THE FLAGYL ANALOGUE OF NICOTINAMIDE ADENINE DINUCLEOTIDE

G. H. COOMBS* and B. R. RABIN
Biochemistry Department, University College London, Gower Street, London WC1E 6BT, England

Received 7 March 1974

1. Introduction

Flagyl [1] is the trade name of metronidazole, 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole, the drug most commonly employed in the treatment of infections of the vagina by *Trichomonas vaginalis*. Recently it has been widely used against entamoebal infections. Flagyl has also been demonstrated to produce aversion to ethanol, and was suggested as a represent of compulsive consumption of alcohol [2].

The ability of mammalian nicotinamide adenine dinucleotide nucleosidases (EC 3.2.2.5) to catalyse transglycosylation reactions is well established, and has been elegantly exploited for the synthesis of a multitude of analogues, largely in the laboratories of Kaplan, working with pyridine derivatives [3–13], and Alivisatos, working with imidazole derivatives [14–25].

Analogue formation has been implicated in the action of drugs [26–32] and in synthetic pathways [14,18,25,33]. Imidazolysis may be involved in the mechanism of anaphylactic shock [15,19,21,34,35] and also the mechanism of action of NAD nucleosidase itself [24].

We report in this communication new analogues, in which the nicotinamide moiety of NAD and NADP is replaced by Flagyl. These we will refer to as FlagAD and FlagADP respectively.

* Present address: Biological Laboratory, University of Kent, Canterbury, Kent, England.

2. Materials and methods

The method of synthesis and purification of FlagAD and FlagADP, the analogue of NADP, is shown in Fig. 1. The conditions for the actual transglycosylation reaction are based on those of Kaplan [11], whereas the column chromatographic separation of the analogue from the other reaction products is an adaptation of the method of Chaykin et al. [36], which utilises the volatile ammonium bicarbonate both to maintain the appropriate pH and to provide

1. Dissolve 1000 mg Flagyl in 50 ml phosphate buffer (0.05 I, pH 7.2) using heat.
2. Cool to 37°C, add 50 mg NAD and 125 mg calf spleen NADase and incubate at 37°C for 3 hr.
3. Centrifuge at 27 000 *g* for 10 min at 4°C, discard pellet.
4. Add supernatant to an ion-exchange column, DE 52, 20 cm × 2.1 cm, 30 ml/hr, equilibrated with 0.005 M ammonium bicarbonate.
5. Elute with a linear gradient of ammonium bicarbonate from 0.005 M to 0.5 M, monitoring effluent at 260 nm.
6. Bulk fractions of each peak and freeze dry.
7. Measure the UV spectrum of the material from each peak.
8. Store the 'FlagAD', the mixed product including FlagAD, at −15°C.
9. Run 20 mg of this product in a TLC system consisting of a 0.75 mm layer of MN300 with a solvent system of tertiary amyl alcohol, formate and water (3:2:1).
10. Identify spots with UV light, elute in water, and freeze-dry products.
11. Measure the UV spectrum of each product.
12. Store the FlagAD at −15°C.

Fig. 1. Methodology of the synthesis of FlagAD.

the elution gradient. The thin layer chromatographic method employed cellulose powder MN300, with a solvent system consisting of tertiary amyl alcohol, formic acid and water in the ratio 3:2:1. The spots were located with ultraviolet light. To scale up this method, grooved plates (May and Baker Ltd) were used with a thicker (0.75 mm) cellulose layer.

Spectrophotometric measurements were made on a Cary 14 spectrophotometer. Flagyl was obtained from May and Baker Ltd., Dagenham, Essex, England. DE52 column chromedia was obtained from Whatman, MN300 cellulose powder was supplied by Camlab, Cambridge, England. Calf spleen NADase was obtained from Boehringer Co. (London) Ltd.

