Behaviour of some Derivatives of 7-Aminocephalosporanic Acid and 6-Aminopenicillanic Acid as Substrates, Inhibitors and Inducers of Penicillinases

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(Received 9 August 1961)

Abraham & Newton (1956) showed that cephalosporin C was highly resistant to hydrolysis by purified penicillinase from *Bacillus cereus* (strain NRRL 569; see Pollock, 1959, 1960) but was a competitive inhibitor of the action of this penicillinase on benzylpenicillin. More recently, however, cephalosporin C was reported to have no significant inhibitory action on the hydrolysis of benzylpenicillin by penicillinase from a strain of *Staphylococcus aureus* (Abraham & Newton, 1961*a*). Rolinson, Stevens, Batchelor, Wood & Chain (1960) reported that 2,6-dimethoxyphenylpenicillin was a competitive inhibitor of penicillinase from *B. cereus*, but not of penicillinase from *Staph. aureus*.

These findings suggested that penicillinase from *B. cereus* and penicillinase from *Staph. aureus* differed with respect to structural features which influenced the combination of enzyme and substrate. They also drew attention to the fact that the affinity for penicillinase of a member of the cephalosporin C or penicillin family might partly determine the rate of enzymic hydrolysis of the substance in the concentration at which it was used as an antibacterial agent.

The results of further experiments in this field are given here. The substances studied were *N*-acyl derivatives of 7-aminocephalosporanic acid which has structure (I; R = H, R' = CH₃·CO·O) (Abraham & Newton, 1961*b*; Loder, Newton & Abraham, 1961), or of 6-aminopenicillanic acid which has structure (II; R = H) (Sheehan, Henery-Logan & Johnson, 1953; Batchelor, Doyle, Nayler & Rolinson, 1959). The derivatives of 7-aminocephalosporanic acid include cephalosporin C, deacetylcephalosporin C (Jeffery, Abraham &

Newton, 1961) and cephalosporin C₄ (pyridine) (Hale, Newton & Abraham, 1961) in which

and R' = CH₃·CO·O, HO, and C₆H₅N⁺ respectively. Cephalosporin C₆ is the lactone of deacetylcephalosporin C. The derivatives of 6-aminopenicillanic acid are penicillins, R being C₆H₅·CH₂·CO in benzylpenicillin, C₆H₅·O·CH₂·CO in phenoxy-methylpenicillin and

in cephalosporin N. The enzymes used were crude or highly purified extracellular penicillinase from several strains of *B. cereus* and crude extracellular penicillinase from a strain of *Staph. aureus*.

Estimates have been made of: (1) the relative rates of hydrolysis of different substrates, where possible in concentrations sufficient to saturate the enzyme (to give *V*_{max}); (2) the relative affinities for the enzymes of different competitive inhibitors and the relative Michaelis constants of different substrates, as revealed by values for *K*_i/*K*_m, where *K*_i is the dissociation constant of the enzyme-inhibitor complex and *K*_m is the Michaelis constant; (3) the relative activities of different substances as enzyme inducers.

METHODS

Abbreviations. The following abbreviations are used: 7-ACA for 7-aminocephalosporanic acid; 6-APA for 6-aminopenicillanic acid.

Penicillinase preparations. These were: (1) a highly purified preparation of the enzyme from *B. cereus* 569/H [preparation (1) of Kogut, Pollock & Tridgell, 1956], a preparation from *B. cereus* 569, believed to be almost pure, and a crystalline preparation from *B. cereus* 5/B, all kindly provided by Dr M. R. Pollock; (2) crude induced enzyme in culture filtrates of *B. cereus* 569 and crude constitutive enzyme in culture filtrates of *B. cereus* 569/H (Pollock, 1959); (3) crude induced enzyme in culture filtrates of

Staph. aureus R1 (a strain isolated from a patient in the Radcliffe Infirmary, Oxford). Stock solutions of the purified enzymes (200 $\mu\text{g./ml.}$) were made up in 0.5% gelatin. Further dilutions of these solutions and dilutions of the crude enzymes were made in 0.2% gelatin.

Substrates and inhibitors. Crystalline benzylpenicillin (sodium salt) (Boots Pure Drug Co. Ltd.) and 2,6-dimethoxyphenylpenicillin (sodium salt) (Beecham Research Laboratories Ltd.) were used. Phenoxymethylpenicillin [The Distillers Co. (Biochemicals) Ltd.] was converted into the potassium salt and the latter crystallized from aqueous acetone. α -Phenoxyethylpenicillin (potassium salt) [The Distillers Co. (Biochemicals) Ltd.] was a mixture of two diastereoisomers differing in configuration about the asymmetric centre of the side chain. The potassium salt of α -phenoxypropylpenicillin was provided by Pfizer Ltd. in three forms: (a) a mixture of two diastereoisomers (with D- and L-side chains respectively) in approximately equal proportions; (b) a mixture containing mainly the D-isomer (about 85% pure); (c) a mixture containing mainly the L-isomer (about 85% pure). Crystalline cephalosporin C (sodium salt) and a preparation of the sodium salt of cephalosporin N (about 25% pure) were provided by the Medical Research Council's former Antibiotics Research Station, Clevedon, Somerset. The concentrations of cephalosporin N quoted refer to the estimated concentrations of pure sodium salt. Cephalosporin C_A (pyridine) (Hale *et al.* 1961), deacetylcephalosporin C (Jeffery *et al.* 1961) and cephalosporin C₆ (Abraham & Newton, 1961*b*) were prepared from cephalosporin C. The N-phenylacetyl and N- α -phenoxypropionyl derivatives of 7-ACA were prepared by acylation of 7-ACA (Loder *et al.* 1961).

Enzyme induction. This was studied by a procedure similar to that used by Pollock (1957). A culture of *B. cereus* 569 or *Staph. aureus* R1 was grown overnight at 37° in stationary Difco heart-infusion broth (pH 7.4). This culture was used to provide an inoculum (10%, v/v) for a larger quantity of the same medium in a flask which was rocked at 35° in the case of *B. cereus* and at 37° in the case of *Staph. aureus*. After about 2 hr., when the extinction of the larger culture had reached 0.6 [as measured with a Spekker absorptiometer (Adam Hilger Ltd.) with a neutral-grey filter (H 508) in a tube of 2 cm. diameter], gelatin was added [2 ml. of a 10% (w/v) solution/100 ml.] and samples of the culture (10 ml.) were distributed in inverted T-tubes of the type devised by Dr J. Monod (see Kay & Fildes, 1950). Varying amounts of inducer (in 0.1 ml. of water) were added to some of the different samples and 0.1 ml. of water to control samples. The tubes were then rocked, normally for 1 hr., at 35° (for *B. cereus*) or at 37° (for *Staph. aureus*). 8-Hydroxyquinoline (1 ml. of 0.01M solution) was then added to each tube to stop enzyme induction and the organisms were removed by centrifuging. Enzyme present in samples (2 ml.) of the suitably diluted supernatant was estimated manometrically. For some experiments the solution of the extracellular staphylococcal enzyme (pH adjusted to 7.0) was concentrated 20-fold by freeze-drying and dissolving the residue in a volume of 0.5% gelatin equal to 5% of the original volume of culture supernatant.

