Chemical Changes following γ-Irradiation of Benzylpenicillin in Aqueous Solution

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The effects of γ-irradiation on aqueous solutions of sodium benzylpenicillin have been investigated. Pulse radiolysis indicates that both hydrated electrons and hydroxyl radicals are responsible for the degradation between 10^{-4} and 10^{-2}M solute concentrations. Reaction of hydroxyl radicals with the benzene ring of the penicillin side-chain leads to the formation of *o*-, *m*-, and *p*-hydroxybenzylpenicillins. Hydroxyl radicals also induce cleavage of the β-lactam ring, yielding benzylpenilloic and benzylpenicilloic acid. Reaction of hydrated electrons with penicillins generally lead to molecular rearrangements yielding products such as benzylpenillic acid.

CONSIDERABLE interest centres on the sterilisation, by ionizing radiations, of pharmaceuticals which cannot be readily sterilised by heat because they undergo decomposition by this treatment.[1] The main concern with the use of ionising radiations is that products may be formed which might adversely affect the value of the drug. Whereas it is inevitable that certain chemical changes accompany treatment of a drug with a sterilising dose of radiation (*ca.* 2·5 Mrad), these changes may be minimal or can, in some way, be effectively reduced. A notable feature of the subject is that little is known about the chemical changes induced by irradiation or organic compounds which have a direct relevance to the pharmaceutical industry. Attention has mainly been directed towards colour and pH changes, loss of potency, formation of readily detectable u.v.-absorbing products and other superficial changes. Our intention is to examine quantitatively the extent of degradation in order to provide data which will assist in a more scientific evaluation of the method. We shall seek also probable degradative paths for a range of compounds which are basic to the pharmaceutical industry. The effects of γ-radiation on sodium sulphacetamide,[2,3] indoles,[4] and vitamin B_{12} [5] have already been described. In this series we examine the radiation degradation of sodium benzylpenicillin, which is basic to a range of penicillins now in common use.

EXPERIMENTAL

The absence of decomposition products in the benzylpenicillin used (Glaxo Laboratories Ltd.) was established by t.l.c. All other chemicals were AnalaR grade. Details of the 10,000 Ci ^{60}Co source, dosimetry, and irradiation procedures have been described.[6,7] Solutions in triply distilled water were adjusted to pH 7 and equilibrated with argon, and N_2O before for 30 min and during irradiations. A dose rate of $6{\cdot}0 \times 10^{17}$ eV ml^{-1} min^{-1} was used throughout. The total irradiation time was routinely less than 10 min in order to minimise decomposition of benzylpenicillin which is known to occur in aqueous solution with time. Aliquots were removed after increasing doses and in each instance compared with unirradiated controls which had been similarly treated. Pulse radiolysis was carried out at

[1] Radiosterilisation of medical products and recommended code of practice. International Atomic Energy Agency, Vienna (1967).
[2] G. O. Phillips, D. M. Power, and M. Sewart, *Radiation Res.*, 1971, **46**, 236.
[3] D. J. Trigger and A. D. S. Caldwell, *J. Hosp. Pharm.*, 1968, 269.
[4] B. Iddon, G. O. Phillips, K. E. Robbins, and J. V. Davies, *J. Chem. Soc.* (*B*), 1971, 1887.
[5] R. Blackburn, D. L. Cox, and G. O. Phillips, *J.C.S. Faraday I*, 1972, 1687.
[6] J. J. Weiss, J. T. Allen, and B. Schwarz, Proceedings First Conference Peaceful Uses of Atomic Energy, Geneva, 1955, **14**, 179.
[7] H. A. Dewhurst, *Trans. Faraday Soc.*, 1952, **48**, 905.

the Christie Hospital and Holt Radium Institute, Manchester, using a 12 MeV accelerator and the techniques have been described.[8,9]

To determine relative rates of reaction of OH radicals with benzylpenicillin and benzylpenicilloic acid, *p*-nitroso-*NN*-dimethylaniline (RNO) was used as a competitor [10] according to the procedure of Anbar, Myerstein, and Neta.[11] Care is required if meaningful results are to be obtained by this method.[2]

Relative rates only were obtained using the RNO method and these were related to the absolute OH rate constant, independently determined from the rate of growth of the OH adduct to phenylacetic acid ($k_2 = 3{\cdot}8 \times 10^9$ l mol^{-1} s^{-1}). Irradiated samples were analysed by t.l.c. using 0·5 mm layers of silica gel GF_{254} buffered at pH 5·8 with phosphate buffer (10^{-1}M). The best separation of benzylpenicillin and its degradation products was obtained with irrigant (I) which contained acetic acid, pH 5·8 phosphate buffer (10^{-1}M), butyl acetate, butan-1-ol, and ethanol, 40 : 24 : 80 : 15 : 5 (by vol.). Degradation products were visible as a result of fluorescence quenching. After elution from the plate with water each compound was recrystallised and rechromatographed in irrigant (II). (Benzene–acetone–acetic acid (5%) 60 : 35 : 5 (v/v/v).