3. Results

Typical profiles of the ion exchange column effluents are shown in fig. 2. A better separation of peaks is obtained in the FlagADP experiment, due to the extra negative charge of the product. Freeze-drying of the bulked fractions corresponding to each peak removed most of the contaminating ammonium bicarbonate, leaving white residues. The absorbance spectrum of the analogue-containing peak, in each experiment, showed a maximum at 320 nm as well as the usual 260 nm nucleotide absorption. This contrasts with the spectra of Flagyl itself, NAD, and a theoretical spectrum of FlagAD, calculated assu-

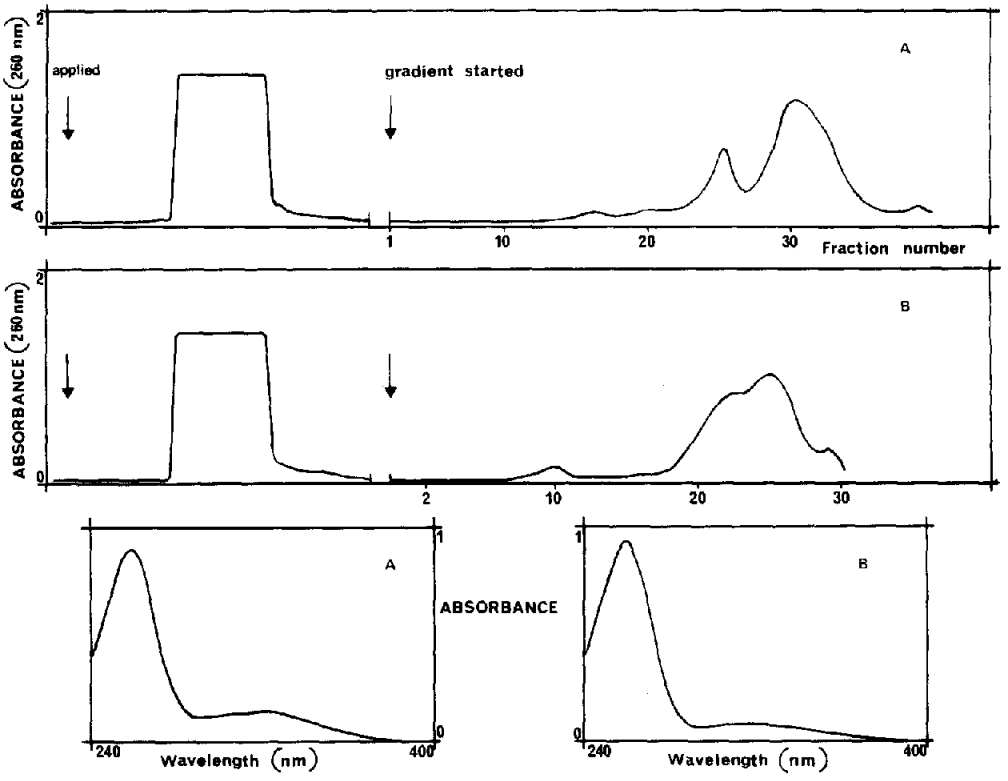

Fig. 2. Chromatographic purification of 'FlagAD' and 'FlagADP' on DEAE cellulose, and the spectra of the analogue-containing peaks. A, 'FlagADP'; B, 'FlagAD'.

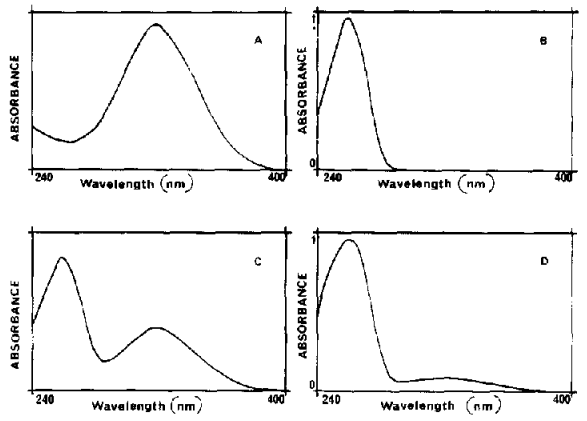

Fig. 3. Spectrum of 'FlagAD', as eluted from the DEAE cellulose column, compared with the spectra of the components and the theoretical spectrum of the analogue. A, 114 μM Flagyl; B, 62.6 μM ADPR; C, theoretical 50 μM FlagAD; D, peak, containing FlagAD, eluted from the DE52 column.