In each experiment with *B. cereus* 569 the amounts of extracellular enzyme formed in the presence of different concentrations of a particular inducer were compared with that formed in the presence of an optimum concentration

(1.0 $\mu\text{g./ml.}$) of benzylpenicillin. The amount formed in the presence of benzylpenicillin was about 180 units/ml.; the amount formed in the absence of inducer was about 4 units/ml., the unit being the quantity hydrolysing 1 μ -mole of benzylpenicillin in 1 hr. at pH 7.0 and at 30° (Pollock & Torriani, 1953). In experiments with *Staph. aureus* R1 the amounts of extracellular enzyme were compared with that formed in the presence of an optimum concentration (1.0 $\mu\text{g./ml.}$) of 2,6-dimethoxyphenylpenicillin. The amount formed in the presence of 2,6-dimethoxyphenylpenicillin was about 14 units/ml., whereas the amount formed in the absence of inducer was too small to measure by the manometric method used.

In the absence of inducer the extinction of the cultures of both *B. cereus* and *Staph. aureus* approximately doubled during 1 hr. With *B. cereus* the increase in extinction during this period was not significantly affected by any of the inducers in the highest concentration in which they were used. With *Staph. aureus* the increase was not reduced by more than 50% when the inducers were used in their growth-inhibitory concentration as determined by a tube-dilution method.

With *B. cereus* the lag period preceding the rapid formation of enzyme (cf. Pollock, 1952) was 15–20 min. when benzylpenicillin, cephalosporin C or cephalosporin C_A (pyridine) was used as inducer. With *Staph. aureus* there was a similar lag period when benzylpenicillin, 2,6-dimethoxyphenylpenicillin, cephalosporin C or N-phenylacetyl-7-ACA was the inducer.

In some induction experiments with *B. cereus* the induction period was extended to 2 or 7.5 hr. After 2 hr. the extinction was about four times and after 7.5 hr. (when growth was no longer logarithmic) about five times the original value.

Crude enzymes from *B. cereus* 569/H and 5/B. These organisms were grown in the manner described for *B. cereus* 569 except that no inducer was used. The organisms were removed by centrifuging and the resulting supernatant was used as a source of the enzyme. With each organism the activity of the supernatant was about 100 units/ml.

Manometric determinations of enzyme activity. These were carried out at 30° and at pH 7.0 in a Warburg apparatus by the method used for penicillinase by Pollock (1952). In experiments with members of the penicillin or cephalosporin C families the main compartment of each vessel contained 0.5 ml. of 0.043M-NaHCO₃ and 2.0 ml. of enzyme solution. The side bulb contained 0.1 ml. of 0.043M-NaHCO₃ and 0.4 ml. of a solution of substrate (1.5–12 mg.) or of substrate and inhibitor. In experiments with triacetin as substrate the latter [2 ml. of a 7% (w/v) solution] was placed in the main compartment of the vessel and the enzyme in the side-bulb. The vessels were gassed with N₂+CO₂ (95:5, v/v). Measurements were made during periods up to 30 min. Where possible, the concentration of the enzyme solution was such that the initial rate of evolution of CO₂ was 10²–10³ $\mu\text{l./hr.}$

In each series of experiments with a given enzyme preparation the rates of hydrolysis of various substrates were compared with that of 2.8 mM-benzylpenicillin (3 mg. of the sodium salt/Warburg vessel). In most cases derivatives of 7-ACA were tested as substrates at concentrations varying from about 1.6 to 12.8 mM. With the staphylococcal enzyme, however, cephalosporin C and 2,6-dimeth-

oxyphenylpenicillin were tested at concentrations as high as 100 mM.

When the initial velocity, v , increased significantly with increase in substrate concentration, s , over the range of concentrations used, an attempt was made to estimate K_m and V_{max} by plotting v against v/s as described by Eadie (1942) and Hofstee (1959). For example, with cephalosporin C₀ and purified penicillinases from *B. cereus*, data were plotted corresponding to substrate concentrations of 1.6, 3.2, 6.4 and 12.8 mM.

In studies of competitive inhibition initial velocities were measured in most cases with three mixtures which contained a single appropriate concentration of inhibitor but different concentrations of substrate. The substrate concentrations were usually about 2.8, 5.6 and 11.2 mM. The results were plotted in the manner of Hunter & Downs (1945).

Alkaline hydrolysis. The rates of alkaline hydrolysis of benzylpenicillin, 2,6-dimethoxyphenylpenicillin and deacetylcephalosporin C were compared at 31° by use of a pH Stat (type SBR 2/SBU 1 Titirgraph in conjunction with type TTT1 Automatic Titrator made by Radiometer, Copenhagen, Denmark) in the manner described by Jeffery *et al.* (1961). The pH of the solution (4 ml., 7 mM) was maintained at 11.1 (glass electrode) by automatic addition of 0.1N-NaOH.

Antibacterial activity. Minimum concentrations of various substances required to inhibit the growth of *Staph. aureus* R1 were determined by the tube-dilution method with a large inoculum. Each tube contained heart-infusion

broth (0.5 ml.) inoculated with 0.02 ml. of a 24 hr. broth culture of the organism diluted 1:40. Readings were taken after 24 hr. and on some occasions also after 6 days.

RESULTS

Maximum rates of enzymic hydrolysis

According to the simple Michaelis-Menten equations

and

$$V_{max.} = k_3[E],$$

where E is the enzyme, S the substrate, P the product of the reaction and $V_{max.}$ the reaction velocity in the presence of sufficient substrate to saturate the enzyme.