Quantitative Determination of G(—Benzylpenicillin) Values.—Irradiated samples (10 ml) were freeze dried and reconstituted by addition of water to give 10^{-2}—10^{-1}M-solutions. Aliquots (1 μl) of the 10^{-1}M-irradiated benzylpenicillin solution or 10 μl of 10^{-2}M-solution were applied to a t.l.c. plate together with six portions (1 μl) of standard penicillin solutions (10—70 μg ml^{-1}). The plates were irrigated (solvent I), dried at 100° for 15 min, and scanned using a Vitatron densitometer. A standard-curve relating densitometer-counts to penicillin concentration obeyed Beers Law over the range 10—60 international units per ml (I.U. ml^{-1}) (1660 I.U. = 1 mg). From the plot of penicillin remaining *versus* radiation dose, *G*(—benzylpenicillin) values were calculated. These data compared with the results of a biological assay of benzylpenicillin using well established techniques.[12]

Another estimate of the extent of benzylpenicillin degradation was obtained by an iodine titration. Benzylpenicillin was separated from radiation degradation products by t.l.c.; compounds were removed from the plate and transferred to a test tube. 2N-Sodium hydroxide (1 ml) was added to them and the slurry so formed was set aside for 45 min with occasional shaking. Hydrochloric acid (1 ml; 1·1M) was then added to the mixture followed by a known excess of 5×10^{-4}N iodine solution; after 30 min the excess iodine was titrated against $2{\cdot}5 \times 10^{-4}$N-sodium thiosulphate solution (starch as indicator). Under the experimental conditions one molecule of benzylpenicillin reacts with eight molecules of iodine.

Quantitative Estimation of Radiation Products.—The iodine titration procedure was employed to determine yields of benzylpenilloic acid; 1 molecule of this compound reacts with 5·5 molecules of iodine.

To estimate benzylpenillic acid, 1 molecule of which reacts with 6 molecules of iodine, the titration procedure was modified. After the addition of N-NaOH, the slurry was heated at 65° for 30 min. Yields of penicilloic acid were measured by densitometry.

Separation of *o*-, *m*-, and *p*-hydroxybenzylpenicillins by t.l.c. is not possible. The total yield of hydroxybenzylpenicillins was estimated by measurement of the phenolate anion absorption at 295 nm. A portion (0·5 ml) of sodium hydroxide solution (10^{-1}M) was added to the irradiated sample 10^{-2}—10^{-4}M and the absorption (295 nm) determined against a reagent blank. The total phenol content of the irradiated sample was calculated by reference to a standard curve relating absorption at 295 nm to concentration.

Gibbs reagent reacts with phenolic hydroxy-groups only in the *o*- or *m*-position.[13] A stock solution was prepared containing 2,6-dichloroquinone chlorimide (0·1 g) in ethanol (100 ml). Gibbs reagent was prepared by mixing a portion (4·5 ml) of this solution with 10 ml borate buffer (pH 9·4) containing boric acid (3·1 g), potassium chloride (3·5 g), and M-sodium hydroxide (32 ml) in 1 l of solution.

A sample (5 ml) of irradiated penicillin solution (10^{-2}—10^{-4}M) was mixed with pH 9·4 borate buffer solution (1·0 ml) and Gibbs reagent (0·1 ml). The solution was set aside in the dark for 30 min and the optical density determined at 610 nm against a reagent blank. A standard curve was constructed relating optical density at 610 nm to concentration.

Nitrosation of hydroxyphenylacetic acids yields the corresponding hydroxynitrosophenylacetic acids.[14] We employed this method to facilitate determination of hydroxybenzylpenicillins. Sulphuric acid (1 ml; 6N) and 4% sodium nitrate solution (0·1 ml) were added to a portion (5 ml) of the irradiated solution (10^{-2}—10^{-4}M) and the solution was heated at 100° for 10 min. Ammonia (*d* 0·880) was added slowly with mixing to the cool solution. The optical density was determined at 406 nm against a reagent blank. A standard curve relating optical density at 406 nm against concentration was also prepared.

The extinction coefficients of the *o*-, *m*-, and *p*-derivatives at 406 nm are 11,050, 5750, and 2150 l mol^{-1} cm^{-1} respectively. Optical density values determined and nitrosation of solutions containing a mixture of *o*-, *m*-, and *p*-hydroxybenzylpenicillins will, therefore, be given by the sum of (*o*-hydroxybenzylpenicillin) × 1 + (*m*-hydroxybenzylpenicillin) × 0·52 + (*p*-hydroxybenzylpenicillin) × 0·195. The yield of hydroxybenzylpenicillins was estimated by each of the three methods and the values plotted against radiation dose. From the initial slope of these curves three *G* values were obtained, namely G_T = total phenol ($o + m + p$), G_G = Gibbs method ($o + m$) and G_N = nitrosation method = ($o + 0{\cdot}52m + 0{\cdot}195p$). The data was used to calculate *G* values for the formation of each individual isomer. Thus, $G(p\text{-hydroxybenzylpenicillin}) = G_T - G_G$.

$$G(m\text{-hydroxybenzylpenicillin}) = 2{\cdot}1\left\{\begin{matrix} G_G - (G_N - 0{\cdot}195\,.\,G_p \\ \text{hydroxybenzylpenicillin}) \end{matrix}\right\}$$

$$G(o\text{-hydroxybenzylpenicillin}) = G_G - G(m\text{-hydroxybenzylpenicillin})$$

[8] J. P. Keene, *J. Sci. Instr.*, 1964, **41**, 493.
[9] E. A. Balazs, J. V. Davies, G. O. Phillips, and M. D. Young, *Radiation Res.*, 1967, **31**, 243.
[10] I. J. Kraljic and C. N. Trumbore, *J. Amer. Chem. Soc.*, 1965, **87**, 2546.
[11] M. Anbar, D. Myerstein, and P. Neta, *J. Phys. Chem.*, 1966, **70**, 1660.
[12] W. M. Hoskins, '*Kirk-Othmer Encyclopedia Chem. Technol.*,' 2nd edn., 1964, **3**, 489.
[13] M. B. Ettinger and C. C. Rucholt, *Analyt. Chem.*, 1948, **20**, 1191.
[14] S. C. Pan, *Analyt Chem.*, 1955, **27**, 65.