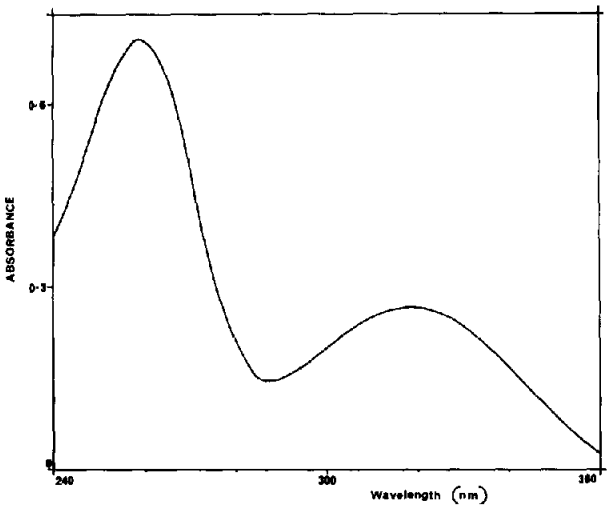

Fig. 5. Spectrum of FlagAD.

ming that both components contribute their 'free' spectrum to the new nucleotide (fig. 3).

This material ('FlagAD') of the analogue-containing peak when run in the TLC system was found to split into two major and two minor spots (fig. 4). None of these corresponded to Flagyl or NAD, but one of the major spots, 1, ran with the marker ADPR, and one of the minor spots, 3, ran as ADP. Spot 2 corresponded to none of the added marker nucleotides. The compound eluted from this spot was found to possess a spectrum (fig. 5) very similar to the calculated, theoretical spectrum of FlagAD. We concluded that this was our pure analogue.

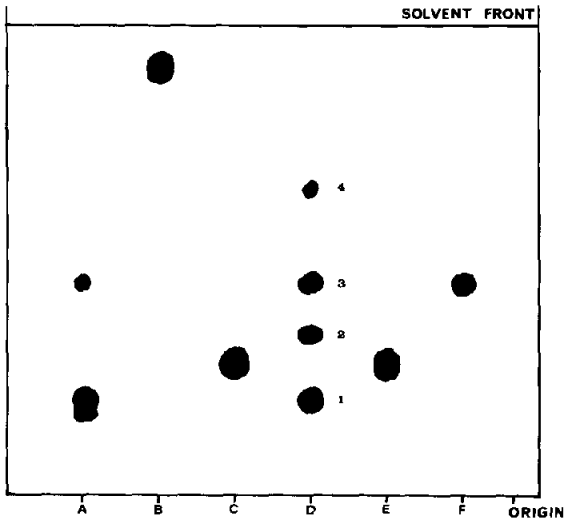

Fig. 4. Purification of FlagAD by Thin Layer Chromatography. Spots: A, ADPR; B, Flagyl; C. peak 1 from DE52; D, peak containing FlagAD from DE52; E, NAD; F, ADP.

Imidazolysis is reported to be irreversible [22,24, 25]. However this new nucleotide was found to be slowly hydrolysed to release Flagyl (as identified by its absorbance and acid difference spectra), in a reaction catalysed by NAD nucleosidase (fig. 6). The reaction in the hydrolytic direction is very slow compared with the synthesis.

4. Discussion

The demonstration that Flagyl will exchange with the nocotinamide moiety of NAD or NADP to produce new nucleotides raised the question as to whether this process may be an integral part of the toxity of Flagyl to various anaerobic organisms. This possibility has been fully tested and the results of these experiments [37] do not suggest that the analogue is involved.