With the penicillinase-sensitive penicillins as substrates the reactions were of zero order over most of their course, as judged by the rate of liberation of CO₂ from sodium hydrogen carbonate, with all the enzyme preparations tested. But with some of the substrates that were relatively resistant to penicillinase, an initial period of 5–10 min. in which the rate of reaction appeared to be constant was followed by a period in which the rate declined, sometimes almost to zero, despite

Table 1. *Behaviour of derivatives of 6-aminopenicillanic acid and 7-aminocephalosporanic acid as substrates for purified penicillinase from B. cereus 569, 569/H and 5/B*

k_3 (relative) is defined as $100 \times k_3$ for a given substrate/ k_3 for benzylpenicillin with a given enzyme solution. The enzymes from strains 569 and 569/H are believed to be identical (Pollock, 1960).

Substrate	Purified enzyme from <i>B. cereus</i>	k_3 (relative)	K_m (mM)
Benzylpenicillin	569/H	100*	—
	5/B	100*	0.043†
α-Phenoxypropylpenicillin (D-)	5/B	41	—
(L-)	5/B	67	—
(DL-)	5/B	50	—
2,6-Dimethoxyphenylpenicillin	5/B	1.8	—
Cephalosporin N	569	54*‡	0.049†‡
	5/B	50	—
Cephalosporin C	569/H	0.01	—
	5/B	0.02	—
Deacetylcephalosporin C	569/H	<0.01§	—
	5/B	<0.01§	—
Cephalosporin C _A (pyridine)	569/H	0.4	—
	5/B	1.0	—
Cephalosporin C ₀	569/H	25 ± 5	1.2 ± 0.3
	5/B	90 ± 10	1.5 ± 0.3
N-Phenylacetyl-7-ACA	5/B	0.06	—
Triacetin	569/H	<0.01	—

* $V_{max.}$ values for benzylpenicillin (moles of substrate hydrolysed/mole of enzyme/min.) with the enzymes from strains 569, 569/H and 5B have been reported to be 1.60×10^5 , 1.62×10^5 and 1.48×10^5 respectively (Kogut *et al.* 1956).

† Value reported by Pollock (1960).

§ Provided that $K_m < 2$ mM.

‡ Abraham & Newton (1956).

|| Provided that $K_m < 0.1$ mM.

the fact that the reaction had not approached completion and that the enzyme was not inactivated in the absence of substrate under the conditions used. This phenomenon was most marked with cephalosporin C and the crude preparation from *B. cereus*, but it was also observed with 2,6-dimethoxyphenylpenicillin and this preparation, with *N*-phenylacetyl-7-ACA and highly purified penicillinase from *B. cereus* 5/B, and with 2,6-dimethoxyphenylpenicillin and crude penicillinase from *Staph. aureus* R1. In such cases the initial rate has been described as $V_{\max}$, provided that this rate did not increase with an increase in the substrate concentration.

Purified penicillinases from B. cereus. Table 1 shows the relative values of k_3 for different substrates with highly purified penicillinases from *B. cereus* 569, 569/H and 5/B. In most cases the concentration of enzyme, $[E]$, used was such that there was an easily measurable reaction velocity and the concentration of substrate was such that the initial velocity corresponded to $V_{\max}$. The relative values of k_3 were then calculated on the assumption that $k_3 = V_{\max}/[E]$. With deacetylcephalosporin C, however, the velocities were too low to measure even with the highest concentrations of enzyme and substrate that it was practicable to use. In these cases an approximate upper limit could be set to the relative value of k_3 on the assumption that the concentration in which the substrate had been tested exceeded the value for the K_m of the substrate-enzyme system concerned.

For a given substrate the relative values of k_3 with the two purified enzymes were of the same order, although in the case of a number of derivatives of 7-ACA the values were significantly higher with the penicillinase from strain 5/B than with that from strain 569/H.

The values of k_3 varied considerably for the different derivatives of 7-ACA that were tested, the value for cephalosporin C_A (pyridine) being significantly higher than that for cephalosporin C. But with one exception the values appeared to be very small relative to that for benzylpenicillin. The exception was the value for cephalosporin C_c (deacetylcephalosporin C lactone). With this compound k_3 was of the same order as k_3 with benzylpenicillin.

Two of the derivatives of 6-APA (α -phenoxypropylpenicillin and cephalosporin N) showed values of k_3 which were about half that for benzylpenicillin, and for α -phenoxypropylpenicillin k_3 was affected significantly by a change in the configuration about the asymmetric carbon atom of the side chain. The value of k_3 for 2,6-dimethoxyphenylpenicillin was lower than that for the other derivatives of 6-APA which were tested, but higher than that for cephalosporin C.

With most of the compounds tested K_m was too small to be determined by the manometric method. But K_m for cephalosporin C_c was of a higher order than that given by Pollock (1960) for benzylpenicillin and was estimated approximately, together with $V_{\max}$, from a plot of v against v/s (Eadie, 1942; Hofstee, 1959).

Crude penicillinases from B. cereus. Table 2 shows the relative values of k_3 with culture supernatants of *B. cereus* 569 (induced), 569/H and 5/B as sources of penicillinase. The values for cephalosporin C and deacetylcephalosporin C, relative to those for benzylpenicillin, are much higher with the culture supernatants than with the purified enzymes, whereas the values for cephalosporin C_A (pyridine) and cephalosporin C_c are not greatly changed. However, the value of k_3 for cephalosporin C, relative to that for benzylpenicillin, decreased with the age of the culture. This appeared to be due to the continued rapid formation of enzyme active against benzylpenicillin after the rate of formation of enzyme active against cephalosporin C had declined to a relatively low value.

Table 2. *Behaviour of derivatives of 6-aminopenicillanic acid and 7-aminocephalosporanic acid as substrates for crude extracellular penicillinase from B. cereus 569, 569/H and 5/B*

The crude enzyme from *B. cereus* 569 was induced (for details see text). The induction period, and the corresponding period of growth for *B. cereus* 569/H and 5/B, was 1 hr. unless otherwise stated. k_3 (relative) is defined as in Table 1. For the absolute activities of the crude enzyme solutions used see text.

Substrate	Crude enzyme from <i>B. cereus</i>	k_3 (relative)
Benzylpenicillin	569	100
	569/H	100
	5/B	100
2,6-Dimethoxyphenylpenicillin	569	5.0
	569/H	8.0
Cephalosporin N	569	41
Cephalosporin C	569	5.0
	569*	2.7
	569†	0.1
	569/H	2.5
	569/H‡	0.3
	5/B	4.0
Deacetylcephalosporin C	569	1.0
Cephalosporin C_A (pyridine)	569	0.6
	569/H	1.1
Cephalosporin C_c	569	25
	569/H	29
<i>N</i> -Phenylacetyl-7-ACA	569	5.0
Triacetin	569	0.25

* Induction period of 2 hr.