Carbon dioxide evolved during irradiation was absorbed in 10^{-4}N-barium hydroxide solution (20 ml) which was then titrated with 10^{-4}N-HCl using phenolphthalein as

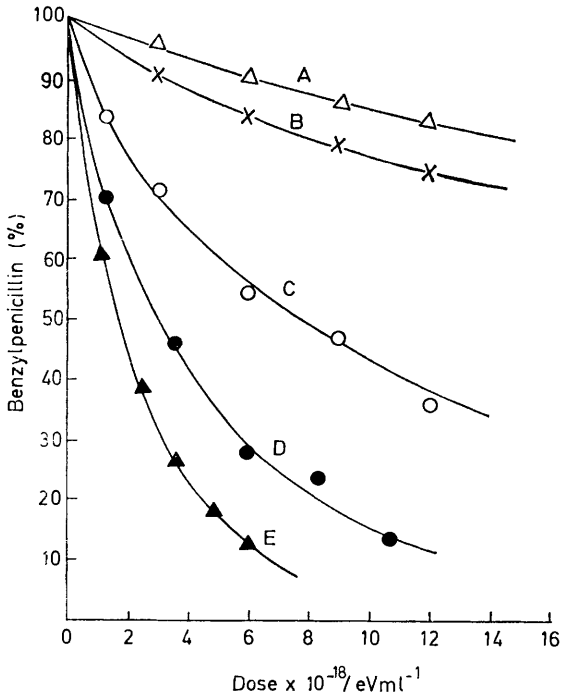

FIGURE 1 Effect of γ-irradiation on benzylpenicillin in argon-saturated solution: A = 10^{-2}M; B = 5×10^{-3}M; C = 10^{-3}M; D = 5×10^{-4}M; E = 10^{-4}M

indicator. Rigorous precautions were taken to minimise atmospheric contamination under the experimental conditions. Hydrogen sulphide evolved during irradiation was measured by reaction with *NN*-dimethyl-*p*-phenylenediamine to yield Methylene Blue.[15]

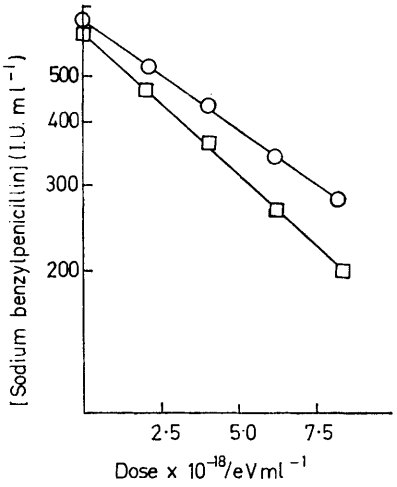

FIGURE 2 Effect of γ-irradiation on benzylpenicillin: ○ = argon-saturated solution, □ = N_2O-saturated solution, benzylpenicillin = 10^{-3}M. 1 mg = 1660 international units

RESULTS

G(—Benzylpenicillin) Values.—The effect of solute concentration (10^{-4}—10^{-2}M) on the degradation of benzylpenicillin by γ-irradiation in argon and in nitrous oxide saturated solution was determined by densitometry bioassay and chemical assay. Typical curves are shown in Figure 1. log(benzylpenicillin) against dose is linear (Figure 2). Initial *G* values, obtained from graphs of residual benzylpenicillin concentration *versus* dose are summarised in Figure 3.

TABLE 1

Products formed during γ-irradiation of sodium benzylpenicillin: * [sodium benzylpenicillin] = 10^{-3}M

	Solvent (I) [a]	Solvent (II) [b]	Argon pH 7	N_2O pH 7	Argon pH 7 + 2-methylpropan-2-ol (10^{-1} M)
Unknown	107	—	+	+	—
Unknown	90	—	+	+	+
Dethiobenzylpenicillin	82	—	+	—	+
Unknown	72·5	—	+	+	—
Benzylpenilloic acid	63	34	+	+	+
Benzylpenillamine †	53	2	+	—	+
Benzylpenicilloic acid	45	3	+	+	—
Penicillamine †	40	49	+	—	+
Benzylpenillic acid	35	0	+	—	+
'Thiazepine' †	25	—	+	—	+
Unknown	17	—	+	+	+
Unknown	4	—	+	+	—

[a] Solvent (I): acetic acid : pH 5·8 phosphate buffer (10^{-1} M) : butyl acetate : butan-1-ol : ethanol 40 : 24 : 80 : 15 : 5 (v/v/v/v/v). [b] Solvent (II): benzene : acetone : acetic acid (5%) 60 : 35 : 5 (v/v/v)

* Chromatographic mobilities (R_p values) are expressed relative to sodium benzylpenicillin (= 100). † Tentative identification only.

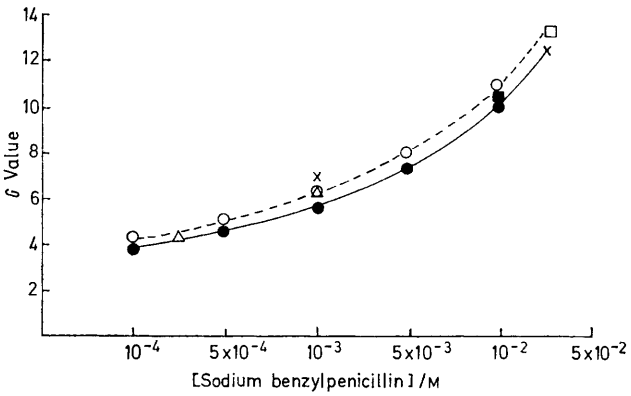

FIGURE 3 Values of *G*(—benzylpenicillin) measured by different methods: —— argon-saturated solution and - - - - N_2O-saturated solution. Densitometry ● = argon, ○ = N_2O; bioassay △ = argon, × = N_2O; iodine titration ■ = argon, □ = N_2O

Radiation Products.—Examination of irradiated aqueous solutions of benzylpenicillin (10^{-3}M) by t.l.c. in irrigants (I) and (II) revealed 12 products in addition to unchanged benzylpenicillin (Table 1), many of which could be identified by reference to known materials.

