AN INVESTIGATION INTO THE EFFECTS OF GAMMA-RAYS ON AQUEOUS SOLUTIONS OF *p*-AMINOBENZOIC ACID

BY P. H. MARRIOTT*

From the Department of Pharmacy, University of Manchester

Received April 29, 1963

Aqueous solutions of *p*-aminobenzoic acid (0·3 per cent) were irradiated with gamma-rays under both oxygen-saturated and oxygen-free conditions. By means of paper chromatography, *p*-nitrobenzoic acid has been isolated from the irradiated, oxygenated aqueous solution and 4-amino-3-hydroxybenzoic acid isolated from the irradiated, oxygen-free aqueous solution.

These products were probably produced by indirect action of the Co-60 gamma-rays.

THE ability of high-energy electromagnetic and particulate radiation to destroy (within certain limits of probability) micro-organisms without appreciable temperature rise in the substrate might offer a means of sterilising heat-labile drugs.

In June, 1960, a report was published (A.B.P.I. Report) on the use of γ-radiation sources for the sterilisation of pharmaceutical products. This report served to emphasise the need for more work in this field.

An investigation into the effects of γ-irradiation on aqueous solutions of *p*-aminobenzoic acid (PABA) should yield information about the action of the radiation on esters of PABA, e.g. procaine, in aqueous solution.

The major primary path by which any high-energy particles or photons interact with matter is by interaction with electron shells of molecules. Energy is absorbed, electrons are ejected to produce ions, and bonds are broken to produce free-radicals. The particles produced are often unstable and decompose with the result that much of the energy is quickly dispersed with the formation of excited molecules and radicals. It is these species that undergo the postulated reactions and "account" for many of the products that are ultimately observed (Hart, 1954a,b).

Initial radical production appears to be a highly indiscriminate process, the number of radicals produced from any component being roughly proportional to the fraction of the component, by weight, present in the system (Henley and Barr, 1956). Accordingly, the reactions of a dilute solution of a reactive substrate in an unreactive solvent are chiefly those of the solvent radicals with the substrate and it may be relatively easy to interpret these. Irradiation of a single complex organic molecule, or mixtures such as those found in biochemical systems, may give results which are of almost meaningless complexity.

Products to be expected on γ-irradiation of aqueous solutions of PABA are those produced from the original solute by oxidation, reduction, deamination and decarboxylation. The relative amounts of the products cannot be predicted.

* Present address: School of Pharmacy, University of London, 29/39 Brunswick Square, London, W.C.1.

EXPERIMENTAL METHODS

Radiation Source and Dosimetry

The radiation source was a 250c Cobalt–60 source, which was calibrated as described by Davies (1960).

Solutions Prepared and Dose Given

p-Aminobenzoic acid (0·3 g.) was dissolved in glass-distilled water (100 ml.). The pH of the solution was not specially adjusted. The solution was divided into two equal portions (by volume), each was contained in a large glass tube, of approximately 70 ml. capacity, fitted with a ground-glass stopper.

Oxygen was bubbled through one of the portions, designated I_o, and oxygen-free nitrogen gas through the other portion, designated I_n, for 2 hr., after which the tubes were well stoppered. The air above solution I_o was assumed to be saturated with oxygen whereas the enclosed air above the second solution, I_n, contained little oxygen. Each portion of the solution was colourless and free from solid particles. The tubes containing the solutions were then irradiated. Solution I_o received a dose of approximately 2 Megarads and solution I_n a dose of approximately 4 Megarads.

Detection of Breakdown Products Using Paper Chromatography

The solvent was a mixture of n-butanol and aqueous ammonium hydroxide solution (1·5N) (35:65 v/v). Whatman No. 1 paper 57 × 46 cm., or smaller sizes cut as required, was used and after equilibration (at 24° ± 1° for 4 to 6 hr.) developed by the descending technique for 17 to 18 hr. The paper was then dried by warm air.

Substances Applied to the Chromatograms

The irradiated solutions I_o and I_n were concentrated by reduced pressure distillation and then applied as such to the chromatograms.

The following possible products of degradation were applied to the chromatograms as spots in ether. *o*-Nitrophenol, *m*-nitrophenol, *p*-nitrophenol, and 4-amino-3-hydroxybenzoic acid were applied as separate spots. A mixture of *p*-aminobenzoic acid, salicylic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid and *p*-nitrobenzoic acid and a mixture of *p*-aminobenzoic acid, *p*-aminosalicylic acid, *o*-aminophenol, *m*-aminophenol, and *p*-aminophenol were applied to the chromatograms as single spots and run alongside the spots of I_o and I_n.

p-Aminosalicylic acid was prepared from the mono-sodium salt of 4-aminosalicylic acid, and 4-amino-3-hydroxybenzoic acid was prepared from 3-hydroxy-4-nitrobenzoic acid by reduction using tin and hydrochloric acid (Froelicher and Cohen, 1921). All other substances were obtained from commercial sources and purified before use.