† Induction period of 7.5 hr.

‡ Growth period of 18 hr.

Penicillinase from Staph. aureus. Table 3 shows relative values of k_3 with crude penicillinase from *Staph. aureus* R1. Unlike the purified enzymes from *B. cereus*, this penicillinase was not available in a sufficiently concentrated form to allow the measurement of rates of hydrolysis which were much less than 0.2% of the rate observed with benzylpenicillin. However, it appears that the

relative values of k_3 for cephalosporin C, deacetylcephalosporin C, cephalosporin C_A (pyridine) and *N*-phenylacetyl-7-ACA are low, whereas k_3 for cephalosporin C₆ is of the same order as that for benzylpenicillin. In general, the variations in k_3 among the different derivatives of 7-ACA and 6-APA were qualitatively similar to those observed with the purified enzyme from *B. cereus* (Table 1).

The K_m for cephalosporin C₆ appeared to be larger, and K_m for 2,6-dimethoxyphenylpenicillin much larger, than that for the other compounds tested (Table 3).

Table 3. *Behaviour of derivatives of 6-aminopenicillanic acid and 7-aminocephalosporanic acid as substrates for penicillinase from Staph. aureus* R1

k_3 (relative) is defined as in Table 1.

Substrate	k_3 (relative)	K_m (mM)
Benzylpenicillin	100	0.0025*
Phenoxymethylpenicillin	100	—
α -Phenoxyethylpenicillin (DL-)	90	—
α -Phenoxypropylpenicillin (D-)	60	—
(L-)	140	—
(DL-)	66–74†	—
Dimethoxyphenylpenicillin	1.6 ± 0.4	100 ± 20
Cephalosporin N	54	—
Cephalosporin C	0.09	—
Deacetylcephalosporin C	0.1	—
Cephalosporin C _A (pyridine)	1.2	—
Cephalosporin C ₆	15 ± 5	6 ± 2
<i>N</i> -Phenylacetyl-7-ACA	<0.1 ‡	—

* Value reported by Novick (1962) for benzylpenicillin with penicillinase from *Staph. aureus* 524 SC.

† With this substance (DL-) the rate of reaction increased during the course of hydrolysis. This may be due to the fact that the D-isomer shows both a lower value of k_3 and a lower value of K_m than the L-isomer.

‡ Provided that $K_m < 2$ mM.

Alkaline hydrolysis

In contrast with the enzyme-catalysed reactions, the rates of hydrolysis of benzylpenicillin, 2,6-dimethoxyphenylpenicillin and deacetylcephalosporin C in alkaline solution were of the same order. Thus the half-lives of these three compounds at pH 11.2 and at 31° were 40, 43 and 45 min. respectively, as estimated from the times during which the amounts of alkali added to keep the pH constant amounted to 0.5 equiv./mole.

Competitive inhibition

Table 4 shows values of K_i/K_m for mixtures of different substrates and inhibitors with penicillinases from *B. cereus* and *Staph. aureus*. The values were obtained by plotting the data from manometric experiments in the manner of Hunter & Downs (1945). One such plot, showing the behaviour of *N*-phenylacetyl-7-ACA as inhibitor with α -phenoxyethylpenicillin as substrate, is shown in

Table 4. *Behaviour of derivatives of 6-aminopenicillanic acid and 7-cephalosporanic acid as competitive inhibitors of penicillinases from B. cereus and Staph. aureus*

The enzyme from *B. cereus* was a highly purified preparation and that from *Staph. aureus* was a crude preparation.

Inhibitor	Substrate	K_i/K_m	
		<i>B. cereus</i> 5/B penicillinase	<i>Staph. aureus</i> R1 penicillinase
Cephalosporin C	Benzylpenicillin	4	>100
	α -Phenoxypropylpenicillin (DL-)	—	>100
	Cephalosporin N	3	26
	Cephalosporin C ₆	0.15	2
Deacetylcephalosporin C	Benzylpenicillin	3	>50
Cephalosporin C _A	Benzylpenicillin	5	—
	Cephalosporin N	2	7.5
<i>N</i> -Phenylacetyl-7-ACA	Benzylpenicillin	2	1.7×10^{-2}
	α -Phenoxyethylpenicillin (DL-)	—	4.1×10^{-3}
	α -Phenoxypropylpenicillin (D-)	—	6×10^{-3}
	α -Phenoxypropylpenicillin (L-)	—	1×10^{-3}
	α -Phenoxypropylpenicillin (DL-)	—	2.2×10^{-3}
	Cephalosporin N	—	8×10^{-4}
<i>N</i> - α -Phenoxypropionyl-7-ACA	Benzylpenicillin	—	0.1
2,6-Dimethoxyphenylpenicillin	Benzylpenicillin	2	>100
	Cephalosporin N	1	>100

Fig. 1. According to the expression of Hunter & Downs (1945):

$$\frac{i\alpha}{1-\alpha} = K_i + \frac{K_i s}{K_m},$$

where i is the inhibitor concentration, s the substrate concentration and α is initial velocity in presence of inhibitor/velocity in absence of inhibitor. Thus, K_i/K_m equals the ratio i/s required for a 50% reduction in initial velocity provided that $i \gg K_i$. The line in Fig. 1 cuts the ordinate axis at zero because K_i is too small, in this case, to be measured by the method used.

The general pattern of the values for K_i/K_m with purified penicillinase from *B. cereus* was strikingly different from that with penicillinase from *Staph. aureus*. In the case of penicillinase from *B. cereus*, values of K_i/K_m fell within the range 1–5 when a variety of derivatives of 7-ACA, or the 2,6-dimethoxybenzoyl derivative of 6-APA, were used as inhibitors and benzylpenicillin was the substrate. Similar values were also obtained with a single inhibitor, such as cephalosporin C, but different substrates, such as benzylpenicillin and cephalosporin N. The only exception was with cephalosporin C₆ as a substrate, when K_i/K_m was considerably lower than with the other compounds used.

In contrast, the values for K_i/K_m with staphylococcal penicillinase varied over several orders of magnitude among the same series of compounds. With benzylpenicillin as substrate cephalosporin C showed no inhibitory power at the highest concentration in which it was tested, but *N*-phenylacetyl-7-ACA was a powerful inhibitor, K_i/K_m being 1.7×10^{-2} . Thus the value of K_i for cephalosporin C was more than a thousand times that of K_i for *N*-phenylacetyl-7-ACA. Comparison of the values of

K_i/K_m with *N*-phenylacetyl-7-ACA as inhibitor and benzylpenicillin, α -phenoxyethylpenicillin, α -phenoxypropylpenicillin and cephalosporin N as substrates gave the relative K_m values of these substrates shown in Table 5. With cephalosporin N as a substrate, K_i/K_m was as low as 8×10^{-4} .