Identification and Quantitative Determination of Radiation

[15] M. B. Jacobs, M. M. Braverman, and S. Hochheiser, *Analyt. Chem.*, 1957, **29**, 1349.

Degradation Products.—(i) *Benzylpenilloic acid.* A compound chromatographically identical with authentic benzylpenilloic acid in both irrigants (I and II) was formed during irradiation in argon and N_2O saturated solution. The

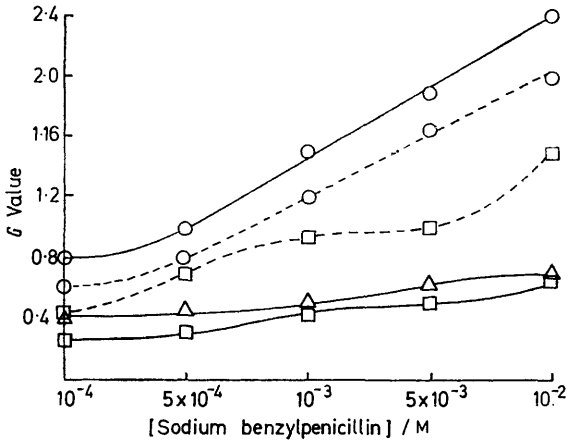

FIGURE 4 Yields of certain products formed during γ-irradiation of benzylpenicillin: ○ benzylpenilloic acid (——) argon, (– – –) N_2O; □ benzylpenicilloic acid (——) argon, (– – –) N_2O; △ benzylpenillic acid in argon

addition of 2-methylpropan-2-ol (10^{-1}M) during irradiation did not inhibit the formation of this compound. Purification by preparative t.l.c. and crystallisation from hot water produced a white compound, m.p. 85° (lit.,[16] 86°). The i.r. spectrum of this product (10 mg) as a KBr disc was identical with that previously obtained for benzylpenilloic acid.[17]

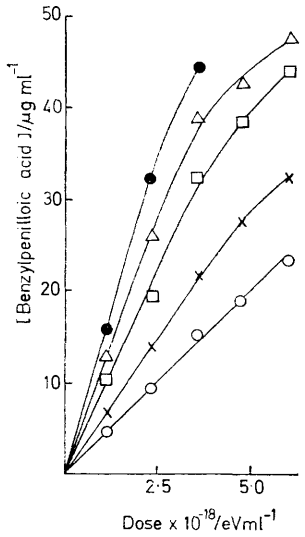

FIGURE 5 Formation of benzylpenilloic acid during irradiation in argon-saturated solution. [Benzylpenicillin]: ● = 10^{-2}M; △ = 5 × 10^{-3}M; □ = 10^{-3}M; × = 5 × 10^{-4}M; ○ = 10^{-4}M

The n.m.r. spectrum of the product showed singlets at δ = 7·37, 4·36, 3·67, 1·60, and 1·28 which contained 5, 1, 2, 3, and 3 protons respectively. These were assigned to the protons from the following groups: C_6H_5, C_3, $-CH_2C{=}O$, and the *gem*-dimethyl group. A triplet and doublet centred at δ = 3·93 containing 1 and 2 protons were assigned to the $-CH_2-CH$ group attached to the thiazolidine ring. The characteristic absorption of β-lactam ring protons at δ = 5·9 was absent.

The yields of benzylpenilloic acid formed during irradiation were estimated by iodine titration. *G*(Benzylpenilloic acid) values (Figure 4), were calculated from the initial slope of the yield dose curves shown for argon-saturated solutions in Figure 5.

(ii) *Benzylpenillic acid.* Following irradiation of benzylpenicillin, a compound was isolated by preparative t.l.c. which was chromatographically indistinguishable in two solvent systems from benzylpenillic acid. The u.v. absorption spectrum ($\lambda_{max.}$ 240 nm, $\Sigma_{max.}$ 6500 l mol^{-1} cm^{-1}) was identical with that of an authentic sample. The i.r. spectrum of the product agreed with those previously

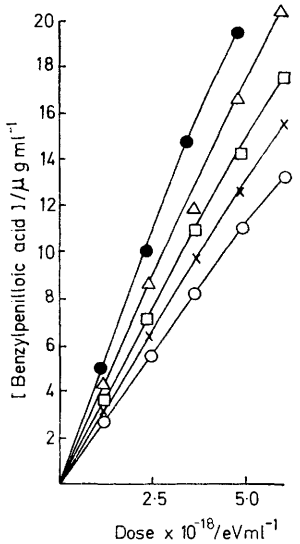

FIGURE 6 Formation of benzylpenillic acid during irradiation in argon-saturated solution. [Benzylpenicillin]: ● = 10^{-2}M; △ = 5 × 10^{-3}M; □ = 10^{-3}M; × = 5 × 10^{-4}M; ○ = 10^{-4}M

published for benzylpenillic acid.[17] The product had m.p. 188° (lit.,[18] 189°). The identity of the compound was confirmed by elemental analysis (Found: C, 57·1; H, 5·5; N, 8·6; S, 9·8. $C_{16}H_{18}N_2O_4S$ requires C, 57·5; H, 5·4; N, 8·4; S, 9·6%).