Reagents for Spot Detection

Ehrlich reagent. *p*-Dimethylaminobenzaldehyde (2 g.) is dissolved by shaking in 20 per cent aqueous hydrochloric acid (100 ml.).

Diazotised p-*nitroaniline and sodium carbonate reagent.* 0·5 per cent (w/v) *p*-nitroaniline (2 ml.) in cold aqueous hydrochloric acid (2 *N*) is just decolourised with 5·0 per cent sodium nitrite (5 drops) in cold water, 20 per cent sodium acetate (8 ml.) in cold water added and the total volume adjusted to 40 ml. with iced cold water. This is used immediately to spray the paper, which when dry, is sprayed with 15 per cent sodium carbonate in water.

Stannous chloride reagent. 2 per cent stannous chloride in aqueous hydrochloric acid.

TABLE I

R_F VALUES OF POSSIBLE DEGRADATION PRODUCTS, USING THE SYSTEM n-BUTANOL AND AQUEOUS AMMONIUM HYDROXIDE (35 : 65 v/v)

Compound	R_F measured to the front of the spot	R_F measured to the centre of the spot
p-Aminobenzoic acid	0·09	0·06
p-Aminosalicylic acid	0·16	0·12
4-Amino-3-hydroxybenzoic acid.. ..	0·05	0·04
Salicylic acid	0·45	0·42
m-Hydroxybenzoic acid	0·20	0·16
p-Hydroxybenzoic acid	0·13	0·08
p-Nitrobenzoic acid	0·43	0·37
o-Aminophenol	0·81	0·77
m-Aminophenol	0·72	0·68
p-Aminophenol	0·57	0·51
m-Nitrophenol	0·74	0·68
p-Nitrophenol	0·51	0·46

RESULTS

After irradiation, solution I_0 had a dark-brown colouration but was free from solid particles. Solution I_n was also a dark-brown colour and contained fine particles of what appeared to be a dust on the surface of the solution and also a black precipitate. The glass of both tubes was tinted brown.

Spots from I_0 were identified as *p*-aminobenzoic acid and *p*-nitrobenzoic acid using the stannous chloride reagent and Ehrlich reagent.

Spots from I_n were identified as *p*-aminobenzoic acid and 4-amino-3-hydroxybenzoic acid using diazotised *p*-nitroaniline and sodium carbonate reagent.

DISCUSSION

Solution I_0 contained *p*-nitrobenzoic acid. The compound stained with Ehrlich reagent only after the paper had been sprayed with stannous chloride reagent. The spot had the same R_F value as authentic *p*-nitrobenzoic acid, both when the *p*-nitrobenzoic acid was separated from the

mixture of compounds applied as a single spot and also when applied on its own. No other compounds were separated from solution I_o by this technique.

4-Amino-3-hydroxybenzoic acid was separated from solution I_n. In contrast to solution I_o, the unchanged PABA in I_n moved as a distinct spot. This could be due either to there being less PABA in I_n or some products present in I_o that are not separated by this system, or both. The spot produced from I_n had a similar R_F value to that of authentic 4-amino-3-hydroxybenzoic acid in the same solvent system. It also stained the same colours with both the Ehrlich reagent (with or without previously spraying the paper with stannous chloride reagent), and the diazotised *p*-nitroaniline and sodium carbonate reagent.

These results agree qualitatively with those of Muto (1961), who employed an optical method for the analysis of his irradiated solution. However, I could find no different absorption peaks in the ultra-violet absorption curves of the irradiated solutions, when compared with the absorption curve of the original solution of PABA.

Nakayama (1961) irradiated weak aqueous solutions of PABA with both X- and γ-rays with similar effects, as measured by loss of PABA, the loss being dependent only on the total dose given.

The mechanism of formation of products from irradiated aqueous solutions should only be postulated on the basis of quantitative results (Minder, 1951; Cadogan, 1961; Scharf and Lee, 1962). However, qualitative experiments often suggest a possible pathway leading to the products.

