgaris*, the products include both lactic acid and acetic acid.

We have worked only with *C. vulgaris* and have attempted to account quantitatively for the products of glucose fermentation. We find, in agreement with Weis & Mukerjee (1958), that both acetic acid and lactic acid are important products, but a considerable quantity of formic acid is also produced as well as small quantities of carbon