The yields of benzylpenillic acid formed during irradiation were measured by iodine titration (Figure 6). *G*-(benzylpenillic acid) values are summarised in Figure 4.

(iii) *Benzylpenicilloic acid.* Chromatographic analysis of the products of benzylpenicillin irradiation revealed a compound which gave a blue colour immediately when sprayed with arsenomolybdate–mercuric chloride reagent. This reaction is specific for benzylpenicilloic acid under the experimental conditions.[14,19] Addition of mercuric chloride solution (0·07%) alone to an alkaline solution of the product led to a strong absorption at 282 nm, which was short lived. This absorption is characteristic of the unstable penamaldic acid formed by rearrangement of the product, benzylpenicilloic acid.[20] The recrystallised product had

[16] R. Mozingo and K. Folkers, in ' The Chemistry of Penicillins,' eds. H. T. Clarke, J. R. Johnson, and R. Robinson, Princeton University Press, 1949, p. 536.
[17] A. H. Cook, ref. 16, p. 124.
[18] K. W. Merz, H. Knieps, and H. Lehmann, *Pharmazie*, 1965, **20**, 764.
[19] N. C. Green and P. R. Monk, *Chem. and Ind.*, 1959, 1210.
[20] R. B. Woodward, A. Neuberger, and N. R. Trenner, ref. 16, p. 427.

m.p. 120° (lit.[18] 126°). Moreover, the u.v. and i.r. spectra corresponded exactly with published data.[21]

Elemental analysis confirmed the identity of this product as benzylpenicilloic acid (Found: C, 51·3; H, 6·1; N, 7·8. $C_{16}H_{20}O_5N_2S,H_2O$ requires C, 51·9; H, 6·0; N, 7·55%).

The yields of benzylpenicilloic acid formed were measured by densitometry and a typical yield–dose curve is shown in Figure 7. *G*(Benzylpenicilloic acid) values are summarised in Figure 4.

(iv) *Hydroxybenzylpenicillins.* A marked increase in absorption at 275 nm was observed in benzylpenicillin solutions after irradiation. Addition of NaOH resulted in a shift to 292 nm, which is characteristic of phenols. The absorption at 275 nm was considerably enhanced when irradiations were carried out in N_2O. Separation of hydroxybenzylpenicillins from benzylpenicillin could not be achieved by t.l.c. in the solvent systems tested. Accordingly, the hydroxylated products formed during irradiation were hydrolysed with 3N-H_2SO_4 for 1 h at 100°. Subsequent t.l.c. analysis of the hydrolysed sample in irrigant (III) hexane : diethyl ether : acetic acid 90 : 25 : 4 indicated the presence of *o*-, *m*-, *p*-hydroxyphenyl-acetic acids, and phenylacetic acid. After elution and freeze-drying the identity of the hydroxy-compounds was confirmed by mass spectrometry. All three hydroxy-compounds showed a molecular ion peak at $m/e = 152$; further peaks were observed at $m/e = 106$, 91, and 84 confirming their identity as mono-hydroxylated products. The mass spectrum of one compound after storage over phosphorus pentoxide gave a molecular ion peak at $m/e = 134$. which could arise from

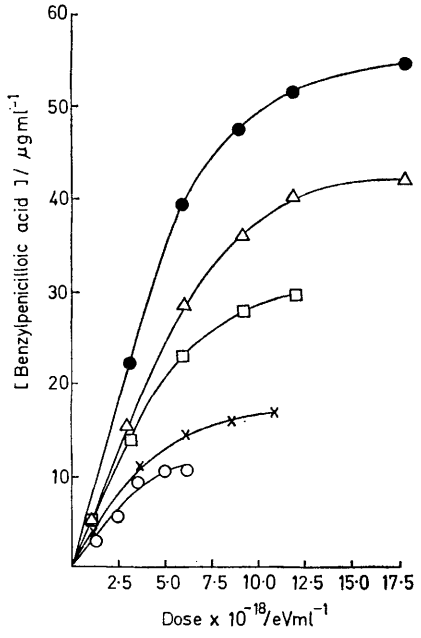

FIGURE 7 Formation of benzylpenicilloic acid during irradiation in argon-saturated solution. [Benzylpenicillin]: ● = 10^{-2}M; △ = 5×10^{-3}M; □ = 10^{-3}M; × = 5×10^{-4}M; ○ = 10^{-4}M

the formation of a lactone by dehydration of *o*-hydroxyphenylacetic acid. U.v. absorption spectra revealed $\lambda_{max.}$ 271, 273, and 276 nm which shifted to 291, 292, and 294 nm respectively in alkali in a manner which is characteristic of the phenolate ion. On spraying with Gibbs reagent, two of the products gave a blue colour in the manner of *o*- and *m*-hydroxyphenylacetic acids. The formation of these compounds by hydrolysis of irradiated solutions of benzylpenicillin was accompanied by the evolution of CO_2 and NH_3 and the production of penicillamine; *G*(hydroxybenzylpenicillin) values are shown in Figure 8.