Derivatives of 6-aminopenicillanic acid and 7-aminocephalosporanic acid as inducers of penicillinase

Figs. 2 and 3 show the relative amounts of penicillinase formed during 1 hr., by *B. cereus* 569 and *Staph. aureus* R1 respectively, in the presence of varying concentrations of different inducers. All the experiments were carried out under similar conditions. The amount of enzyme formed in the presence of a given inducer showed some variation from one experiment to another, but the amount shown for each substance in Figs. 2 and 3 is relative to the amount formed in the presence of an optimum concentration of benzylpenicillin or 2,6-dimethoxyphenylpenicillin in the same experiment.

Induction in B. cereus. Pollock (1957) found that cephalosporin C, in optimum concentration, induced the formation of more penicillinase in *B. cereus* 569 than did benzylpenicillin, but that the concentration of cephalosporin C required to produce its maximum effect was much higher than that of benzylpenicillin. The experiments recorded here confirmed these findings qualitatively. They also showed that the behaviour of deacetylcephalosporin C was similar to that of cephalosporin C (Fig. 2) but that cephalosporin C_A (pyridine) had a higher maximum inducing activity than cephalosporin C.

Table 5. *Relative values of K_m and relative values of K_i for substrates and inhibitors respectively with penicillinase from Staph. aureus R1*

The relative values of K_m for different substrates are calculated from values for K_i/K_m with *N*-phenylacetyl-7-ACA as inhibitor (Table 4). The relative values of K_i are calculated from values for K_i/K_m with benzylpenicillin as substrate (Table 4). In each case the value of K_m for benzylpenicillin has been arbitrarily taken as unity.

Substrate or inhibitor	K_m (relative)	K_i (relative)
Benzylpenicillin	1.0	—
α -Phenoxyethylpenicillin (DL-)	4.1	—
α -Phenoxypropylpenicillin (D-)	2.8	—
(L-)	17	—
(DL-)	7.7	—
Cephalosporin N	20	—
<i>N</i> -Phenylacetyl-7-ACA	—	0.017
<i>N</i> - α -Phenoxypropionyl-7-ACA (DL-)	—	0.1
Cephalosporin C	—	>100
2,6-Dimethoxyphenylpenicillin	—	>100

Fig. 1. Effect of *N*-phenylacetyl-7-ACA on the rate of hydrolysis of α -phenoxyethylpenicillin in the presence of penicillinase from *Staph. aureus* R1. Plot according to Hunter & Downs (1945).

Pollock (1957) pointed out that the relationship between the concentration of an inducer and the amount of an enzyme induced per unit time might show a formal resemblance to the relationship between the concentration of a substrate and the velocity of an enzyme reaction, and he introduced the term 'induction constant' for the concentration of an inducer at which the latter showed half its maximum inducing activity. The term 'apparent induction constant', rather than 'induction constant', has been employed here for presenting the data (Table 6), since the inducers tested were

Fig. 2. Induction of penicillinase in *B. cereus* 569. The curves show the results with different initial concentrations of the following inducers: ○, benzylpenicillin; ▲, 2,6-dimethoxyphenylpenicillin; △, cephalosporin C; ×, deacetylcephalosporin C; □, cephalosporin C_A (pyridine); ●, cephalosporin C₆; ■, *N*-phenylacetyl-7-ACA. The maximum amount of enzyme formed in the presence of benzylpenicillin was about 180 units/ml.

antibacterial compounds which varied greatly in their sensitivity to penicillinase and hence the numerical relationship between the concentrations in which different inducers were used did not remain constant during the induction period. Moreover, only the amounts of extracellular enzyme were determined. With *B. cereus* 569 the apparent induction constants for cephalosporin C_A (pyridine), cephalosporin C₆ and *N*-phenylacetyl-7-ACA were nearer to the apparent induction constant for benzylpenicillin than to that for cephalosporin C (Table 6). The apparent induction constant for 2,6-dimethoxyphenylpenicillin had an intermediate value.

Induction in Staph. aureus. All the compounds tested which had induced the formation of peni-

Fig. 3. Induction of penicillinase in *Staph. aureus* R1. The curves show results with different initial concentrations of the following inducers: ○, benzylpenicillin; ▲, 2,6-dimethoxyphenylpenicillin; △, cephalosporin C; ×, deacetylcephalosporin C; □, cephalosporin C_A (pyridine); ■, *N*-phenylacetyl-7-ACA. Growth-inhibitory concentrations (readings at 24 hr.; for method of determination see text) are indicated by ||. The maximum amount of enzyme formed in the presence of 2,6-dimethoxyphenylpenicillin was about 14 units/ml.

Table 6. *Apparent induction constants and activity against Staph. aureus* R1 *of derivatives of 6-aminopenicillanic acid and of 7-aminocephalosporanic acid*

The apparent induction constant is arbitrarily defined as the initial concentration of an inducer required to induce half the maximum amount of extracellular penicillinase obtainable with that inducer during 1 hr. under the conditions used. The minimum growth-inhibitory concentrations against *Staph. aureus* were determined with a large inoculum (for details see text). Readings were taken after 1 day and 6 days.

Inducer	Apparent induction constant (µg./ml.)		Minimum growth-inhibitory concentration (µg./ml.) against <i>Staph. aureus</i> R1	
	with enzyme from <i>B. cereus</i>	with enzyme from <i>Staph. aureus</i> R1		
			After 1 day	After 6 days
Benzylpenicillin	0.07	> 30	500	4000
2,6-Dimethoxyphenylpenicillin	0.3	0.3	4	8
Cephalosporin C ₆	0.03	> 50	900	2000
Cephalosporin C	6.6	10	125	250
Deacetylcephalosporin C	6.6	13	500	1000
Cephalosporin C _A (pyridine)	0.05	0.9	8	32
<i>N</i> -Phenylacetyl-7-ACA	0.06	0.01	0.5	2
<i>N</i> -α-Phenoxypropionyl-7-ACA	0.1	0.03	1	1

icillinase in *B. cereus* also acted as inducers in *Staph. aureus* (Fig. 3). There were striking differences, however, between the relative behaviour of the compounds in the two systems with respect to their maximum inducing activity and, more particularly, their apparent induction constants (Table 6). In general, the apparent induction constants varied roughly in parallel with the minimum concentrations of the compounds required to inhibit the growth of *Staph. aureus* from a large inoculum. In contrast with the situation with *B. cereus*, the apparent induction constants of benzylpenicillin and cephalosporin C₆ were higher than the apparent induction constant of cephalosporin C and much higher than that of *N*-phenylacetyl-7-ACA.