Flagyl is known to have anti-alcohol activity, and the main research into the mode of this action has

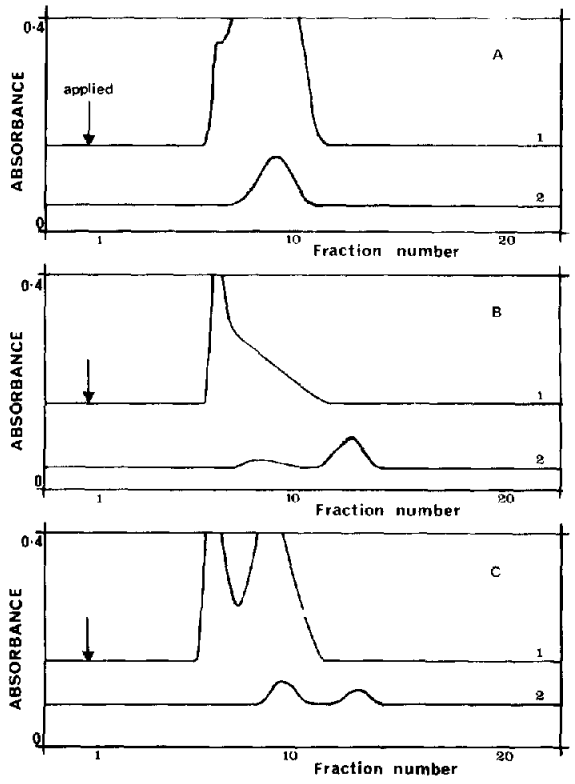

Fig. 6. Hydrolysis of FlagAD. Incubation mixture contained 20 mg 'FlagAD', as eluted from the DE52 column, 45 mg calf spleen NADase, and 1.5 ml phosphate buffer (0.05 I, pH 7.2), and was at 37° C. The reaction was stopped by the addition of 0.1 ml 5M TCA, and the mixture was then centrifuged. The supernatant was neutralised with 1 M NaOH, and 0.75 ml of it applied to a G-10 sephadex column, 30 cm by 2.1 cm, flowing at 30 ml hour, and eluted with phosphate buffer (0.05 I, pH 7.2). Length of incubation: A, 48 hr, control, no enzyme; B, 19.5 hr; C, 6.3 hr. 1, absorbance at 260 nm; 2, absorbance at 320 nm.

involved the study of the inhibition by Flagyl of various of the enzymes involved in the oxidation of ethanol [38–42]. The validity of some of this work has been questioned [43], and in some cases it is likely that the apparent inhibitions are simply due to spectrophotometric artefacts. It has recently been shown [44] that a reported chemical reaction between Flagyl and NADH [43] was just this. As yet no satisfactory mechanism has been proposed for the antagonism between Flagyl and ethanol although FlagAD or FlagADP might well be involved.

This is the first time to our knowledge that an imidazolytic reaction has been demonstrated to be reversible. This reversibility can possibly be explained by the possession of a substituent, in Flagyl, on the second nitrogen of the imidazole ring. As a consequence of this substitution the positive charge produced on the combination of Flagyl with the ADPR moiety cannot be lost by ionisation of a proton as with analogues containing non-substituted imidazoles.

Acknowledgement

During the period of this research one of us (GHC) was supported by a Science Research Council studentship.

References

[1] May & Baker Ltd., Dagenham, Essex, England.
[2] Taylor, J. A. T. (1964) Bull. Los Ang. Neurol. Soc. 29, 158.
[3] Zatman, L. J., Kaplan, N. O., Colowick, S. P. and Ciotti, M. M. (1954) J. Biol. Chem. 209, 453.
[4] Zatman, L. J., Kaplan, N. O., Colowick, S. P., and Ciotti, M. M. (1954) J. Biol. Chem. 209, 1467.
[5] Anderson, B. M., Ciotti, M. M., and Kaplan, N. O. (1959) J. Biol. Chem. 234, 1219.
[6] Anderson, B. M., and Kaplan, N. O. (1959) J. Biol. Chem. 234, 1226.
[7] Windmueller, H. G., and Kaplan, N. O. (1962) Biochim. Biophys. Acta 56, 388.
[8] Kaplan, N. O., and Stolzenbach, F. E. (1957) Methods in Enzymology, (Colowick, S. P., and Kaplan, N. O. eds.), Vol. 3, p. 902, Academic Press, New York and London.
[9] Kaplan, N. O., and Ciotti, N. M. (1956) J. Biol. Chem. 221, 823.
[10] Ciotti, N. M., Humphreys, S. R., Venditti, J. M., Kaplan, N. O., and Goldin, A. (1960) Cancer Res. 20, 1195.
[11] Kaplan, N. O. (1955) Methods in Enzymology, (Colowick, S. P., and Kaplan, N.O., eds), Vol. 2, p. 660, Academic Press, New York and London.
[12] Kaplan, N. O., Ciotti, M. M., and Stolzenbach, F. E. (1956) J. Biol. Chem. 221, 833.
[13] Humphreys, S. R., Venditti, J. M., Ciotti, M. M., Kline, T., Goldin, A., and Kaplan, N. O. (1962) Cancer Res. 22, CS 483.
[14] Abdel-Latif, A. A., and Alivisatos, S. G. A. (1962) J. Biol. Chem. 237, 500.