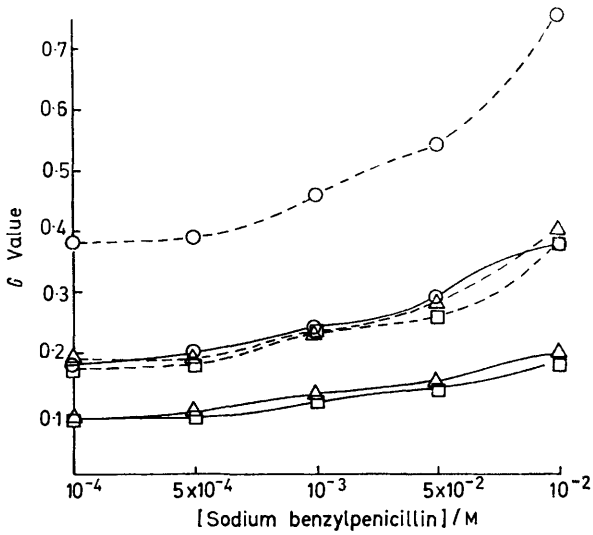

FIGURE 8 Yields of *o*-, *m*-, and *p*-hydroxybenzylpenicillins during γ-irradiation. ○ = *ortho*, (——) argon, (– – – –) N_2O; △ = *meta*, (——) argon, (– – – –) N_2O; □ = *para*, (——) argon, (– – – –) N_2O

(v) *Gaseous products.* The evolution of CO_2 and H_2S was detected during irradiation of benzylpenicillin. $G(CO_2)$ and $G(H_2S)$ values are shown in Table 2.

TABLE 2

Yields of gaseous products formed during irradiation of sodium benzylpenicillin in argon and nitrous oxide saturated solution

Benzylpenicillin concentration (M)	10^{-4}	5×10^{-4}	10^{-3}	5×10^{-3}	10^{-2}
$G(CO_2)$ in argon	0·6	0·6	1·0	1·0	1·1
$G(CO_2)$ in nitrous oxide	0·5	0·5	0·6	0·7	0·6
$G(H_2S)$ in argon	0·04	0·05	0·06	0·08	0·10
$G(H_2S)$ in nitrous oxide	<0·02	<0·02	<0·02	<0·02	<0·02

(vi) *Minor products.* (a) Dethiopenicillin. T.l.c. analysis of irradiated solutions of benzylpenicillin revealed a compound with the same mobility in irrigants (I) and (II) as dethiobenzylpenicillin. No reaction occurred on spraying with potassium hydroxide–starch iodine reagent, indicating the absence of sulphur. A positive reaction occurred with ninhydrin reagent. This evidence, together with the evolution of H_2S during irradiation is strongly indicative that the compound was dethiopenicillin. The low yields of this compound precluded unequivocal identification.

(b) 2,2-Dimethyl-7-oxo-6-phenylacetamido-1,4-thiazepine-3-carboxylic acid. Compound $R_p = 25$ (Table 1) failed to react with arsenomolybdate–mercuric chloride reagent; it did not, therefore, contain a β-lactam ring and was not structurally related to benzylpenicilloic acid. No immediate reaction occurred on spraying with starch iodine reagent,

[21] R. B. Woodward, A. Neuberger, and N. R. Trenner, ref. 16, p. 442.

indicating the absence of thiol groups and thiazolidines with a replaceable hydrogen atom in the 3-position. The compound absorbed in the u.v. region ($\lambda_{max.}$ 320 nm; $\Sigma_{max.}$ 8000 l mol^{-1} cm^{-1}). Studies on benzylpenicillenic acid [22] indicate that absorption at 320 nm may be attributed to the group RNH·CH:C·C:O·. The absorption arises from conjugation, when the group substituted in the oxazalone ring is RNH·CH= and R is aliphatic. The i.r. spectrum of the crude product showed absorption bands at 3300, 1730, 1690, 1620, and 1550 cm^{-1} which may be attributed to NH, amide, carboxylic acid, ring carbonyl group, and C=C respectively. These data are consistent with the recently published properties of thiazepines derived from penicillin.[23-25]

Irradiation of benzylpenicillin solutions resulted in the formation of trace quantities of several compounds which

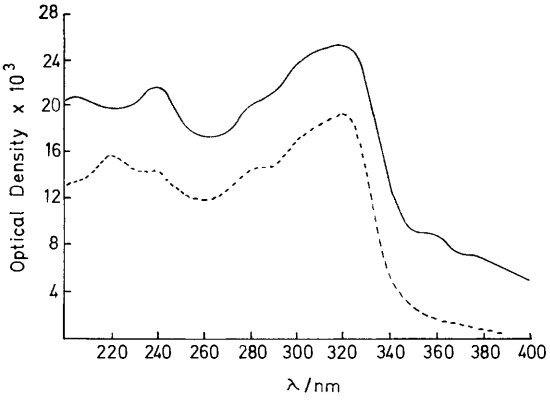

FIGURE 9 Transient species produced during pulse radiolysis of benzylpenicillin solutions. [Benzylpenicillin] = 10^{-3}M; (——) argon, (– – – –) N_2O. Dose ~1·5 × 10^{17} eV ml^{-1} and pH = 6·5

have been tentatively identified from their chromatographic mobility and reaction with certain selected spray reagents. Among these could be penicillamine and penillamine. The minute quantities formed precluded positive identification.

A summary of the *G* values obtained for the major products formed during irradiation of benzylpenicillin (10^{-3}M) is given in Table 3.

TABLE 3

Yields of major products formed during γ-irradiation of benzylpenicillin in dilute aqueous solution (10^{-3}M)

	G Value	
	Argon	H_2O
Benzylpenilloic acid	1·5	1·2
Benzylpenillic acid	0·5	<0·05
Benzylpenicilloic acid	0·47	0·97
o-Hydroxybenzylpenicillin	0·24	0·46
m-Hydroxybenzylpenicillin	0·13	0·23
p-Hydroxybenzylpenicillin	0·12	0·23
CO_2	1·0	0·6
H_2S	0·06	<0·02

Reactivity with OH *Radicals.*—The second-order rate constants for the reactivity of OH radicals with benzylpenicillin determined by the RNO competition method was 3·4 × 10^9 mol l^{-1} s^{-1}. An identical value was obtained for benzylpenicilloic acid.