Penicillinase and cephalosporinase

Abraham & Newton (1956) reported that the ratio 'activity against cephalosporin C/activity against benzylpenicillin' was much larger with crude penicillinase from *B. cereus* 569 than with highly purified penicillinase from the same organism. They suggested that the crude product contained a C-cephalosporinase which differed from the enzyme responsible for much of the activity of this product against benzylpenicillin. The question whether the cephalosporinase was also a penicillinase remained unresolved.

The present findings confirm the earlier results and indicate that both cephalosporinase and penicillinase are formed by *B. cereus* strain 569/H and

strain 5/B as well as by strain 569 (Tables 1 and 2). Further evidence for the presence of two distinct enzymes in a culture fluid of *B. cereus* 569 was obtained from determinations of the activity of the cell-free culture fluid against cephalosporin C and benzylpenicillin respectively after it had been kept for varying times at 60°. The values of $V_{\max}$ obtained with a standard volume of culture fluid were used as a measure of enzymic activity. Fig. 4 shows that the activity against benzylpenicillin fell rapidly to about 15% of its original value, whereas 90% of the original activity against cephalosporin C was retained under the same conditions. After 30 min., $V_{\max}$ for cephalosporin C was about one-third of that for benzylpenicillin, whereas initially it was only one-twentieth as great (Table 2). It is possible that the remaining activity against benzylpenicillin represented penicillinase activity shown by the cephalosporinase.

The formation of cephalosporinase in *B. cereus* 569 was induced not only by cephalosporin C but also by deacetylcephalosporin C, cephalosporin C_A, cephalosporin C₆ and benzylpenicillin. No significant differences were detected in the relative activities against different substrates of the culture fluids obtained after induction for 1 hr. by different inducers.

The inactivation of cephalosporin C by the cephalosporinase in a crude preparation of induced penicillinase from *B. cereus* 569 had been shown to be accompanied by loss of the characteristic absorption band of the antibiotic with $\lambda_{\max}$ at 260 m μ (Abraham & Newton, 1956). A similar change in absorption occurred when a solution of cephalosporin C (sodium salt) (0.5 mg./ml.) in 0.01 M-sodium phosphate buffer, pH 7.0, containing 0.5% of gelatin and a relatively high concentration of highly purified penicillinase from *B. cereus* 5/B (25 μ g./ml.) was kept at 30°. Under these conditions the extinction at 260 m μ fell to 50% of its initial value in 2 hr. Under similar conditions, but with an enzyme concentration of 5 μ g./ml., the extinction of cephalosporin C_A (pyridine) at 257 m μ fell to 30% of its initial value in 30 min. With an enzyme concentration of 0.1 μ g./ml. the absorption band of cephalosporin C₆ ($\lambda_{\max}$, 256 m μ) was replaced in 30 min. by a new band ($\lambda_{\max}$, 265 m μ) whose maximum extinction was 48% of that of the original band.

DISCUSSION

N-Acyl derivatives of 7-ACA (I) contain both a labile β -lactam ring and a labile *O*-acetyl group. The ability of purified penicillinase and of cephalosporinase from *B. cereus* to hydrolyse derivatives of 7-ACA has been estimated from the acid liberated during the enzymic reactions. None of the products of these reactions has been isolated.

Fig. 4. Change in activities of a solution of crude enzyme from *B. cereus* 569 (pH 7.4) when kept at 60°. ○, Activity against cephalosporin C; ●, activity against benzylpenicillin. The initial activity against benzylpenicillin was 200 units/ml. The initial value of $V_{\max}$ for cephalosporin C was one-twentieth of that for benzylpenicillin.

Nevertheless, it is a reasonable assumption that the enzymes are acting as β -lactamases. The enzymic hydrolysis of cephalosporin C appears to terminate when approximately 1.2 equiv. of acid/mole has been liberated and is accompanied by a change in ultraviolet absorption resembling that which occurs on treatment of the compound with dilute alkali. The change in alkaline solution is thought to involve opening of the β -lactam ring, removal of the *O*-acetyl group, and further degradation of the resulting unstable product (Abraham & Newton, 1961b). Removal of the *O*-acetyl group alone, by citrus acetylsterase, leads to deacetylcephalosporin C, whose ultraviolet absorption spectrum is similar to that of cephalosporin C (Jeffery *et al.* 1961). Thus the action of the preparations of penicillinase and cephalosporinase could not be satisfactorily accounted for by the assumption that these preparations contained an acetylsterase. Other findings gave support to the view that the reactions studied were not catalysed by an acetylsterase: (a) the acetylsterase activity of the preparations, with triacetin as a substrate, was extremely small; (b) the formation of cephalosporinase in *B. cereus* 569 was induced not only by cephalosporin C but by a number of compounds which do not contain an acetoxy group, such as deacetylcephalosporin C and benzylpenicillin; (c) the cephalosporinase appeared to show activity against deacetylcephalosporin C as well as against cephalosporin C.

With the purified enzymes from *B. cereus* all the compounds of structure (I) that were hydrolysed at a measurable rate in the concentrations used showed very low values of k_3 . For cephalosporin C

k_3 was about 10^{-4} times that for benzylpenicillin. A change in R' to HO appeared to reduce k_3 still more, but a change in R' to $\text{C}_6\text{H}_5\text{N}^+$ raised k_3 to nearly 10^{-2} times and a change in R to $\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CO}$ raised it to about 6×10^{-4} times that for benzylpenicillin. In contrast, k_3 for cephalosporin C₆ approached that for benzylpenicillin, though the K_m for cephalosporin C₆ was considerably higher than that for benzylpenicillin. The high value of k_3 for cephalosporin C₆ may be correlated with the fact that the β -lactam ring of this compound is particularly susceptible to nucleophilic attack. Thus cephalosporin C₆ is hydrolysed much more readily than cephalosporin C in weakly alkaline solution in the absence of enzyme (Abraham & Newton, 1961b). On the other hand, the great differences between the rates of enzymic hydrolysis of benzylpenicillin and deacetylcephalosporin C are not paralleled by comparable differences in the

susceptibility of these compounds to attack by hydroxyl ions. The relatively high value of K_m with cephalosporin C₆ (deacetylcephalosporin C lactone) raises the question whether the carboxyl group attached to C-4 of the dihydrothiazine ring in compounds with structure (I) plays a part in the formation of the enzyme-substrate complex.