The production of *p*-nitrobenzoic acid from PABA can be explained by general oxidation of the amine by the radiolysis products of oxygen-saturated water. Hydroxyl radicals abstract hydrogen away from nitrogen in preference to the abstraction of hydrogen from carbon in the benzene ring (Riesz and Burr, 1962), thus producing a free radical and one molecule of water. Proskurnin and Kolotyrkin (1958) suggest that the hydroxyl radicals add to the benzene ring producing a hydrogen radical and a phenol.

Boyland and Sims (1954) have investigated the action of alkaline persulphate on substituted aromatic amines. They found that 4-amino-3-hydroxybenzoic acid was the only product formed by persulphate oxidation of PABA. Since 4-amino-3-hydroxybenzoic acid (and not 4-amino-2-hydroxybenzoic acid) could be detected only after an aqueous solution of PABA had been irradiated with γ-rays, it is suggested that this action can be explained as oxidation of the PABA by peroxide produced from the water, and not by direct action of the γ-rays. Similar effects have been reported with other solutions. Irradiation of aqueous solutions of tyrosine produced 3,4-dihydroxyphenylalanine and not the 2,4-isomer (Rowbottom, 1955). But irradiation of aqueous solutions of phenylalanine produced *o*-, *m*-, and *p*-hydroxyphenylalanines (Vermeil and Lefort, 1957). Irradiation of aqueous solutions of nitrobenzene gave *p*-hydroxynitrobenzene (*p*-nitrophenol) as a major product (Loebl, Stein and Weiss, 1950).

No *p*-nitrobenzoic acid was detected in solution I_n and no 4-amino-3-hydroxybenzoic acid detected in solution I_o. The lack of *p*-nitrobenzoic acid in I_n indicates that dissolved oxygen gas plays a part in oxidation procedures that occur on γ-radiolysis of oxygenated aqueous solutions. The degree of oxidation is controlled by the tensions of oxygen, nitrogen and other gases present. In oxygen-free solutions monophenolic compounds would be stable whereas in oxygen-saturated solutions further oxidation would almost certainly occur giving rise to poly-phenolic compounds or nitro-compounds from PABA. In oxygen-free solutions any free-radicals produced would tend to combine, forming stable compounds such as diphenyl derivatives. The black precipitate in solution I_n could well be a polyphenyl derivative but identification of the precipitate has not been completed.

The future prospects for γ-radiation sterilisation of drugs in aqueous solution would seem to lie with compounds that decompose, into non-toxic products, in preference to the breakdown of the drug.

Acknowledgements. The author wishes to express his gratitude to Professor K. Bullock for the encouragement and help given him, and to the Department of Scientific and Industrial Research for the award of a Research Studentship, throughout the course of this work.

REFERENCES

A.B.P.I. Report (1960). "The use of Gamma-radiation Sources for the Sterilisation of Pharmaceutical Products," Report of a Working Party Established by The Association of British Pharmaceutical Industry in collaboration with The School of Pharmacy, University of London and The Isotope Division, Atomic Energy Research Establishment. London: A.B.P.I., Tavistock Square.

Boyland, E. and Sims, P. (1954). *J. chem. Soc.*, 980–985.

Cadogan, J. I. G. (1961). *The Royal Institute of Chemistry, Lecture Series*, No. 6, 1–28.

Davies, D. J. G. (1960). *M.Sc. Thesis*, University of Manchester.

Froelicher, V. and Cohen, J. B. (1921). *J. chem. Soc.*, 1425–1431.

Hart, E. J. (1954a). *Rad. Res.*, **1**, 53–61.

Hart, E. J. (1954b). *Ann. Rev. Phys. Chem.*, **5**, 139–162.

Henley, E. J. and Barr, N. F. (1956). *Advances in Chemical Engineering*, 1, pp. 1–76, New York: Academic Press Inc.

Loebl, H., Stein, C. and Weiss, J. (1950). *J. chem. Soc*, 2704–2709.

Minder, W. (1951). *Brit. J. Radiol.*, **24**, 435–440.

Muto, T. (1961). *Nuclear Science Abstracts*, 20739.

Nakayama, Y. (1961). *Ibid.*, 20765.

Proskurnin, M. A., and Kolotyrkin, Ya. M. (1958). *Proc. U.N. Intern. Conf. Peaceful Uses At. Energy*, 2nd., Geneva, P/2022, 29, 52–61.

Riesz, P. and Burr, B. E. (1962). *Rad. Res.*, **16**, 661–667; 668–673.

Rowbottom, J. (1955). *J. biol. Chem.*, **212**, 877–885.

Scharf, K. and Lee, R. M. (1962). *Rad. Res.*, **16**, 115–124.

Vermeil, C. and Lefort, M. (1957). *C.R. Acad. Sci. Paris*, **244**, 889–891.