Pulse Radiolysis.—Transient spectra. Irradiation of aqueous benzylpenicillin solutions (10^{-3}M), which had been equilibrated with argon, using a 2 μs pulse of 12 MeV electrons led to a transient species which has absorption maxima near 320 and 240 nm (Figure 9). The effects of irradiating in N_2O on the transient absorption spectra is also shown. The species were observed using an absorption cell with a 2·5-cm light-path using a Tektronix model 554 oscilloscope.

Reactivity with hydrated electrons (e_{aq}^-). Second-order rate constants for the reaction of e_{aq}^- with solutes may be obtained using the oscilloscope trace of the decay of e_{aq}^- as described previously.[26] For benzylpenicillin k_2 = 2·7 × 10^9 l mol^{-1} s^{-1} and for penicilloic acid, k_2 = 1·4 × 10^9 l mol^{-1} s^{-1}.

DISCUSSION

Extensive degradation of sodium benzylpenicillin accompanies γ-irradiation in dilute aqueous solution. The *G* value of 3·8 in argon for 10^{-4}M solutions indicates that both the major species of water radiolysis e_{aq}^- and OH participate in the degradation. Moreover, pulse radiolysis demonstrates the high reactivity of benzylpenicillin towards e_{aq}^- (k_2 = 2·7 × 10^9 l mol^{-1} s^{-1}) and an even greater reactivity towards OH radicals (k_2 = 3·4 × 10^9 l mol^{-1} s^{-1}). The total yield of primary radical species formed during radiolysis of water is *ca.* 5·7 molecules/100 eV. Values for *G*(—benzylpenicillin) in excess of this figure were obtained at benzylpenicillin concentrations greater than 10^{-3}M. Therefore, either a transient product produced by radical attack reacts with unchanged penicillin or direct action may contribute to decomposition at the higher concentrations. The latter possibility is unlikely, since the highest concentration used was only 10^{-2}M. *G*(—Benzylpenicillin) values obtained by chemical and biological assays are in close agreement (Figure 3) which indicates that radiation degradation of benzylpenicillin does not produce compounds which, although chemically similar, are without antibacterial activity as sometimes occurs during fermentation.

The major product formed during irradiation of benzylpenicillin is benzylpenilloic acid. The yields of this compound (Figure 4) are similar in both argon and N_2O. Moreover, benzylpenilloic acid is formed both in the presence of an OH radical scavenger, 2-methylpropan-2-ol and an electron scavenger, N_2O (Table 1). These observations indicate that reaction with both e_{aq}^- and OH radicals can lead to the formation of this product. Benzylpenilloic acid could result from e_{aq}^- attack on the carbonyl group of the β-lactam ring of benzylpenicillin, followed by loss of carbon monoxide. The hydrogen atom at C-6 of benzylpenicillin is activated by the 6-amido-group. Abstraction of this hydrogen by OH radicals would further weaken the already strained β lactam ring resulting in cleavage of the C–N bond. Subsequent loss of carbon monoxide would then yield benzylpenilloic acid by this path also.

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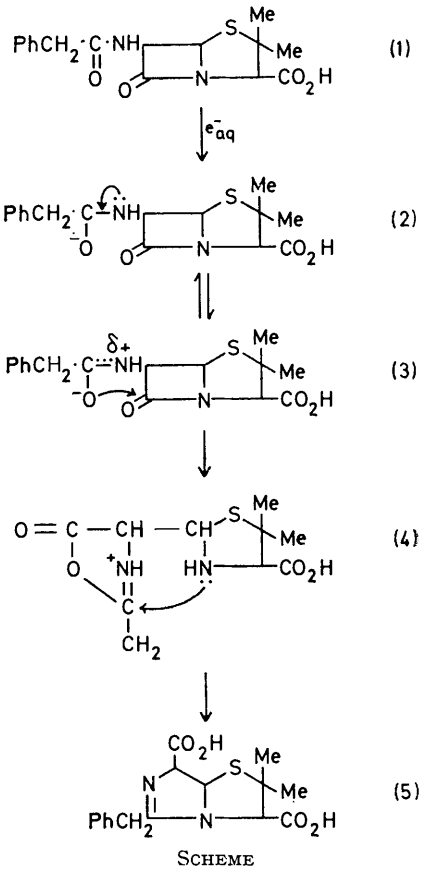
The comparatively large increase in *G*(benzylpenilloic acid) with increase in benzylpenicillin concentration cannot readily be explained in terms of increased radical scavenging. These data also (Figure 4) support the previous suggestion that a short chain-reaction is initiated by the radicals produced by reaction with the primary products of water radiolysis. This secondary reaction must lead also to penilloic acid.