Cephalosporin C₆ also showed a higher value of k_3 than did compounds of structure (I) when it was used as a substrate for staphylococcal penicillinase. With the penicillins (II) and penicillinase from *Staph. aureus*, k_3 decreased significantly in some cases when the *N*-acyl group (R) was derived from a di- instead of a mono-substituted acetic acid, and decreased considerably when it was derived from a substituted benzoic acid as in 2,6-dimethoxyphenylpenicillin. In the case of α -phenoxypropylpenicillin, which was available in different diastereoisomeric forms, k_3 was found to vary with the optical configuration of the side chain.

The use of different compounds of structure (I) as competitive inhibitors showed that changes in the *N*-acetyl group (R) of such compounds could result in changes of several orders of magnitude in affinity for the staphylococcal enzyme. Thus, K_i for cephalosporin C

was more than 10^3 times that for *N*-phenylacetyl-7-ACA (I; $R = \text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CO}$). The use of the same inhibitor for different substrates showed that K_m for a number of substrates of structure (II) varied, with a given change in R, in the same direction as K_i for different inhibitors of structure (I) (Table 5). The variations in K_m , however, were smaller than those in K_i . On the basis of simple Michaelis-Menten kinetics this was not inconsistent with the assumption that a given change in R had a similar influence on the affinities for the enzyme of an inhibitor of structure (I) and a substrate of structure (II) respectively.

Novick (1962) has reported that K_m for benzylpenicillin with a strain of penicillinase from *Staph. aureus* 524 SC (Rogers, 1953) is $2.5 \mu\text{M}$. If this is also the value of K_m for benzylpenicillin with penicillinase from *Staph. aureus* R1, the corresponding value for cephalosporin N with penicillinase from *Staph. aureus* R1 (calculated from the data in Table 5) is $50 \mu\text{M}$. This is close to the value of K_m for both cephalosporin N and benzylpenicillin with purified penicillinase from *B. cereus* 569 [$43 \mu\text{M}$ derived from the data of Abraham & Newton (1956) and Pollock (1960)]. Thus when

in structure (II), K_m appears to have a similar value with the enzymes from *B. cereus* and *Staph. aureus*, but, when $R = C_6H_5 \cdot CH_2 \cdot CO$, K_m has a considerably higher value with the former enzyme than with the latter.

Although the results of these experiments have been expressed in terms of Michaelis-Menten kinetics the true mechanism of the enzymic reactions may well be more complicated, involving, for example, acyl-enzyme intermediates in addition to Michaelis complexes. Thus k_3 and K_m may be overall constants dependent on several different processes. The relative values for K_i shown in Table 5 have been assumed to relate to competitive inhibition, since they were obtained from data which were consistent with this type of inhibition when plotted in the manner of Hunter & Downs (1945). However, the failure of the reaction to continue at its initial rate for more than about 10 min. when some of the inhibitors were used as substrates in concentrations apparently high enough to saturate the enzyme (crude cephalosporinase or a high concentration of purified penicillinase from *B. cereus*) suggests that inhibition other than that of the simple competitive type may occur.

The relative activities of the compounds tested as inducers of penicillinase changed greatly when *Staph. aureus* was used in place of *B. cereus* to synthesize the enzyme. The interpretation of the results is complicated by the fact that cell growth occurred, as judged by the increase in opacity of the cultures, during the induction period of 1 hr. Thus an inducer which was readily destroyed by penicillinase would be expected to be less effective, other things being equal, than one which was not. This would be so even if only brief contact between inducer and cell were sufficient to stimulate enzyme formation, as was found by Pollock (1950) to be the case with *B. cereus* 569, since the penicillinase-sensitive inducer would not survive in the culture fluid to react with newly formed cells. In accordance with this expectation it was found that 1.5 times as much penicillinase was induced in *B. cereus* when a given total amount of benzylpenicillin (100 μ g./ml.) was added in six equal portions at intervals of 10 min. as was induced when it was all added at the beginning of the induction period. Moreover, cephalosporin C_A (pyridine), which showed a lower value of k_3 in the presence of the crude enzyme from *B. cereus* than any of the other compounds tested except deacetylcephalosporin C, was a particularly good inducer in optimum concentration. However, factors other than stability to penicillinase appear to operate in this connexion. Pollock (1957) reported that cephalosporin C (in optimum concentration) induced more penicillinase than benzylpenicillin in *B. cereus*

569 when the 'cold pre-treatment' method was used. In this method free penicillin is not present in the medium during most of the time in which induced enzyme is being synthesized (Pollock, 1950).

The apparent induction constants for benzylpenicillin and cephalosporin C_e with *Staph. aureus* were more than 400 and 1000 times respectively greater than those with *B. cereus*, although the constants for each of the other compounds tested (which were highly resistant to hydrolysis by penicillinase) were of the same order of magnitude with the two enzymes. This suggests that the continued presence of benzylpenicillin in the medium is more important for the induction of the staphylococcal enzyme than for the induction of the enzyme from *B. cereus*. In general, the apparent induction constants of different inducers of the staphylococcal enzyme varied in the same direction as their minimum inhibitory concentrations for the growth of *Staph. aureus* R1 from a large inoculum. The decrease, in some cases, in the amount of enzyme formed when the concentration of inducer exceeded the minimum inhibitory concentration (Fig. 3) may well have been due to inhibition of cell growth, but the extent to which the antibacterial action of the inducers influenced the values obtained for the apparent induction constants cannot be assessed from the present data. The minimum inhibitory concentration depends in part on the stability of the compound concerned to staphylococcal penicillinase and probably in part on the affinity of the compound for a specific centre, or centres, in the cell. With a penicillinase-sensitive compound it is greater for a large inoculum than for a small one, since the former produces more enzyme. Thus the low value for the apparent induction constant of *N*-phenylacetyl-7-ACA, compared with that of cephalosporin C, may be a reflexion of the high affinity of the former substance for the induction site in the staphylococcus, while the high value for the apparent induction constant of benzylpenicillin may be due to the difficulty of keeping the induction site saturated in the presence of penicillinase produced by the large inoculum used in the induction experiments and not to an intrinsically low activity on the part of this inducer.