[15] Alivisatos, S. G. A., Ungar, F., Lukacs, L., and Lamantia, L. (1960) J. Biol. Chem. 235, 1742.
[16] Alivisatos, S. G. A., Lamantia, L., and Matijevitch, B. L. (1962) Biochim. Biophys. Acta 58, 201.
[17] Alivisatos, S. G. A., Lamantia, L., and Matijevitch, B. L. (1962) Biochim. Biophys. Acta 58, 209.
[18] Alivisatos, S. G. A., and Woolley, D. W. (1956) J. Biol. Chem. 221, 651.
[19] Alivisatos, S. G. A. (1958) Nature 181, 27.
[20] Alivisatos, S. G. A., and Lamantia, L. (1960) Biochem. Biophys. Res. Commun. 2, 164.
[21] Abdel-Latif, A. A., and Alivisatos, S. G. A. (1961) J. Biol. Chem. 236, 2710.
[22] Alivisatos, S. G. A., and Woolley, D. W. (1955) J. Amer. Chem. Soc. 77, 1065.
[23] Alivisatos, S. G. A., and Woolley, D. W. (1956) J. Biol. Chem. 219, 823.
[24] Alivisatos, S. G. A. (1954) Nature 183, 1034.
[25] Alivisatos, S. G. A., Lamantia, L., Ungar, F., and Savich, B. (1958) Biochim. Biophys. Acta 30, 660.
[26] Kaplan, N. O., Goldin, A., Humphrey, S. R., Ciotti, M. M., and Venditti, J. M. (1954) Science 120, 437.
[27] Hicks, S. P. (1955) Amer. J. Pathol. 31, 189.
[28] Burton, R. M. (1957) J. Neurochem. 2, 15.
[29] Windman, T., and Bekierkunst, A. (1969) Life Sci. 8, pt. 1, 73.
[30] Johnston, W. J. (1963), Metabolic Inhibitors, (Hochster, T. M., and Quastel, J. H. eds), 2, 1.
[31] Halliday, S. L., Sloboda, A., Will, L. W., and Oleson, J. J. (1957) Federation Proc. 16, 190.
[32] McColl, J. D., Rice, W. B., and Anamkiewicz, V. W. (1957) Canad. J. Biochem. Physiol. 35, 795.
[33] Alivisatos, S. G. A., Lamantia, L., Ungar, F., and Matijevitch, B. L. (1962) J. Biol. Chem. 237, 1212.
[34] Paton, W. D. M. (1955) Int. Arch. Allergy Appl. Immunol. 6, 203.
[35] Alivisatos, S. G. A. (1959) Mechanisms of Hypersensitivity, Shaffer, J. H., Lo Grippo, G. A., and Chase, M. W. eds.), p. 259, Little Brown & Co., Boston.
[36] Chaykin, S., Dagani, M., Johnson, L., Samli, M., and Battaile, J. (1965) Biochim. Biophys. Acta 100, 351.
[37] Coombs, G. H., in press.
[38] Fried, R., and Fried, L. W. (1966) Third Int. Congr. Pharmac. San Paulo, 23.
[39] Fried, R., and Fried, L. W. (1966) Biochem. Pharm. 15, 1890.
[40] Fried, R. (1966) Int. Symp. Alcohol, Santiago.
[41] Edwards, J. A., and Price, J. (1967) Nature 214, 190.
[42] Metronidazole and alcoholism, 2nd edition, (1966) May and Baker Ltd.
[43] Fried, R., and Fried, L. W. (1968) Experientia 24, 56.
[44] Coombs, G. H., and Rabin, B. R., in press.