Benzylpenillic acid is formed only during irradiation of argon-saturated solutions of benzylpenicillin; none is formed in N_2O. Moreover, the presence of an effective OH radical scavenger does not prevent its formation (Table 1). Benzylpenillic acid, therefore, results of e_{aq}^- attack. Yet, no trace of this compound was formed after irradiation of benzylpenicilloic acid solutions. This observation suggests that the β-lactam ring is necessary in order that rearrangement to benzylpenicillic acid can be effected after initial e_{aq}^- attack. Dennen and Davis [27] have shown that benzylpenillic acid is formed in acid solutions of benzylpenicillin, and the rate of formation was dependent on the nature of the side chain in the penicillins studied. Therefore, it was postulated that penillic acid formation was dependent on enolisation of the amide group, followed by an intramolecular rearrangement in which the side chain reacted directly with the carbon atom of the β lactam carbonyl with simultaneous cleavage of the lactam bond. Since the β-lactam ring in benzylpenicillin is strained to the extent of *ca.* 80 kJ, it is probable that attack of e_{aq}^- on the carbonyl group of the β-lactam ring would result in ring cleavage and preclude the possibility of any intramolecular rearrangement *via* the side chain. Formation of benzylpenillic acid during irradiation is, therefore, more likely to result from initial attack on the amide carbonyl group by e_{aq}^- followed by rearrangement (Scheme). Evidence for the oxazolone–thiazolidine intermediate (4) may be adduced from the experimental observation that an analogous oxazolone compound benzylpenicillenic acid may be converted into benzylpenillic acid in good yield.[28]

G(Benzylpenicilloic acid) values obtained in nitrous oxide-saturated solution are double those obtained in argon-saturated solution (Figure 4). Benzylpenicilloic acid is, therefore, formed exclusively by OH attack. The probable mechanism for production of this compound is formation of an OH adduct with the carbonyl group of the β-lactam ring. However, the increase in *G*(benzylpenicilloic acid) with increase in penicillin concentration (Figure 4) is greater than would be anticipated simply from increased OH radical scavenging by the penicillin.

Hydroxylation of the benzene ring of benzylpenicillin occurs in *o*-, *m*-, and *p*-positions during γ-irradiation. The yield of these products is in the ratio 2 : 1 : 1 (Figure 8). A two-fold increase in yield occurred in N_2O compared with argon. Moreover, these compounds were not formed in the presence of 2-methylpropan-2-ol and are, therefore, formed exclusively by OH radical attack. Previous studies on OH substitution in benzoic acid by radicals generated radiolytically, photochemically, and



SCHEME

from the ion-pair complex Fe^{3+} OH^- have yielded conflicting data concerning the ratio of *o*-, *m*-, and *p*-substitution.[29-34] However, the most consistently obtained ratio is 2 : 1 : 1,[33,34] which is in agreement with the data of the present study. The variation between investigators mainly reflects the difficulty in estimating yields of the individual isomers. The reactivity of monosubstituted benzene derivatives towards radicals is greater in the *ortho*- and *para*-positions than in the *meta*-position.[35] Studies on radiation-induced hydroxylation [36,37] confirm that 75% of the OH radicals appear in the *ortho*- and *para*-positions, which is in agreement with our results. When electron-donating substituents are present in the benzene ring the electrophilic OH

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radical may be expected to be directed mainly to the *ortho-* and *para*-positions. However, selectivity is considerably less marked in homolytic aromatic substitution compared in heterolytic aromatic substitution, and for the former all three isomers are normally found in substantial yields. Our yields of hydroxylated products formed during γ-irradiation of benzylpenicillin solutions indicate that the substituent CH_2·CO·NHR has little effect in directing the OH radical attack. Substitution appears to be random, giving yields in the ratio 2 : 1 : 1.

Carbon dioxide is formed during irradiation of penicillin in both argon and nitrous oxide saturated solution, Table 2, and its production is not prevented by 2-methylpropan-2-ol. Comparison of the *G* values for formation of benzylpenicilloic acid, benzylpenilloic acid, and carbon dioxide indicates that benzylpenilloic acid is not formed from benzylpenicilloic acid by liberation of CO_2. The carbon dioxide, therefore, arises from the 3-carboxy-group of the thiazolidine ring in benzylpenicillin. Liberation of CO_2 by direct radical attack on carboxy-groups does not generally occur.[34] Formation of CO_2, therefore, probably involves e_{aq}^- and OH attack at other sites. However, it has been suggested[30] that decarboxylation of benzoic acid by OH radicals can occur by the following mechanism:

$$PhCO_2^- + \cdot OH \longrightarrow OH^- + PhCO_2\cdot \longrightarrow Ph\cdot + CO_2$$

For benzylpenicillin, abstraction of a hydrogen atom from C-3 by OH radicals could also result in loss of carbon dioxide, since C-3 is activated by the adjacent CO·N group in the β-lactam ring.[38-41]

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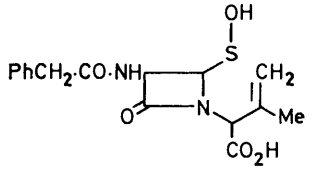
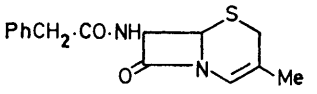
It is possible that CO_2 could arise following attack of e_{aq}^- or OH on the sulphur atom of benzylpenicillin. The initial reaction might be expected to result in β elimination of a hydrogen atom from the *gem*-dimethyl group leading to an intermediate similar in structure to (1). This compound would then lose CO_2 with con-



(1)



(2)

commitant ring extension to yield Δ^3-cephem (2). A similar mechanism has been proposed[42] for the interaction of Raney nickel with benzylpenicillin, which results in ring extension and loss of CO_2.

The formation of H_2S and dethiopenicillin in argon-saturated solution during irradiation indicates that reaction occurs between e_{aq}^- and the sulphur atom of the thiazolidine ring in benzylpenicillin.

Extensive degradation of benzylpenicillin occurs during irradiation. The wide variety of products identified and extreme sensitivity clearly indicate that radiation sterilisation of aqueous solutions of benzylpenicillin is impracticable.

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