The finding that cephalosporin C and *N*-phenylacetyl-7-ACA have similar affinities for penicillinase from *B. cereus*, whereas the latter has a much higher affinity than the former for penicillinase from *Staph. aureus*, is reminiscent of the fact that a change in the group R of structures (I) and (II) from

to $C_6H_5 \cdot CH_2 \cdot CO$ has relatively little effect on activity against *Salmonella typhi* but greatly increases activity against *Staph. aureus*.

Crawford & Abraham (1957) found that cephalosporin C and benzylpenicillin acted synergistically against one penicillinase-producing strain of *Staph. aureus* (strain D3R), but a significant degree of synergism has not been found with other strains (Moat, Ceci & Bondi, 1961). It was suggested that the effect with strain D3R might be attributed, at least in part, to a competitive inhibition of the action of penicillinase on benzylpenicillin by cephalosporin C (Crawford & Abraham, 1957). If it is assumed that penicillinases from *Staph. aureus* D3R and *Staph. aureus* R1 have similar properties, the finding with the enzyme from strain R1 that K_i for cephalosporin C is high, relative to K_m for benzylpenicillin, renders this explanation untenable. The much smaller value of K_i for *N*-phenylacetyl-7-ACA with staphylococcal penicillinase might appear to indicate that this derivative would show a significant synergistic effect with benzylpenicillin by inhibiting enzymic destruction of the latter. However, calculation shows that, with concentrations of such an inhibitor which are lower than those at which the inhibitor alone is bactericidal, the degree of protection of the substrate may not be very large. On the assumption that K_m for benzylpenicillin is $2.5 \mu M$ (Novick, 1962), K_i for *N*-phenylacetyl-7-ACA can be assigned the value $0.043 \mu M$, since K_i/K_m is 1.7×10^{-2} (Table 4). From the expression of Hunter & Downs (1945) it is then possible to calculate α (rate of inactivation of benzylpenicillin in the

presence of inhibitor/rate in the absence of inhibitor) for various concentrations of inhibitor and substrate (Fig. 5). With very low concentrations of substrate

$$\alpha_{\text{lim}} (s \rightarrow 0) = \frac{K_i}{i + K_i}$$

i.e. $\alpha = K_i/i$ if i is large compared with K_i . With *N*-phenylacetyl-7-ACA concentrations of 1, 0.5, 0.25 and $0.125 \mu g./ml.$, the corresponding limiting values of α when $s \rightarrow 0$ are 1.8, 2.5, 6.7 and 12.5 % respectively. As the substrate concentration is increased the values of α increase at a rate which depends on the K_m for the substrate (Fig. 5). Since the minimum inhibitory concentration of *N*-phenylacetyl-7-ACA alone is $0.5 \mu g./ml.$ (about $1 \mu M$) or less for *Staph. aureus* R1, it may not be possible, under conditions in which synergism could be demonstrated with this derivative, to reduce the rate of inactivation of a penicillinase-sensitive substance to less than about 10 % of its original value.

When derivatives of 7-ACA or 6-APA are used in concentrations which are of the order of their K_m values or less, their rate of inactivation by penicillinase will fall at a significant rate with a decrease in concentration. In other words, the effective resistance of a substance of this type to staphylococcal penicillinase should rise not only with a decrease in k_3 but also with a rise in K_m to a value approaching or exceeding that for the concentration at which the substance is able to exert a bactericidal effect. For this reason it seemed possible that *N*- α -phenoxypropionyl-7-ACA would be somewhat more effective than the phenylacetyl derivative against penicillinase-producing staphylococci *in vitro* when the compounds were tested under such conditions that a further decrease in even a very low sensitivity to the enzyme could be important. The finding that the minimum inhibitory concentration of *N*- α -phenoxypropionyl-7-ACA was unchanged when readings were taken at 6 days instead of at 24 hr., whereas that of the phenylacetyl derivative increased about fourfold, was consistent with this possibility.

SUMMARY

1. The maximum rates of hydrolysis of several derivatives of 7-aminocephalosporanic acid in the presence of purified penicillinase from strains of *Bacillus cereus* or crude extracellular penicillinase from a strain of *Staphylococcus aureus* are very low. In contrast, the rate of hydrolysis of cephalosporin C_6 (deacetylcephalosporin C lactone) is of the same order as that of benzylpenicillin.

2. Several derivatives of 7-aminocephalosporanic acid behave as competitive inhibitors of the

Fig. 5. Calculated inhibition of hydrolysis of different penicillins by different concentrations of the *N*-phenylacetyl derivative of 7-ACA (I) in the presence of penicillinase from *Staph. aureus* R1. The curves refer to the following substrates: —, benzylpenicillin; ---, α -phenoxypropylpenicillin; ····, cephalosporin N. α represents (velocity in presence of inhibitor)/(velocity in absence of inhibitor).

action of penicillinase from *B. cereus* or *Staph. aureus* on penicillins. Different derivatives show a similar ability to inhibit the enzyme from *B. cereus* but differ greatly in their ability to inhibit the staphylococcal enzyme.

3. Culture fluids of strains of *B. cereus* contain a cephalosporinase which may be a β -lactamase.

4. Several derivatives of 7-aminocephalosporanic acid induce the formation of penicillinase by a strain of *Staph. aureus* and a strain of *B. cereus*. Under the conditions used the relative efficiencies of the inducers vary greatly from one organism to the other.

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Studies on the Mode of Action of Excess of Vitamin A

4. THE SPECIFICITY OF THE EFFECT ON EMBRYONIC CHICK-LIMB CARTILAGE IN CULTURE AND ON ISOLATED RAT-LIVER LYSOSOMES*

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(Received 11 August 1961)

Previous work has shown that when embryonic cartilage is grown in medium containing an excess of vitamin A, metachromatic material is progressively lost from the matrix, which rapidly disintegrates (Fell & Mellanby, 1952; Fell & Thomas, 1960); metachromatic material also disappears from the cartilage of young rabbits fed with excessive amounts of the vitamin (Thomas, McLuskey, Potter & Weissmann, 1960). This previous work

suggested that the degradation of the cartilage matrix under the influence of hypervitaminosis A might be due to an enhanced proteolytic activity of the chondroblasts; recent results (Dingle, Lucy & Fell, 1961; Lucy, Dingle & Fell, 1961) have confirmed this view and indicated that the degradation is associated with the release of protease from the lysosomes (Dingle, 1961).

The present experiments were undertaken to determine whether the action of vitamin A on cartilage in culture and on isolated lysosomes is specific, or whether a similar effect can be produced by other chemically-related compounds. The

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